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# MicroRNA profiling in prostate cancer and prostate derived holoclone cell model

A thesis submitted for the Degree of

PhD in Philosophy of Science

By

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**March 2012**

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**Declaration**

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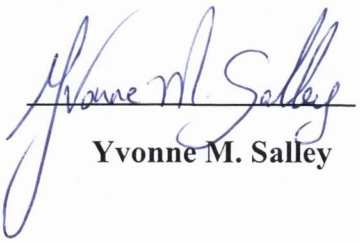

**Yvonne M. Salley**

*To my parents*

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| Abbreviation | Description |
|---|---|
| AFP | Alpha-fetoprotein |
| AGCC | Affymetrix GeneChip Command Console |
| AR | Androgen receptor |
| AUC | Area under the curve |
| bp | Base pair |
| BPH | Benign Prostatic Hyperplasia |
| °C | Degrees Celsius |
| CD | Cluster of differentiation |
| cDNA | Complementary DNA |
| Ct | Threshold cycle |
| DAVID | Database for annotation, visualisation and integrated discovery |
| EC | Expression console™ |
| EDTA | Ethylenediaminetetraacetate |
| EMT | Epithial-mesenchymal transition |
| ENO3 | Enolase 3 |
| DNA | Deoxyribonucleic Acid |
| dNTP | Deoxynucleoside triphosphate |
| dTTP | Deoxy Thymidine Triphosphate |
| FBS | Foetal Bovine Serum |
| FC | Fold change |
| FDR | False discovery rate |
| F12-K | F12-K Nutrient Mixture Kaighn's Modification |
| g | Gram |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |

| | |
|---|---|
| $H_2O$ | Water |
| IMS | Industrial Methylated Spirits |
| IVT | In vitro transcription |
| K | Keratin |
| LNCaP H | LNCaP holoclone |
| mM | Milimolar |
| mins | Minutes |
| mRNA | Messanger RNA |
| miRNA | MicroRNA |
| $MgCl_2$ | Magnesium chloride |
| NaCl | Sodium chloride |
| Nanog | Tir na nOg |
| NCAM1 | Neural cell adhesion molecule1 |
| ng | Nanogram |
| nt | Nucleotides |
| NTC | Non-template control |
| Oct4 | Octamer-binding transcription factor |
| OD | Optical density |
| PC-3 H | PC-3 holoclone |
| PIN | Prostatic intra-epithelial neoplasia |
| PM | Perfect match |
| PSA | Prostate Specific Antigen |
| RA | Retinoic Acid |
| RISC | RNA-induced silencing complex |
| RLE | Relative log expression |

| | |
|---|---|
| RMA | Robust Multi-array Analysis |
| RNA | Ribonucleic Acid |
| rpm | Revolutions per minute |
| RPMI | Royal Park Memorial Institute (culture medium) |
| RT | Reverse Transcription |
| PBS | Phosphate buffered saline |
| PCR | Polymerase Chain Reaction |
| PreAmp | Preamplification |
| Pre-miRNA | Precursor miRNA |
| Pri-miRNA | Primary miRNA |
| TE | Tris-EDTA |
| TGF-β | Transforming growth factor beta |
| TGF-β-R1 | Transforming growth factor beta-receptor 1 |
| TGF-β-R2 | Transforming growth factor beta-receptor 2 |
| TLDAs | TaqMan Low Density Arrays |
| TRIS | Tris(hydroxymethyl)methylamine |
| UTR | Untranslated region |
| UV | Ultra Violet |
| V | Version |
| WT | Whole Transcript |
| µg | Microgram |
| µl | Microliter |
| β-ME | β-Mercaptoethanol |
| 3’ | three prime |
| 5’ | five prime |

**Publications, Abstracts and Poster Presentations**

**Y Salley**, M Gallagher, S Elbaruni, C Spillane, P Symth, C Martin, W Watson, O Sheils J O'Leary Genome Expression Profiling of Holoclones Derived from Prostate Cancer. United States and Canadian Annual Pathology Conference 2012 (Vancouver, BC, Canada). **(A,P)**

**Y Salley**, M Gallagher, P Symth, C Martin, O Sheils J O'Leary. Expression profiling of holoclone and non-holoclone derived cell lineage in prostate cancer. United States and Canadian Annual Pathology Conference 2011 (San Antonio, USA). **(A,P)**

**Y Salley,** M Gallagher, P Smyth, S Elbaruni, C Martin, O Sheils J O'Leary. Holoclone and Non-Holoclone Derived Cell Lineage Characterisation Analysis in Prostate Cancer. United States and Canadian Annual Pathology Conference 2010 (Washington, USA). **(A,P)**

Michael F Gallagher, Richard J Flavin, Salah A Elbaruni, Jamie K Mc Inerney, Paul C Smyth, **Yvonne M Salley**, Sebastian F Vencken, Sharon A O'Toole, Alexandros Laois, Mathia YC Lee, Karen Denning, Jinghuan Li, Sinead T Aherne, Kai Q Lao, Cara M Martin, Orla M Sheils and John J O'Leary. 2010. Regulation of MicroRNA Biosynthesis and Expression in 2102Ep Embryonal Carcinoma Stem Cells is Mirrored in Ovarian Serous Adenocarcinoma Patients. *Journal of Ovarian Cancer.* **(J).**

**Y Salley**, M Gallagher, P Symth, C Martin, O Sheils J O'Leary. Holoclone and non-holoclone derived cell lineage miRNA analysis in prostate cancer. United States and Canadian Annual Pathology Conference 2009 (Boston, Massachusetts, USA). **(A,P)**

**Y Salley,** M Gallagher, P Smyth, C Martin, O Sheils, J O'Leary. Holoclone and non-holoclone derived cell lineage miRNA analysis in prostate cancer. 7$^{th}$ International Cancer Conference 2009 (Dublin, Ireland). **(A,P)**

**Y Salley**, M Gallagher, P Smyth, C Martin, O Sheils, J O'Leary. Holoclone and non-holoclone derived cell lineage miRNA analysis in prostate cancer. Coombe Symposium 2008 (Dublin, Ireland). **(A,P)**

**Abstract**

Prostate cancer is a heterogeneous disease and is the most commonly diagnosed malignant tumour and the second most common cause of cancer deaths in western males. Prostate cancer is the most commonly diagnosed cancer in Irish males. Stem-like cells have been identified in several malignancies including prostate cancer and are thought to drive primary tumourgenesis through self-renewal and differentiation. Additionally, persistence of stem cells post-therapeutic intervention has been proposed as an explanation for metastasis and recurrence. Holoclones are a tightly packed clone of small cells generally thought to contain stem cells and progenitors. The aim of this study was to generate an expression profile of holoclone derived cell lineage in prostate cancer.

A gene expression profile of the cancer stem cell like holoclones was generated. The analysis characterized the holoclones and also identified specific cellular targets representing stemness, differentiation and epithelial to mesenchymal transition in the holoclones in comparison to the relevant founder cell lines. miRNA populations have been identified and bioinformatically analysed in both holoclones. The analysis indicated that the holoclone population of cells could potentially play a major role in driving tumourgenesis and recurrence and this thesis has identified potentially important cancer stem cell or stem-like, holoclone specific miRNA populations.

In this thesis, a number of significant molecular aberrations have been identified at mRNA and miRNA expression level, in both holoclones and their relevant founder cells. These genetic changes may be potentially useful biomarkers in the screening of patients at risk for prostate cancer and may represent potential markers of disease progression and recurrence for future use in prostate cancer screening.

# Introduction

Chapter 1

## 1.1 Prostate cancer

Prostate cancer is the most commonly diagnosed malignant tumour and the second most common cause of cancer deaths in western males. Prostate cancer is the most commonly diagnosed cancer in Irish males (National Cancer Registry Ireland, 2010). When non-melanoma skin cancer is excluded prostate cancer accounts for 23% of all new cancers in Irish men. During 1994 to 2003, the incidence of prostate cancer rose faster than that of any other cancer. Rates increased by an average of 7.1% annually, however this increase has largely been due to the frequency of prostate specific antigen testing in Ireland over this time period (Drummond *et al.*, 2010). Prostate cancer is predominantly a disease of older aged men, and less than 1% of cases are in those aged 50 and below, however 90% of cases occur in those aged 70 and over and just over 20% of these are in men aged 80 years and older (Drummond *et al.*, 2009).

Localised cases can be treated with radiation therapy and surgery, however many patients relapse with resistance and develop metastatic disease. Currently there is no successful treatment for progressive hormone-refractory metastatic prostate cancer (Miki and Rhim., 2007). The prostate gland is the most common site for male cancer to occur (Jemal *et al.*, 2003). Prostate cancer is a heterogeneous disease. The molecular pathology of prostate cancer involves various genes in pathogenesis and also environmental factors. Prostate cancer is epidemiologically divided into hereditary and sporadic forms, however this is not distinguishable at the molecular level (Hughes *et al.*, 2005).

Prostate cancer epidemiology can be divided into two forms: hereditary and sporadic. At the molecular level it is not possible to identify the difference. As this disease is more common among older men, the incidence rate is expected to increase as the population ages. The main characteristic of prostate tumours is that they are much slower growing than most other tumours, however the actual aggressiveness of these tumours varies greatly (Chodak *et al.*, 1994).

### 1.1.1 Molecular pathology of Prostate Cancer

The molecular pathology of prostate cancer involves multiple genes and environmental factors such as diet and inflammation. Prostate cancer can be divided into two forms termed sporadic and hereditary, however they cannot be identified at the molecular level (Hughes *et al.*, 2007). The heterogeneous nature of prostate cancer exists at various levels such as; anatomical, histopathological and genetic. At the genetic level, the genetic lesions are not consistent even within a single area of the tumour where allelic losses appear random, involving losses of both the maternally and paternally inherited chromosomes (Maitlans and Collins. 2005).

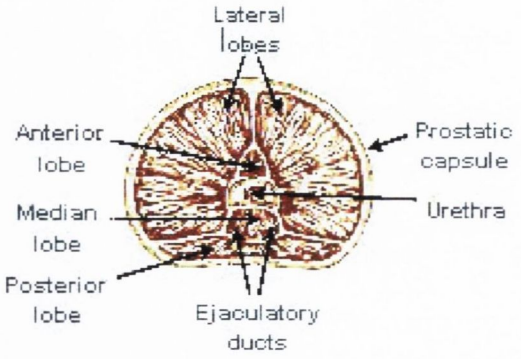
Gleason grading is currently the best histopathological examination to detect prostate cancer however, this method is highly variable. Often biopsies may not correlate with the prostatectomy sample and morphologically identical prostate cancer can act differently (Hughes *et al.*, 2007). The expression and function of many genes have been shown in various studies to be altered in all types of prostate cancer. The alterations of these genes are involved in cell cycle regulation and gene expression and also steroid hormone metabolism (Foley *et al.*, 2004). The consequences of heterogeneity cause much uncertainty in the diagnosis of prostate cancer and in determining the prognosis and treatment decisions. However the study of prostate cancer at the molecular could possibly eliminate much uncertainty currently associated with prostate cancer.

### 1.1.2 Anatomy of the normal human prostate

The prostate gland develops due to stimulation of the hormone testosterone and is a tubule-alveolar gland comprised of an epithelial parenchyma that is embedded in a matrix of connective tissue. Epithelial cells are contained within glands and branch out from the urethra. The cell types contained within a normal mature prostatic epithelium are basal, secretory, luminal and neuroendocrine cells. Anatomically, the prostate gland is divided into "lobe" regions (Meyers., 2000). These regions consist of the anterior / isthmus lobe, posterior lobe, lateral lobe and the median lobe.

The prostate gland is classified into zones first described by McNeal in 1968 and is comprised of four main glandular regions with two of these regions arising from different sections of the prostatic urethra. The four glandular regions are the peripheral zone (posterior lobe), central zone (lateral lobe), transitional zone (anterior lobe) and anterior fibro-muscular / stroma zone. The peripheral zone is the sub-capsular segment of the posterior of the prostate gland and surrounds the urethra. It is from this zone of the gland that almost 80% of prostate carcinomas arise. The central zone surrounds the ejaculatory ducts and approximately 2.5% of prostate carcinomas arise from this zone. However these rare carcinomas tend to be very aggressive and are likely to invade the seminal vesicles (Cohen *et al.*, 2008). The transition zone surrounds the proximal urethra and it is this section of the gland that expands throughout life and causes benigh prostatic hyperplasia (BPH). It is thought around 10-20% of carcinomas arise in this zone. The anterior fibro-muscular / stroma zone is comprised of fibrous and muscle tissue and is usually void of any glandular components.

(A)

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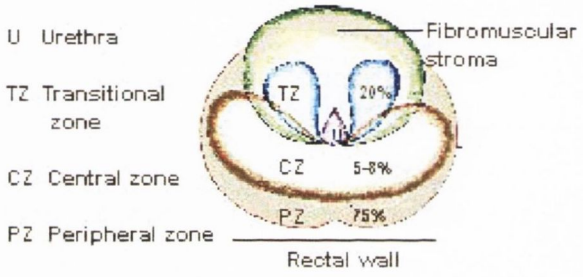


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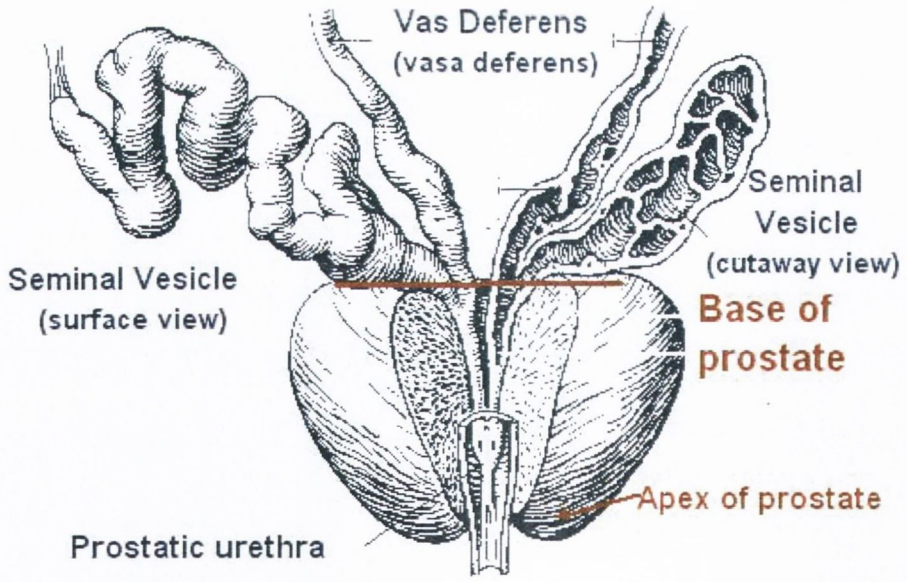



**Figure 1.1: Anatomy of the prostate.** The prostate refers to the gland of the male reproductive system. The size of the prostate is that of a walnut and is conical in shape. **(A)** Lobes of the prostate **(B)** Zones of the prostate **(C)** The base of the prostate is directed upwards and is located close to the inferior surface of the bladder. The apex is directed downwards and makes contact with the superior fascia of the urogenital diaphragm. (National Cancer Institute, www.cancer.gov).

### 1.2 Prostatitis

Prostatitis is described as inflammation of the prostate gland tissue. Prostate biopsies that show levels of inflammation of the prostate are very common in the aging male population. Inflammation is usually associated with the body's response to infection, however is also occurs in the absence of infection. Many studies have shown inflammation of the prostate may increase the chance of developing prostate cancer. Sexually transmitted infections such as; Chlamydia, gonorrhoea and syphilis appear to increase the risk (Dennis *et al.*, 2002).

#### 1.2.1 Prostatic intra-epithelial neoplasia (PIN)

PIN is dysplasia of the lining of the epithelium of the prostate gland and can proceed to prostatic carcinoma as age advances. However it does not invade the surrounding tissue. PIN can remain unchanged for some time, but can also slowly advance to prostate carcinoma over several years. PIN is associated with cells of the epithelium that are irregular and atypically proliferate causing a pseudo multilayer of cells (Bostwick and Qian., 2004). The basal cell layer in PIN is disrupted but still present; this is not the case with prostatic carcinoma. PIN is usually divided or grouped into stages referred to as Low Grade PIN and High Grade PIN. High Grade PIN is thought to be a pre-malignant state and does not require any therapy but is usually monitored with repeat biopsies (Bostwick and Qian., 2004).

### 1.2.2 Benign Prostatic Hyperplasia (BPH)

Hyperplasia refers to the proliferation of cells within an organ or tissue beyond the normal levels of proliferation (Foster., 2000). Hyperplasia can result in the enlargement of the affected organ and can be associated with neoplasia or a benign tumour. The cell morphology appears normal however there is an increase in the number of cells present. BPH is the enlargement of the prostate and is also known as benign prostatic hypertrophy which is the enlargement of the prostate gland, characterised by the proliferation of the cellular elements of the prostate. Prostate growth is hormonally regulated (Page *et al.*, 2006). The incidence of BPH increases with age (Barry *et al.*, 1992) and is also related to ethnicity. In the West, African American men have an earlier onset than Caucasian men.

BPH occurs in over 70% of men aged 70 or older and most cases are symptomatic. Prostatic hyperplasia begins centrally and compresses the urethra and obstructs urinary flow. Stromal tissue is also involved in the obstruction. The stroma to epithelium ratio is 2:7 however in cases where obstruction is present the ratio can be 4:6. This indicates that the stroma makes a significant contribution to the process. The peripheral prostate is compressed by hyperplasia paraurethral into a capsule shape, the consequence of which is urinary obstruction (Foster., 2000).

### 1.2.3 Adenocarcinoma of the prostate

Almost all prostate cancers are adenocarcinomas and they tend to arise from the outer peripheral zone of the prostate and can be multifocal. It is estimated that approximately 80% of prostate cancer cases are adenocarcinomas (Yin *et al.*, 2007). Adenocarcinomas arise in the epithelial cells; prostate cancer develops in the lining cells of the ducts and glands which secrets seminal fluid. If these malignant cells are contained within the individual ducts and glands they are considered to be high grade PIN. However if these cells invade through the surrounding basement membrane is now defined as adenocarcinoma.

Prostatic adenocarcinomas are made up of small glands that are closely packed together with little or no intervening stroma. Cytologic features of adenocarcinoma include enlarged round, hyperchromatic nuclei that have one single prominent nucleolus. Carcinomas that are less differentiated have fused glands called cribriform glands, including solid nests or sheets of tumor cells, and many tumors have two or more of these particular patterns, see Figure 1.2. Prostatic adenocarcinomas almost always arise in the posterior outer zone of the prostate (Pearson *et al.*, 1996). The PC-3 cell line employed in this study represents a Grade 4 adenocarcinoma.

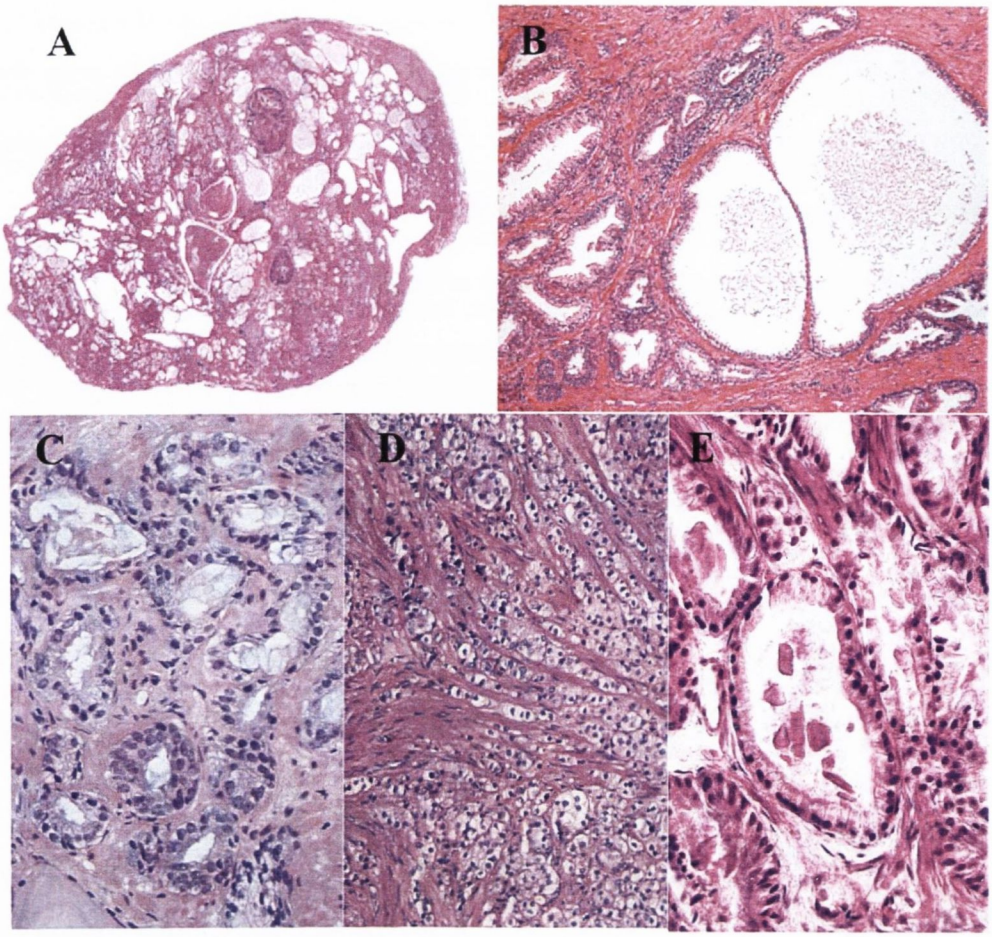



**Figure 1.2: Pathology of prostate cancer. A.** Whole Mount of Nodular hyperplasia of prostate, showing nodular configuration and cystic changes **B.** BPH showing cystic dilation of the glands. Characteristically, the epithelium is tall on one side and flattened on the other **C.** Microscopic appearance of prostatic carcinoma. Well differentiated tumour composed of medium sized glands. Note the irregular shape of the glands and presence of intraluminal basophilic secretion. **D.** Poorly Differentiated tumour growing in a diffuse pattern. **E.** Well differentiated prostatic adenocarcinoma showing intraluminal crystalloids (Rosai and Ackerman., 2004)

### 1.2.4 Metastatic prostate cancer

In prostate cancer, the first site of metastasis is in bone, and is an osteoblastic process, (bone forming). (Logothetis and Lin., 2005). Metastasis uses the process of angiogenesis, where by new blood vessels form and malignant cells are supported (facilating metastasis) and therefore spread to other parts of the body. Firstly, the prostate cancer cells localise at the site of the bone and progression occurs from this point. These malignant cells spread to the bone and or the lymph nodes and then divide uncontrollably. The LNCaP cell line employed in this study represents a metastatic prostate cancer.

A solid tumour's growth is limited by its blood supply. However, to grow beyond a certain size, the tumour must allow for the formation of a network of capillaries. This enables the tumour to invade and expand. Angiogenesis supports the invasion and spread of the tumour. It is also controlled by the balance between positive and negative regulators of microvascular growth (Folkman. 1995). Tumour-associated angiogenesis allows for prostate cancer progression and increased tumour production. Angiogenic factors involved in prostate cancer include; transforming growth factor-alpha and vascular endothelial growth factor (Harper *et al.*, 1996).

Prostate cancer most commonly metastasises to the bones, lymph nodes, and may also invade the rectum, bladder and the lower ureters. Bone metastases cause the greater cancer morbidity and mortality (Norgaard *et al.*, 2010). The ability of circulating prostate cancer cells to establish a metastatic centre involving mutual interactions between the malignant cells and the bone stroma is hugely important in metastasis. It is the extracellular matrix of the bone that supports cellular adhesion and could possibly

contribute to the skeletal localisation of prostate metastases. Cellular adhesion of the extracellular matrix causes an increase in androgen sensitivity and also an increase in androgen responsive gene expression. Adhesion to the extracellular matrix causes an androgen independent expression of some androgen responsive homeobox genes (Robbins *et al.*, 1996). This indicates that extracellular matrix induced gene expression could contribute to androgen independent growth.

Prostate cancer is the only malignancy that produces largely osteoblastic metastases. Prostate cancer cells produce many growth factors that possibly account for the osteoblastic lesions, such as bone morphogenic proteins and endothelin-1. Studies indicate increased endothelin-1 activity could possibly mediate the osteoblastic response of bone to metastatic lesions and possibly drive prostate cancer progression by an autocrine mechanism (Nelson *et al.*, 1996).

### 1.2.5 PC-3 and LNCaP cell models

The LNCaP cell line was chosen as a suitable model because it is androgen-sensitive and has been established from prostate adenocarcinoma cells that are derived from the left supraclavicular lymph node metastasis from a 50 year old, Caucasian male in 1977 (Horoszewicz., 2006). They are loosely adherent epithelial cells that grow in aggregates and also as single cells. These cells attach lightly, acidify the medium in which they are grown, are slow growing and do not become confluent however they tend to cluster instead of forming a monolayer (Horoszewicz., 1983). Highly sensitive androgen receptors are present in the cytosol of LNCaP cells both in culture and in tumors. This makes LNCaP a highly androgen-sensitive cell line and suitable cell model.

In contrast the the PC-3 cell line was chosen as it is an advanced androgen independent bone metastasis of prostate cancer of a Grade 4 prostate adenocarcinoma from a 62 year old, Caucasian male. The PC-3 cells have a very high metastatic potential when compared to the cell line DU145 for example, these cells have a moderate metastatic potential (Alimirah *et al.*, 2006, Pulukari *et al.*, 2005). The characteristics of these cells exhibit low acid phosphatise and testosterone-5-alpha reductase activities (Kaighn *et al.*, 1979). In general, the functional and morphologic characteristics of PC-3 cells are those of a poorly-differentiated Grade 4 adenocarcinoma.

### 1.3 Methods used to diagnose prostate cancer: Gleason Score

The Gleason score is a common method used to grade prostate cancer tissue and is helpful in classifying the stage of cancer. The system is based on the observing the pattern of the glands of the prostate tumour. It evaluates the cells of the cancer in the glands and compares the resemblance of the normal prostate. Gleason grading starts from well differentiated (Grade 1) to extremely poorly differentiated (Grade 5). The most common pattern of cell differentiation is given a Gleason number (1 through to 5). Then the second most common pattern of differentiation is assigned a second number (1 through to 5) and the sum of these two numbers is the Gleason score. More aggressive types of prostate cancer can have scores of 8, 9 or10.

However the Gleason score is always displayed with both individual scores and the final score. This is because both individual scores are as important as the final score. For example a Gleason score of 5+4=9 is a higher score than a Gleason score of 4+5=9 as the first score is the most prevalent or common pattern of differentiation. This method allows for better understanding of each individual prostate cancer case.

Grade 1: Cells are similar to the normal prostate cells; malignant cells closely redembles normal cells and they grow close together. Grade 2: Tissue samples are similar to grade 1 however the abnormal growth that is present is less compact and cells may have started to invade muscle tissue in the surrounding area. Grade 3: The colour of the normal tissue is darker and the cells shape or morphology is different and more invasion of the muscular tissue than grade 2. Grade 4: Cell morphology becomes increasingly abnormal. Grade 5: Malignant growth shows many different patterns at the cellular level (See Figure: 1.3).

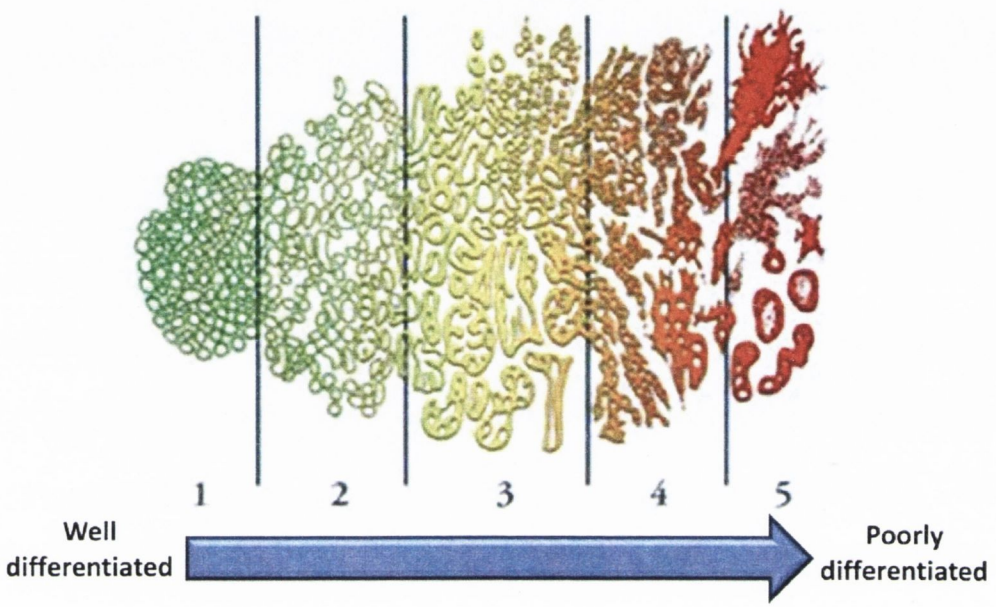



**Figure 1.3: Illustration of Dr. Gleason's conceptual diagram of the five grades of prostate cancer.** Histological grading and clinical staging of prostatic carcinoma drawing from left to right of deteriorating cancer cell architecture; **(1)** Grade 1; small uniform glands and cells are well differentiated, **(2)** Grade 2; more space between glands, **(3)** Grade 3; infiltration of cells from glands at the margins, **(4)** Grade 4; irregular masses of cells with very few glands and **(5)** Grade 5; visible lack of glands and sheets of cells, very poorly differentiated (Gleason, 1977).

### 1.4 Prostatic epithelial stem cells

The prostatic epithelium is comprised of two morphologically different layers, the basal layer and the luminal layer. It is also composed of different types of cells; the basal, secretory and neuroendocrine cells. It is the luminal secretory cells that express the androgen receptor (AR), prostate specific antigen (PSA), CD57 / human natural killer-1 (HNK1), prostatic acid phosphatase, cytokeratins 8 and 18 (Miki and Rhim., 2007). Basal cells are non-secretory and demonstrate CD44, p63 and keratin 5 and 14 (Signoretti *et al.*, 2000). The secretory cells are androgen dependent however the basal cells are androgen independent. Some cells can display a keratin pattern intermediate between the basal and luminal cells and express K5, K8 and K18 only.

Normal prostate stem cells have been shown to exist in the basal layer. Studies have shown that androgen independent stem cells give rise to stem cells and androgen independent "transit amplifying" cells (Collins *et al.*, 2001). These cells can divide rapidly and have proliferative potential and can also differentiate into luminal cells. Neuroendocrine cells are dispersed through the basal and luminal layers and lack AR and PSA, however they do express peptide hormones and chromogranin A. Neuroendocrine cells are thought to arise from dendritic cells and have an expression pattern of basal and luminal markers. Their main role is in growth and differentiation however their precise function remains unknown (Miki and Rhim., 2007).

## 1.5 Cancer Stem cells

Recently it has emerged that solid tumours, tissues and cancer cell lines contain small populations of cancer stem cells. Tumour stem cells are thought to give rise to a clone or clones of cells that are differentiated and maintain a high survival level compared to the other cells contained in the tumour (Figure 1.4). The cancer stem cell paradigm indicates that these differentiated cells will not allow for successful treatment of the tumour and allows for recurrence and metastasis. Therefore the cancer stem cell phenotype also needs to be identified, targeted and treated. Currently these prostate cancer stem cells remain largely uncharacterised and therefore there are no effective treatments to target these stem cells have been developed (Rizzo *et al.*, 2005).

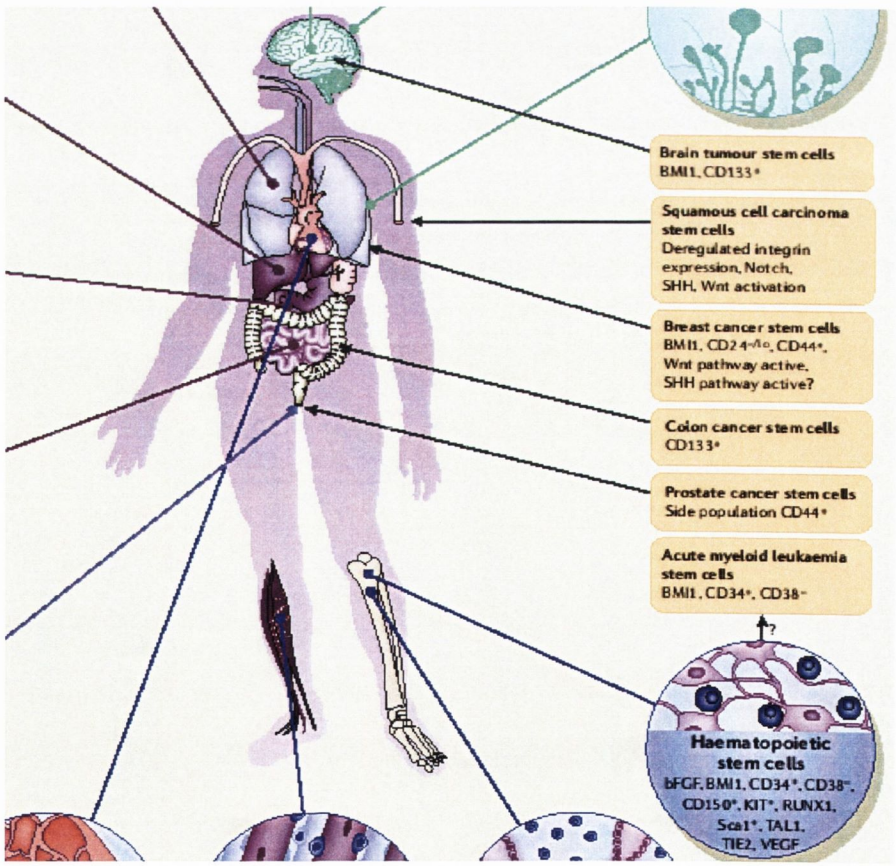



**Figure 1.4: Schematic of Embryonic stem cells, tissue stem cells and cancer stem cells** (www.naturereviewsmolecularcellbiology.com)

### 1.5.1 Prostate cancer stem cells/ progenitor cells

Somatic stem cells generate normal tissue and cancer stem cells generate tumour tissue. Normal tissue is created from stem cells that proliferate and differentiate in order to create progenitors and a mature population of cells (Miki and Rhim., 2007). The cancer stem cell theory suggests that tumours contain a small population of stem-like cells called cancer stem cells/ progenitor/ tumour initiating cells. Tumours may arise from the transformation of progenitor cells rather than stem cells. These cells have been implicated in the solid tumours, such as; breast, brain and prostate and are resistant to conventional chemotherapy or radiation therapy (See Figure 1.5).

Prostate epithelial stem cells can differentiate into three distinct lineages, these include; basal, luminal secretory and neuroendocrine. The basal cells express low molecular weight cytokeratins and support the luminal cells. The luminal cells express low molecular weight cytokeratins and produce prostatic secretions and PSA and are often regulated by androgens. It is the luminal cells that express the androgen receptor. The neuroendocrine cells produce neuropeptides (Kasper, 2008).

Progenitor cells or stem like cells tend to have the ability to differentiate into certain cell types. However stem cells are much more specific than progenitors. Stem cells are forced to differentiate into their target cell. The main significant difference between progenitor's cells and stem cells is that progenitors only divide a limited number of times however stem cells replicate indefinitely if necessary.

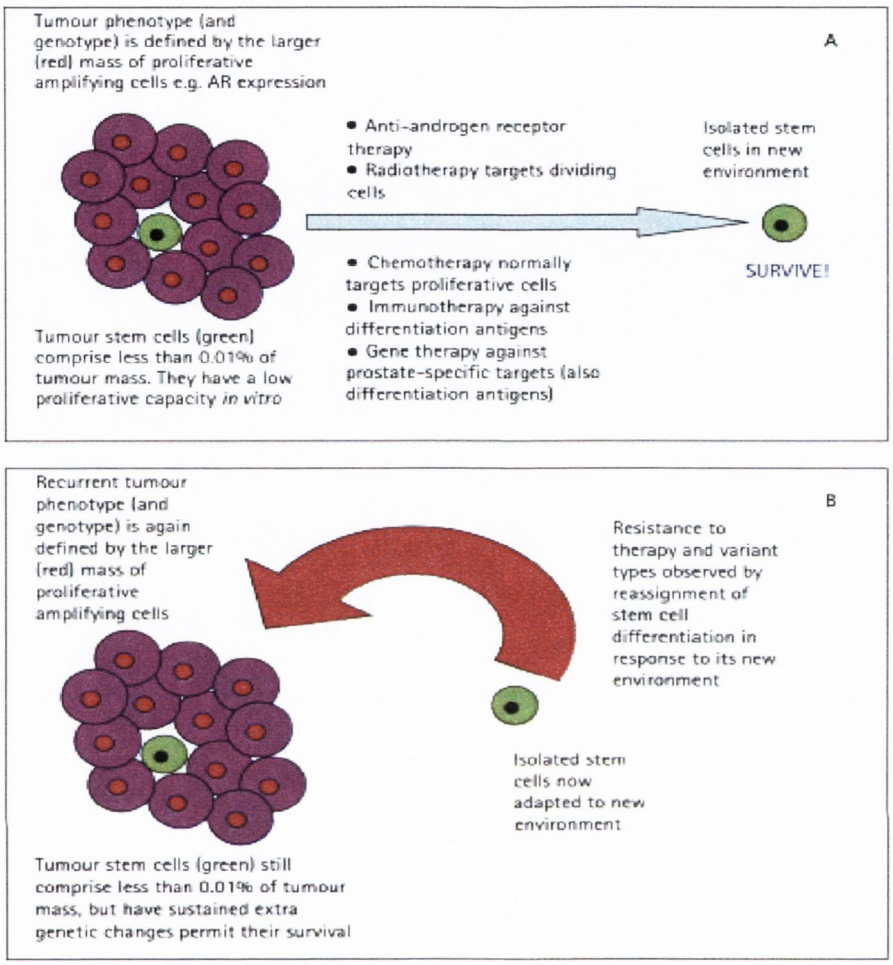



**Figure 1.5: Cancer stem cells and recurrence. A;** Residual stem cells persist after conventional anti-tumour therapies and treatments. **B,** The addition of new properties by the residual tumour stem cells results in re-growth or recurrence of the tumour (Maitland and Collins, 2008).

### 1.6 Holoclones, Paraclones and Meroclones

Prostate cancer stem cells or progenitors derived from cell lines using 3D cultures display three types of clonal morphologies known as holoclones (*holo* meaning entire), paraclones (*para* meaning beyond) and meroclones (*mero* meaning partial). Holoclones are a clone of cells that contain tightly packed small cells and have the greatest ability to replicative capacity with approximately 5% of the cell colonies aborting and terminally differentiating (Barrandon & Green, 1987). These tightly packed clones of cells generally contain both stem cells and progenitors (Li *et al.*, 2008). The holoclone is the only clonal cell type that has the ability to self-renew (Barrandon & Green, 1987).

Paraclones are a loosely packed clone of large cells with a very short replicative life span and after approximately 15 cell generations the paraclones abort and also terminally differentiate (Li *et al.*, 2008). However meroclones contain a combination of cells of different proliferative potential and is a transitional stage between a holoclone and paraclone (Li *et al.*, 2008). Holoclones have a high colony-forming efficiency, an extended life span and also have the ability to generate a more committed progeny such as paraclones and meroclones (See Figure 1.6). Both paraclones and meroclones have a very low colony-forming capacity (Larcher *et al.*, 2007). Within a cell line, only a small number of cells have the ability to generate holoclones however the majority of cells form paraclones and meroclones (Li *et al.*, 2008).

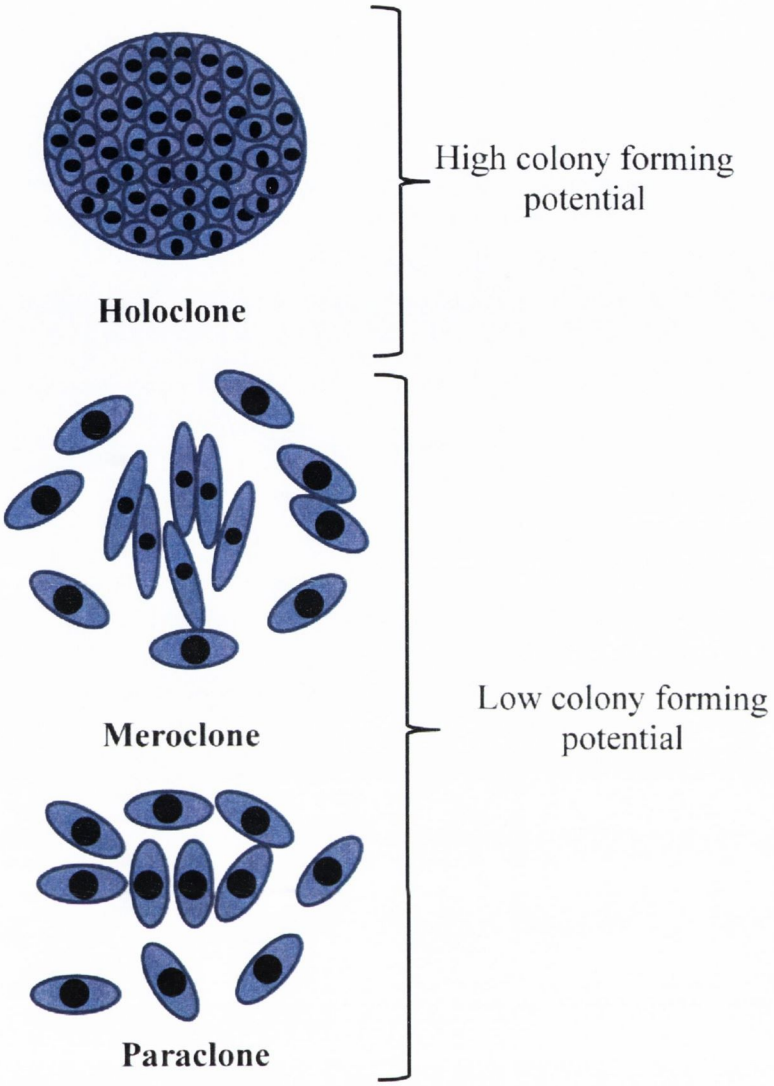



**Figure 1.6: Illustration of Holoclones, Meroclones and Paraclones.** Colony morphologies of holoclones, paraclones and meroclones derived from cell lines PC-3 and LNCap.

### 1.7 Markers of differentiation, stemness genes and pathways

Characterisation of prostate cancer derived holoclones and Retinoic Acid treated differentiated or super-differentiated cell lines were characterised using markers of stemness and differentiation (Hyslop *et al.*, 2005, Fong *et al.*, 2008). All stem cells have the ability to self-renew and differentiate (Gallagher *et al.*, 2009). Stem cells express markers indicative of the three germ layers; endoderm, ectoderm and mesoderm. The expression of differentiation, stemness genes and pathways were assessed by markers of differentiation, such as; NCam1 (ectoderm), ENO3 (mesoderm), AFP (endoderm). Self renewal was determined by genes representing pluripotency, such as Oct4 (Kehler *et al.*, 2004, Looijenga *et al.*, 2003) Sox2 and Nanog (Chambers *et al.*, 2005, Pereira *et al.*, 2006). Signalling pathways involved in regulation of the stemness processes include Hedgehog (Sonic Hedgehog) (Velcheti., 2007), Snail (Li *et al.*, 2006, Paznetas *et al.*, 1999), TGF-beta (TGF-beta-R2), Notch (Bolós *et al.*, 2007, Gaiano *et al.*, 2002) and Wnt (Wnt5a), (Logan and Nusse., 2004, Nusse., 2008).

#### 1.7.1 Neural cell adhesion molecule - Ectoderm germ layer marker

NCAM1 is a gene expression marker indicative of the ectoderm germ layer differentiation (Abeyta *et al.*, 2004). NCAM1 is also known as the cluster of differentiation CD56 or MSK39. It is a homophilic binding glycoprotein and is expressed on the surface of natural killer cells, neurons, glia and skeletal muscles. NCAM1 has been shown to be involved in learning and memory and cell-cell adhesion. It is expressed in neurons (Deak *et al.*, 2005). It has been shown to be expressed during the formation of the neuroectoderm.

### 1.7.2 Alpha-fetoprotein – Endoderm germ layer marker

AFP is a 65-KDa glycoprotein of 59 amino acids that is encoded by the AFP gene in humans (Irony-Tur-Sinai *et al.*, 2006). AFP is produced in the developing embryo and is found in foetal and maternal fluids during pregnancy. AFP is the most abundant serum protein and is secreted by the visceral endoderm (D'Amour *et al.*, 2005). AFP gene regulatory elements are thought to severely effect driver reporter gene expression in the developing tissues of the endoderm (Kwon *et al.*, 2006). AFP is expressed in the genome of mesenchymal stem cells. AFP has been associated with many different functions including anti-cancer active site peptide. Gastric tumours that produce AFP have poor prognosis and are associated with high incidences of liver metastasis and it is also expressed in both immature and mature teratomas (Hiroshima *et al.*, 2001). AFP is unregulated through a cascade that involves the Gata6 gene and thus was employed in this thesis as an endoderm differentiation marker.

### 1.7.3 Enolase 3 - Mesoderm germ layer marker

Enolase 3 is a gene expression marker indicative of the mesoderm germ layer of differentiation and is expressed in the adult muscle of humans. Beta-enolase is the enzyme which encodes the human ENO3 gene. The enzyme catalyses the inter-alterations of 2-phosphoglycerate and phosphoenolpyruvate. In adult muscle, over 90% of enolase activity is accounted for by the enzyme beta-enolase, which is the protein product of the ENO3 gene (Comi *et al.*, 2001). The muscle specific enolase gene contains 12 exons and spans 6Kb approximately; it also encodes a protein of 433 residues (Peshavaria and Day., 1991).

### 1.7.4 Notch Signaling Pathway

The Notch signaling pathway is a highly evolutionarily conserved intercellular signalling system present in all mammals. It plays important functions in cell-cell communication and is involved in gene regulation mechanisms that control multiple cell differentiation processes in both the embryo and the adult. The pathway is involved in neuronal function and development (Gaiano and Fishell., 2002). Notch signalling has been shown to be dysregulated in several cancers including malignancies such as leukemias and lymphomas and carcinomas of the skin, lungs, kidneys, cervix and breast. It is also implicated in many other diseases, such as, Multiple Sclerosis. Notch signalling inhibition has displayed anti-proliferative effects in cultured cell lines and also in animal mouse models (Nemir *et al.*, 2006, Cerdau *et al.*, 2010). Notch proteins are important in the maintenance of stem cells and lineage specification.

There are 4 Notch proteins; Notch1, Notch2, Notch3 and Notch4 and they are activated by membrane bound ligands such as; Delta-like 1-3 and Jagged/ Serrate families (Radtke and Raj., 2003). They are then transported to the membrane of the plasma as intact polypeptides. Ligand interactions cause two proteolytic cleavages that release the Notch intercellular domain from the plasma. The Notch intercellular domain translocates to the nucleus and a complex is formed with binding protein of DNA (CSL) and displaces a histone deacetylase co-repressor complex. Components of the activation complex are recruited to the Notch intracellular domain-CSL complex and therefore allowing for transcriptional activation of target genes of Notch (See Figure 1.7).

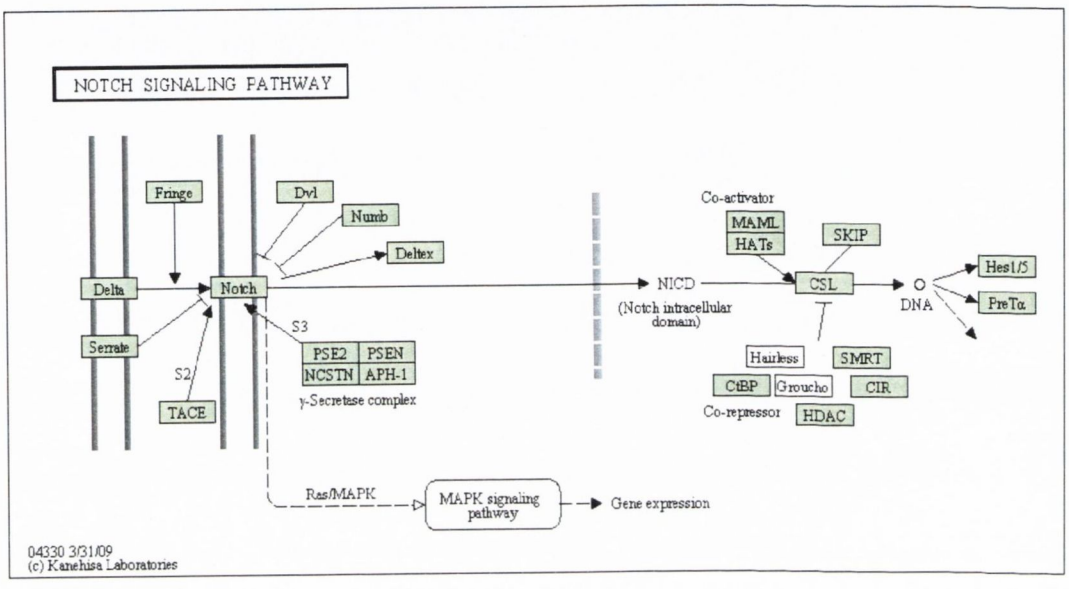



**Figure 1.7: Notch Signaling Pathway.** Notch receptors are expressed on the surface of the cell as heterodimeric proteins and the ligands are membrane bound. Signaling occurs through these receptors and is triggered by the binding of ligands which allows for the induction of cleavage within the transmembrane domain. The second cleavage allows for the translocation of the cytosolic domain of these receptors into the nucleus (www.genome.jp and www.netpath.org).

### 1.7.5 Wnt Signaling Pathway

The Wnt signalling pathway contains many proteins that are secreted morphogens and their main functions are involved mainly in embryogenesis and carcinoma (Lie *et al.*, 2005). Wnt is also a key pathway involved in stem cell development. Wnt is required for many basic developmental processes, such as; cell fate specification, stem-like or progenitor cell proliferation and controls asymmetric cell division (See Figure 1.8).

In the Canonical branch of the pathway, the Wnt ligand binding to its receptor results in the stabilisation of cytoplasmic beta-catenin through the inhibition of beta-catenin degradation complex. This allows for it to enter the nucleus and activate Wnt regulated genes through the interaction of the T-cell factor family of Transcription Factors and concomitant recruitment of co-activators. The Planar cell polarity branch of the pathway signals causing the activation of the RAS homologue gene family member A and RAC1 which are small GTPases. These then activate the stress kinase Jun N-terminal kinase. This allows for changes in cell adhesion and motility and also cytoskeleton remodelling. Signaling through the Wnt-$Ca^{2+}$ branch of the pathway is mediated through the G proteins such as Frizzled and phospholiases (Liu *et al.*, 2005, Wehrli *et al.*, 2000). This leads to an increase in cytoplasmic free calcium and in turn activates protein kinase C, calcium calmodulin mediated kinase 2 and phosphatise calcineurin.

Properties that define pluripotent stem cells are; the cells' ability to self-renew and differentiate (Nussei., 2008). Wnt signaling is significantly involved in stem cell differentiation. Transcription Factor 3 is regulated by the Wnt signaling pathway and inhibits Nanog another gene with a strong association with pluripotency which leads

to beta-catenin activity increasing significantly; also indicating Wnt involvement (Pereiva *et al.*, 2006). Wnt proteins play key roles in the development, maintenance and proliferation of stem and progenitor cell populations (Logan *et al.*, 2004). The Wnt pathway is believed to have a key involvement in the occurrence of cancer stem cells. The secreted Frizzled protein is a regulator of Wnt5 and when it binds to the secreted Frizzled protein activation of the Wnt pathway is prevented. This has been shown in breast and colorectal cancers (Beachy *et al.*, 2004).

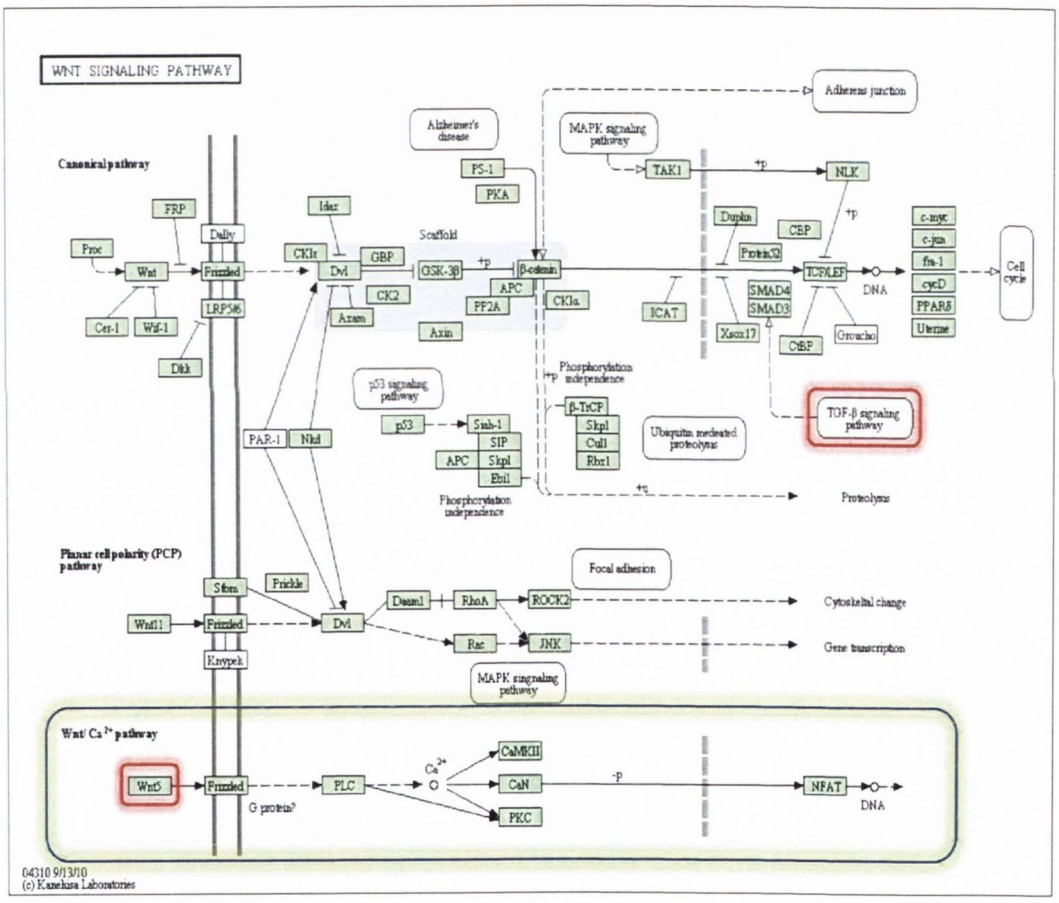



**Figure 1.8: Wnt Signaling Pathway.** The Wnt signalling pathway contains 3 different branches within the Wnt pathway these include; the Canonical pathway, the planar cell polarity pathwas and the Wnt/$Ca^{2+}$ pathway and contains Wnt5. Wnt5 is a signaling ligand that is received by Frizzled, a membrane bound protein. Resulting in activation of the downstream network of modulatory genes, that are not fully characterised (www.genome.jp)

### 1.7.6 Transforming growth factor beta pathway (TGF-β)

TGF-β family member includes TGF-betas, activins, and bone morphogenetic proteins and are also found in many different species. TGF-β family members regulate various cellular functions in both the adult and developing embryo such as differentiation, proliferation, apoptosis and migration. In normal epithelial cells, it is an anti-proliferative factor and also at the early stages of oncogenesis (Bhowmick *et al.*, 2004). TGF-β is a secreted protein that consists of three different isoforms; TGF-β1, TGF-β2 and TGF-β3.

Normally in cells, TGF-β stops the cell cycle at the G1 phase and in turn stops proliferation, induces differentiation or apoptosis through its signalling pathway. However in a cancer cell, TGF-β can no longer control the cell normally and the cancer cell starts to proliferate. Various parts of the pathway are mutated as the cell transforms from a normal cell to a cancer cell; thus the cell is immune to the effects of the TGF-β pathway.

The TGF-β family member binds to the Type II receptor and recruits Type I, in turn the Type II receptor phosphorylates and therefore causes activation of Type I. Type I receptor phosphorylates R-Smads (Smad1, Smad2, Smad3, Smad5 and Smad8) (Moustakas., 2002). After phosphorylation occurs, the R-Smads associate with Smad the co-mediator and the heteromeric complex translocates into the nucleus. Within the nucleus, Smad activates specific genes through interaction with DNA-binding and co-activator or co-repression proteins (See Figure 1.9).

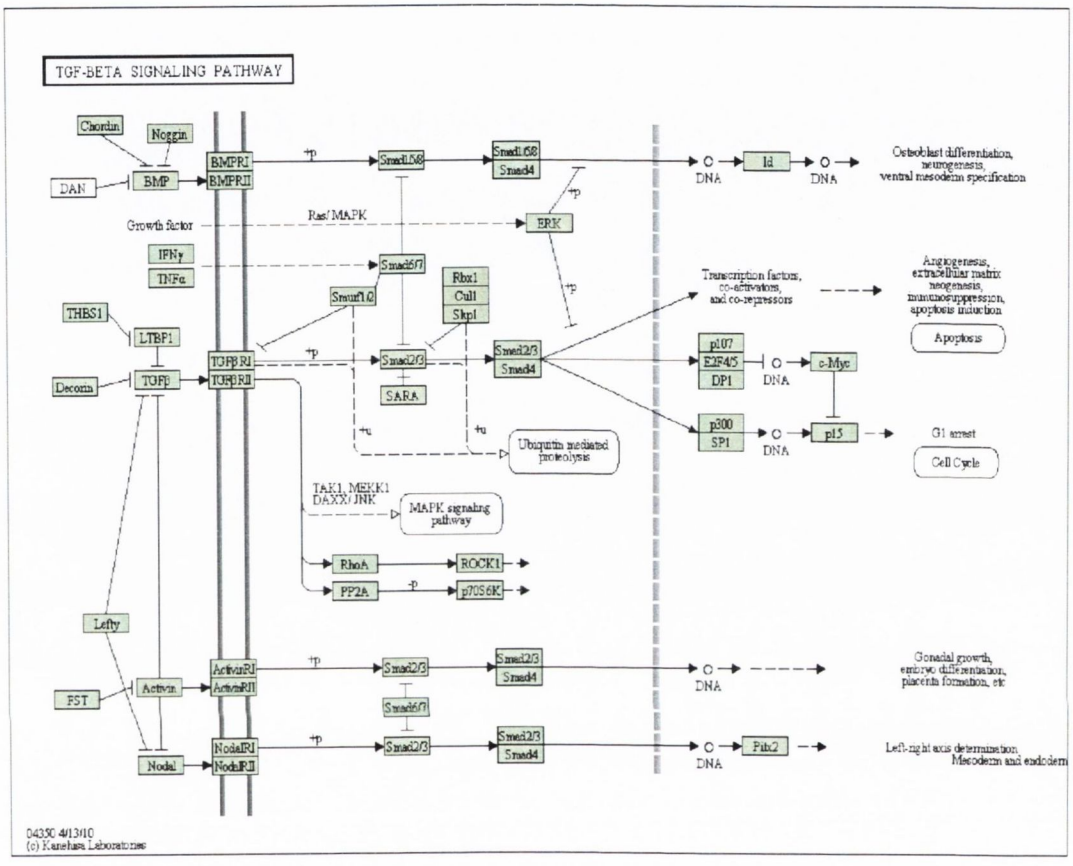



**Figure 1.9: TGF-β signalling pathway.** TGF-β signalling pathway is involved in many cellular processes and therefore mechanisms in this pathway are regulated positively and negatively such as receptor regulation and R-Smad regulation. (www.genome.jp)

### 1.7.7 Sonic Hedgehog and Hedgehog Signalling pathway

Sonic Hedgehog is a protein from the mammalian hedgehog signalling pathway. Of the hedgehog homologues (Desert and Indian hedgehog), SHH plays the most crucial role in development (Dorus *et al.,* 2006). SHH main roles are in the regulation of organogenesis including the growth of digits of limbs and brain organisation. SHH signalling molecules play various different roles in the patterning of the central nervous system during development. The hedgehog signaling pathway regulates processes involved in development and tissue homeostasis (Jiang *et al.,* 2008, Nüsslein-Volhard *et al.,* 1980). The activation of hedgehog signaling has been reported in approximately 30% of human cancers (Xie *et al.,* 2008).

The signaling pathway gives embryonic cells the information to ensure that the embryo develops correctly. SHH affects the cells of the developing embryo depending on the concentration levels being expressed. It also affects adult cells and is involved in processes such as, controlling cell division of adult stem cells and also in the development of various cancers in organs such as prostate, brain, skin, and mammary gland. Upon activation of the hedgehog pathway, the protein expression of Snail is increased, in turn E-cadherin and Tight Junctions are decreased (Li *et al.,* 2006). Hedgehog signalling is a major regulator of angiogenesis which in turn leads to metastasis (Velcheti., 2007). The hedgehog pathway is also involved in the regulations of tumours and the activation of this pathway also leads to an increase in the angiogenic factors angioprotein-1 and 2, cyclins D1 and B1 and anti-apoptoctic genes and a decrease in Fas which is an apoptotic gene (See Figure 1.10).

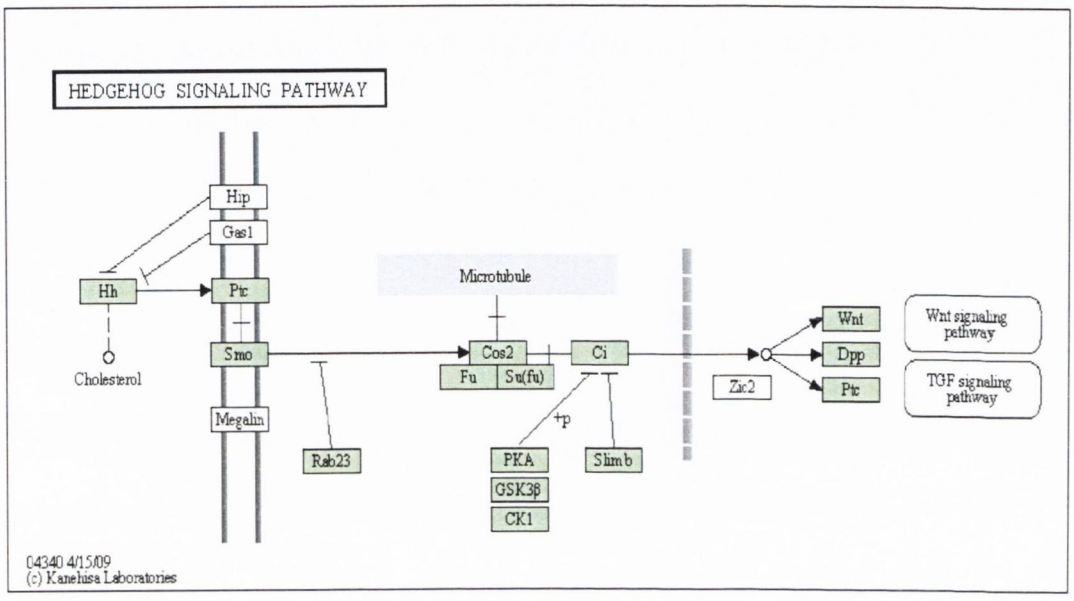



**Figure 1.10: Hedgehog Signaling Pathway.** Hedgehog family are a group of secreted signaling proteins that play a major role in development, regulate morphogenesis in many different tissues and organs. The hedgehog signaling pathway plays a major role in the control of proliferation of stem cell in adults and has been linked to the activation of various different types of cancer. (www.genome.jp).

### 1.7.8 Snail gene super-family

The Snail gene super-family of zinc-finger transcription factors are involved in development of vertebrate and invertebrate embryos and also in the progression of tumours and metastasis (Sefton *et al.,* 1998). The Snail genes encode DNA binding zinc-finger proteins and act as transcriptional repressors. A number of the zinc finger proteins are essential for the formation of mesodermal development and the neural crest. The Snail gene contains three exons and the Snail transcript is 2.0kb in length and is found in the lungs, brain, liver, skeletal muscle, adult heart and the placenta. It codes for a protein 264 amino acids and is 29.1 kDa in size (Paznekas *et al.*, 2002).

Snail expression in tumours has indicated high expression levels in sarcomas and fibro sarcomas (Francí *et al.,* 2006). Snail is highly expressed in patients with breast carcinoma and can result in poor outcome and is also associated with process of breast cancer recurrence (Moody *et al.,* 2005). Snail has been found to cause resistance to cell death which allows embryonic cells to migrate and colonise and also allows for malignant cells to separate from the primary tumour and invade, therefore leading to metastasis (Vega *et al.*, 2004). Epithelial cells that display Snail expression ectopically adapt a fibroblastoid phenotype and develop tumorigenic and invasive properties.

Snail genes are involved in regulating stem cell populations in Drosophila (fruit fly) and vertebrates. Wnt signalling pathway also regulates Snail1 expression. There are three genes in the Snail super-family Snail/Snai1, Snai2/Slug and Snai3 (Lomeli *et al.*, 2009).

### 1.7.9 Octamer-binding transcription factor 4 (Oct4)

Oct4 also known as POU5F1 is a protein that is encoded by the POU5F1 gene and is a homeodomain transcription factor belonging to the POU family (Takeda *et al.*, 1992). The Oct4 protein plays a crucial role in embryonic stem cells as its involved in self renewal of undifferentiated embryonic stem cells and is commonly used as a marker for undifferentiated cells. It can also cause the cells to differentiate if there is a lesser or greater amount present. Oct4 also plays an essential role in the maintance of pluripotency of cell of the inner mass in the embryo (Kehler *et al.*, 2004).

Oct4 is involved in the undifferentiated phenotype in various tumours. Once Oct4 is knocked down in cells it promotes the cell from the stage of cell renewal to a state of differentiation. Therefore Oct4 is critical in the regulation of pluripotency and cell differentiation at an early stage and prevents embryo differentiation. Oct4 has been found to be involved in the tumourgenesis of adult germ cells. It has been shown in mice that intestinal dysplasia causes an increase in progenitor cell population. This results in the upregulation of beta-catenin transcription through the inhibition of cellular differentiation (Hochedlinger *et al.*, 2005).

### 1.7.10 Tír na nÓg (Nanog)

Nanog is a homeobox protein that is encoded by the NANOG gene. The structure of the Nanog protein consists of a 305 amino acid protein that has a conserved homeodomain motif and is confined to the nuclear component of cells. It is the homeodomain region that allows for DNA binding. It is a transcription factor implicated with the self renewal of embryonic stem cells in the undifferentiated state (Chambers *et al.,* 2003). Nanog is one of three genes involved in maintaining pluripotency and expressed by embryonic stem cells. The other two genes are Oct4/POU5F1 and SOX2 which also establish embryonic stem cell identity (Takahashi and Yamanaka., 2006).

The over expression of Nanog in embryonic stem cells (human) allows for the propagation of numerous passages in culture in which the cells have remained pluripotent (Zaehres *et al.*, 2005). Studies have also shown that the knockdown of Nanog allows for the differentiation, suggesting its importance in self renewal of human embryonic stem cells (Zaehres *et al.*, 2005). Oct4 expression has been shown in mouse embryonic stem cells to induce pluripotency in SOX2 null cells. SOX2 plays a major role in induced pluripotent stem cells to control Oct4 expression (Looijenga *et al.,* 2003).

## 1.8 MicroRNAs

MiRNAs are a family of small RNA molecules and miRNA lin-4 was first discovered in the Nematode *C.elegans* in 1993 (Lee *et al.,* 1993). MiRNAs are short single stranded RNAs that are approximately 21-25nt in length. They direct messenger RNA degradation and can also disrupt mRNA translation (Tay *et al.*, 2008). MiRNAs are a group of regulatory molecules of non-coding RNAs that control protein synthesis. MiRNAs are expressed from the genomes of all multi-cellular organisms (Pillai *et al.,* 2006) and there are approximately 800 known miRNAs. However their precise function remains unknown, but they are thought to be involved in the regulation of cellular differentiation, proliferation and apoptosis. They are also thought to be involved or controlled by epigenetic alterations. MiRNAs are generated through a stepwise process that occurs in both the nucleus and cytoplasm, see Figure 1.11. MiRNAs have been shown to be coded for in both intron and exon regions of the genome.

Small RNAs are known to control gene expression and affect transcription at the mRNA level. The turnover of mature miRNA is required for rapid changes in miRNA expression profiles. MiRNAs bind to the 3' untranslated region of target genes with imperfect complementarity in order to prevent translation occurring. The key component of the RNA induced silencing complex known as RISC is an Argonaute protein and miRNAs are loaded onto the Ago protein. Ago proteins bind to the miRNA fragments and directly interact with the miRNA (Pillai *et al.,* 2004 and Behm-Ansmant *et al.,* 2006). One strand of miRNA becomes removed by the bypass mechanism allowing Ago to bind the target mRNA. It is this binding of Ago to the target mRNA that allows for translational inhibition (Bartel *et al.*, 2009). More recently data has

demonstrated that the miRNA loaded RISC can silence translation by targeting sites along the coding region of the mRNA strand complementary to the loaded miRNA (Tay *et al.*, 2008).

However, controversy remains over the actual precise processes involved in mRNA translation silencing by RISC after miRNA-mRNA alignment. One proposed theory is that by inhibiting translation before initiation is carried out by aggregating mRNAs in P bodies or possibly in a different place (Eulalio *et al.*, 2007). Another theory is that of the inhibition of translation by the RISC occurring at the polysomal level after initiation has taken place. A widespread trait of miRNA-mRNA hybridisation is the frequent imperfection of complementarity of the two sequences. This results in bulge formations which are thought to prevent RISC from cleaving the target mRNA. However in its place the target is preserved and therefore appears to be somewhat postponed for expression (Rana, 2007).

MicroRNAs are produced through a stepwise process which takes place in the nucleus and the cytoplasm, see Figure 1.14. miRNAs have also been found to code for in both intronic and exonic regions (Kim *et al.*, 2007). A pre or precursor hairpin miRNA is initally generated in the nucleus; this pre-miRNA is part of a longer primary transcript referred to as the pri-miRNA, which has a 5' 7-methyl guanosine cap and a 3' polyA tail. This is thought to occur through the action of RNA polymerase II (Zeng *et al.*, 2006). In the nucleus the pre-miRNA is generated through interaction with the microprocessor complex which contains the enzyme Drosha, an RNase III like enzyme and its co-factor DGCR8, a double stranded RNA binding protein. Drosha is comprised of 2 RNase II domains, of which one is thought to chop the 3' end of the pre-miRNA while the other cleaves the 5' strand (Han *et al.*, 2004).

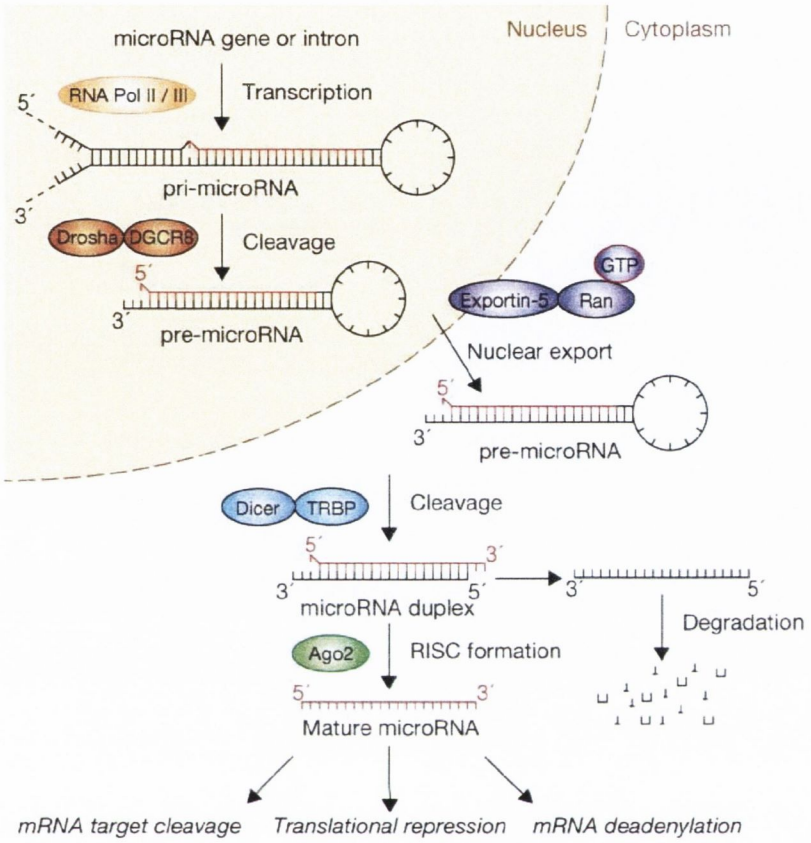



**Figure 1.11: MicroRNA processing pathway.** miRNA processing and activity occurring in the nucleus and the cytoplasm. Excision and activation of active single-stranded miRNAs from precursor transcripts occurs through a multi-step process. The process involves; transcription followed by the hairpin loop released in the nucleus, which is then exported from the nucleus into the cytoplasm, processing by the dicer enzyme and then strands selection by RISC which contains the enzyme Dicer (Winter *et al.*, 2009).

Various different isoforms or families of miRNAs have recently been identified. Mature miRNAs differ in 1-3 nucleotides and these miRNAs are designated using different letters or identical mature miRNAs are produced from different genes, generally located on different chromosomes and these miRNAs are identified by a specific number (Jiang *et al.*, 2005). Single nucleotide polymorphisms or SNPs in miRNAs either pre or mature. It should also be noted that miRNA targets could potentially play an important role in the determination of miRNA function.

### 1.8.1 miRNA processing machinery; Dicer, Drosha and eIF6

Both the production and function of miRNAs required precise processing by proteins of the miRNA machinery such as Dicer, Drosha and RNase III endonucleases as they are essential components of miRNA machinery. Recently eIF6 has been shown to play a role in miRNA mediated post-transcriptional silencing (Flavin *et al.*, 2008). The anti-association factor eIF6 and is a ribosome inhibitory protein which prevents the productive assembly of the 80S ribosome. It is recruited by the RISC complex and enables miRNA complexes to exert their repressive effects on the translation of protein (See Figure 1.12). The miRNA processing machinery of various prostate cancer cell models was examined in this thesis.

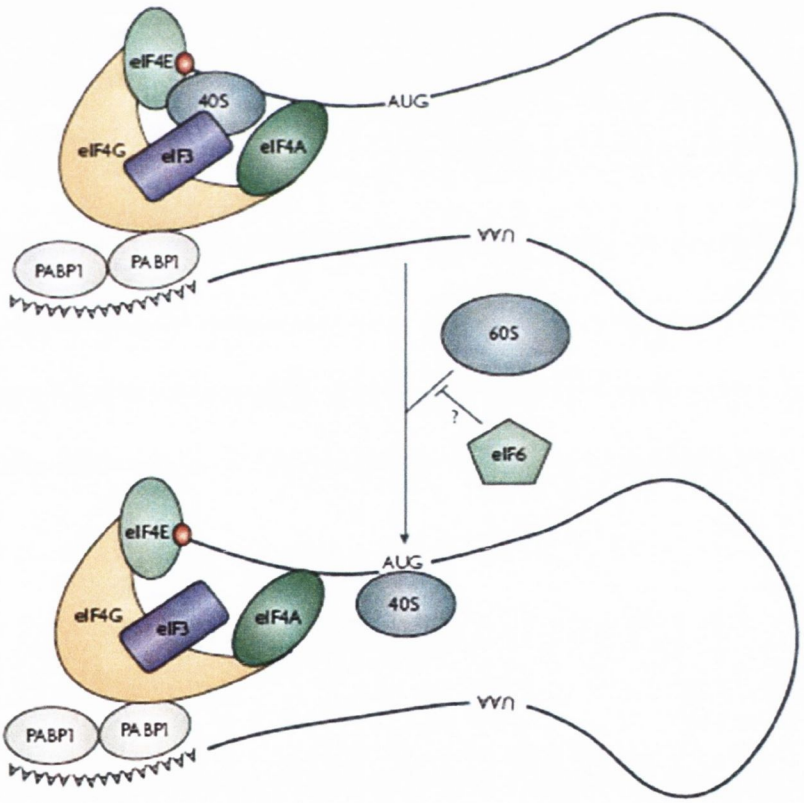



**Figure 1.12: Steps involved in eukaryotic translation.** Translation of mRNA comprises of three main steps; initation, elongation and termination. The ribosomal anti-association factor eIF6 has been found to have a role in miRNA mediated post transcriptional silencing. (Filipowicz *et al.*, 2008)

### 1.8.2 Role of miRNAs

Recently miRNAs have been shown to be crucial for the direction of cell fate, in regulation of development in order to control subtle or non essential regulatory pathways while others may help perfect and adjust the complex genetic network that builds a multicellular organism (Caldas and Brenton., 2005). The importance of miRNAs is becoming more apparent as they are involved in the regulation of numerous fundamental processes. miRNAs have been show to be involved in the regulation of many biological processes and malignancies. Many groups have demonstrated that they function in stem cell differentiation and maintenance; regulate cell cycle and inflammation in many cancers (Tay *et al.*, 2008, Wang *et al.*, 2007).

A role for miRNAs in the development and progression of human cancers has been recently been established. They have been shown to act as tumour suppressors or oncogenes and can be dysregulated through events including; deletion, mutations, amplification and epigenetic events (Caldas and Brenton., 2005). Errors in miRNA processing can occur in either the pri-miRNA sequence variations or problems in the miRNA processing machinery however these events are not fully understood.

### 1.8.3 MicroRNAs and cancer

MiRNAs are known to be involved in normal functioning of cells, however their deregulation is also associated with disease; specifically the relationship between miRNA deregulation and cancer, see Figure 1.13. Various miRNAs have been found to be linked with certain types of cancer (Mraz *et al.*, 2009). Many experiments involving transgenic mice which over express or lack certain miRNAs have proved they play a role in various malignancies (Zanesi *et al.*, 2010). Another study in mice which altered the production of c-Myc to excess showed that miRNAs have effects on the development of cancer. Mice that produced an excess of miRNA types found in lymphoma cells developed this disease within 50 days and died two weeks after this time point. However mice without the excess of this miRNA survived for over 100 days and did not develop this disease (He *et al.*, 2005).

In summary, miRNAs are currently thought to be mechanistically involved in the development of various malignancies and could potentially be a new class of biomarkers. Currently there is evidence for a link between miRNAs and cancer in that they are involved in cell proliferation and apoptosis. MiRNA genes are frequently located near fragile site and the deregulation of miRNA expression has been identified in many cell line models and also in malignant tumours.

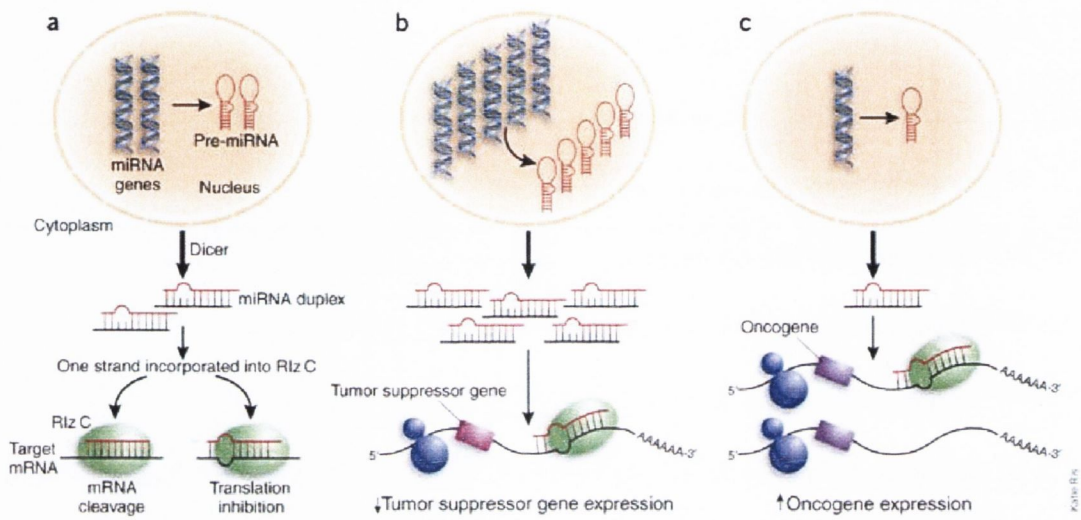



**Figure 1.13: miRNA involvement in cancer by the regulation of expression of oncogenes and tumour suppressor genes. A)** pri-miRNAs are processed into pre-miRNAs which takes place in the nucleus. Pre-miRNAs are exported out of the nucleus to the cytoplasm and are processed by Dicer to produce miRNAs. These miRNAs function by cleaving mRNA or inhibiting translation in concert with RISC. **B)** Overexpression of miRNAs could result in decrease expression of the target- tumor suppressor gene. **C)** Underexpression of miRNAs could result in increased expression of a target- oncogene (Caldas and Brenton., 2005).

### 1.8.4 Types of miRNAs involved in cancer

MiRNAs can act as either oncogenes or tumour suppressor genes. They are differential expressed as either upregulated and/or downregulated in both tumour progression and in metastasis (White *et al.,* 2011). Numerous different miRNAs are known to have effects on various different pathways that can contribute to metastasis such as; migration, invasion, cell proliferation, EMT, angiogenesis and apoptosis, see Figure 1.14. MiRNAs are defined as follows;

- **Oncomir:** is a miRNA that plays a crucial role in cancer and they can function as either oncogenes or tumour suppressors depending on the target.
- **Metastimir:** is a miRNA that is associated with metastasis and can have prometastatic and antimetastatic effets.
- **Apoptomir:** is a miRNA involved in apoptosis.
- **Hypoxamir:** is a miRNA involved in hypoxia.
- **Angiomir:** is a miRNA that can regulate angiogenesis, proangiomirs can promote angiogenesis where as angiangiomirs can inhibit angiogenesis.

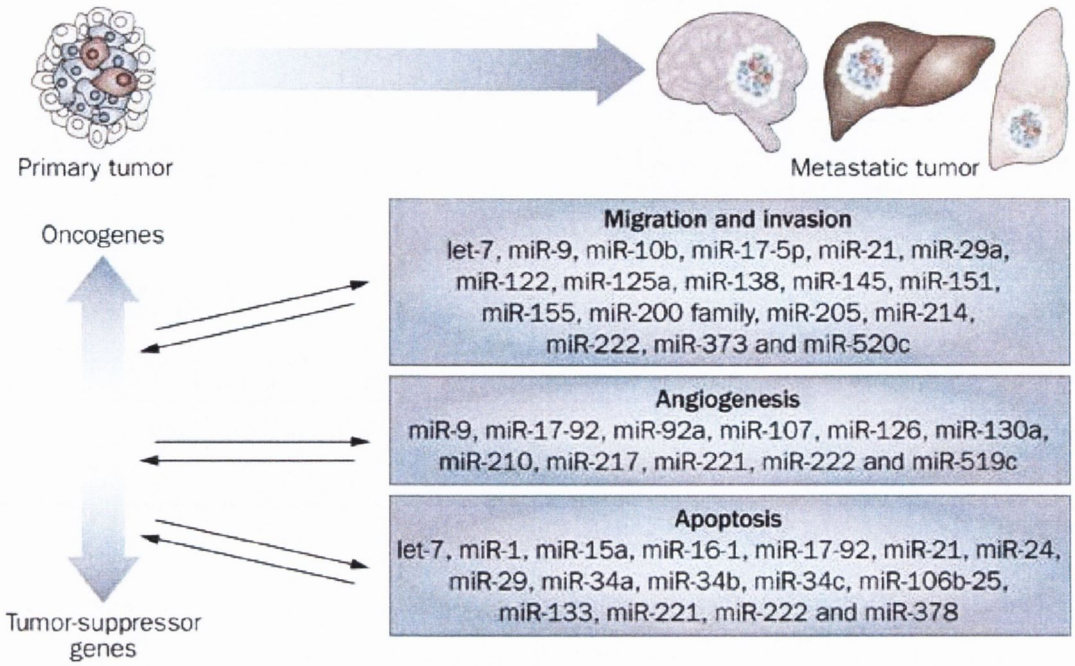



**Figure 1.14: Schematic of the effects of miRNAs on metastasis.** This model displays the interactions between miRNAs and both oncogenes and tumour suppressor genes. MiRNAs are also downstream targets of oncogenes and tumour suppressor genes. They can also directly target then causing migration, invasion, angiogenesis and apoptosis, thus contributing to the progression of the tumour and metastasis (White *et al.*, 2011).

## 1.9 Aims of this study

The initial aim of this project was to optimise and generate prostate cancer holoclones from the cell lines PC-3 and LNCaP. This was achieved by using a 3D culture method known as a high-salt soft-agar assay and analysing of key stemness genes and pathway expression. Cell lines PC-3 and LNCaP were treated with Retinoic Acid in order to generate "super-differentiated" cells. This allowed for the analysis of key stemness gene and pathway expression present in the holoclones and "super-differentiated" cells when compared to that of the resting cell lines and to examine the miRNA processing machinery which include the following; Drosha, Dicer and eIF-6.

The second aim was to genome profile PC-3 and LNCaP cell lines and PC-3 and LNCaP holoclones in order to characterise the holoclones to identify specific cellular targets representing stemness, differentiation and epithelial-mesenchymal transition. This specific aim included:

- Generation a genome wide expression profile of the holoclones.
- Determination of the differences between the holoclones and the resting cell lines.
- Identification of genes and pathways representing stemness in the holoclones.

The third aim was to compile a systemic review and meta-analysis of various different independent studies on miRNA expression profiling in prostate cancer, in cell line models and prostatectomy specimens. The miRNA expression of prostate cancer cell lines LNCaP and PC-3 and derived holoclones was investigated and from this a

miRNA profile for the PC-3 and LNCaP holoclones. From this profile prostate cancer holoclone stem cell specific miRNA populations could be identified.

The final aim of the project was to perform bioinformatic analysis on all generated data from this study. Putative gene targets and metabolic pathways were predicted in this study from various online databases.

# Materials and Methods

Chapter 2

## 2.0 Materials and methods

This chapter provides a full description of the materials and methodologies employed in this thesis. Background information is provided for some newer techniques described. A full background description of the technique appears in the relevant chapters only.

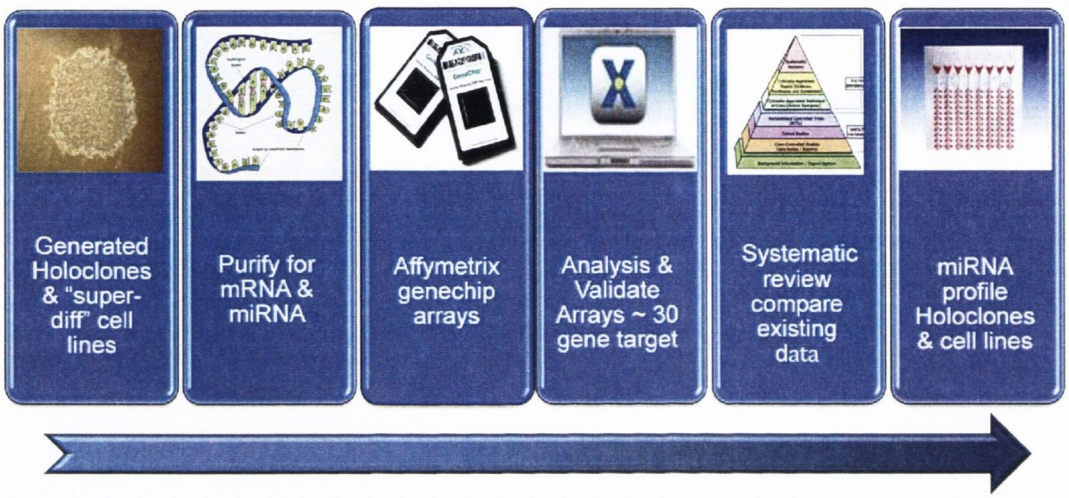



**Figure 2.1:** Schematic representing various steps employed in materials and methods chapter.

## 2.1 Systematic review of miRNA expression profiling on prostate cancer

### 2.1.1 Background: Generating a systematic review of miRNA of prostate cancer

A systemic review is a comprehensive method of combining the results of various different independent studies collected from previously published literature. Summaries and conclusions are drawn to evaluate effectiveness. Merging data from several different technical platforms, tumour sample types and cell line models (versus normal samples) allows for the investigation into specific miRNA profile patterns. This analysis distinguishes common miRNAs that may play a crucial role in prostate cancer. Systemic review was carried out on 6 original studies reporting on miRNA expression profiling on prostate cancer in cell line models and prostatectomy specimens.

### 2.1.2 Review question definition and criteria development for study inclusion

In order to create a clear, defined review a defined question was needed and therefore a broad question was defined as review "objectives" and a detailed specification was defined as "criteria". The review question needed to specify clearly the types of populations in this case miRNAs, comparisons i.e. between the miRNAs and the type of outcomes that were of interest. The components of this specific question with any additional specifications types were included in the study to form the basis of the pre-specified eligibility criteria was included in the review. Outcomes needed to be meaningful and did not include trivial outcomes. The primary outcome was limited to small number included adverse as well as beneficial and relevant outcomes.

### 2.1.3 Identification and selection of published literature

A computerised literature search was carried out using the database PubMed (National Center for Biotechnology Information) for the time from 1970 to March 2011. The general search keywords imputed into PubMed were "miRNA" "prostate" and "cancer". An initial identification of the literature was performed by viewing the titles and the relevant abstracts of the obtained literature from the PubMed search. The citations and references of the relevant selected studies were checked. This was a repetitive process, continued until no new study of relevance could be identified. A series of inclusion criteria was defined, and all relevant literature was then checked for suitable eligibility.

The inclusion criteria considered were;

*a*) Study design, namely, with profiled miRNAs in prostate cancer

*b*) Written in English

*c*) Sufficient data reported to be used in a systematic review

### 2.1.4 Study selection, data assembly and minimising bias

Systemic reviews require a thorough, objective and reproducible search of a range of sources in order to identify as many relevant studies available. This is a very important factor in distinguishing systemic reviews and also helps minimise bias, thus resulting in reliable estimates of effects. The eligibility of studies was assessed and data was extracted. Data from the published literature was reported in diverse formats however it was converted into a suitable format. All collected data that was included in the systematic review, was normalised to a standard format for all studies as follows; all miRNAs profiled in malignant samples and cell lines were compared to that of normal tissue in each study and a fold change value of 0.5 and 2 was considered significant.

### 2.1.5 Comparisons inclusion

The most important step in the analysis was to specify the pair-wise comparisons. The comparisons addressed in the review should clearly relate and directly answer the questions posed when the review is formulated. The systematic review was carried out on 6 original studies reporting on miRNA expression profiling on prostate cancer in cell line models and prostatectomy specimens. Lists of miRNA genes were compiled together from these studies using the following criteria;

- miRNA genes that were upregulated
- miRNA genes that were downregulated
- miRNA genes that were undetected
- miRNA genes that were unchanged
- miRNAs genes that were not profiled

### 2.1.6 Bioinformatics: analysis of significant miRNAs

Bioinformatic analysis of 20 of the most profiled and relevant miRNAs were performed using on line resources. A list of gene targets predicted to interact with each selected miRNA was generated using miRGen, version 3 on line database (http:/www.diana.pcbi.upenn.edu/miRGen/v3/miRGen.html).

Secondly, the relationship between these selected miRNAs and their predicted metabolic pathways were generated using the online database miRNApath (http://lgmb.fmrp.usp.br/mirnapath/query_mirna.php). This search tool provides a relationship between the miRNA list and metabolic pathways.

**2.2 Prostate cell lines: culture and maintance**

All cell culture was performed in a sterile laminar air flow hood and all surfaces were cleaned down with 70% IMS. All cells were grown and maintained in sterile flat bottomed 75cm$^2$ cell culture flasks (Starsted AG and Co, Nümbrecht, Germany).

The PC-3 cell line was established from a bone metastasis of a Grade 4 prostate adenocarcinoma from a 62 year old, Caucasian male. The cell line was obtained from the American Type Culture Collection (CRL-1435). Cells were grown to 70% confluent in a constant atmosphere containing 5% $CO_2$ at 37°C in the following medium: F12-K containing L-Glutamine (Gibco at Invitrogen Life technologies, Maryland, USA), 10% FBS (Lonza Group Ltd. Basel, Switzerland) and 100U/ml Penicillin/Streptomycin (Lonza Group Ltd. Basel, Switzerland).

The LNCaP cell line was established from a carcinoma from the metastatic site of the left supraclavicular lymph node of the prostate from a 50 year old, Caucasian male in 1977. The cell line was obtained from the American Type Culture Collection (CRL-1740). Cells were grown to 70% confluent in a constant atmosphere containing 5% $CO_2$ at 37°C in the following medium: RPMI 1640 containing L-Glutamine and HEPES (Lonza Group Ltd. Basel, Switzerland), 10% FBS and 100U/ml Penicillin/Streptomycin.

The PWR-1E cell line was established from human prostatic epithelial cells, derived from a normal prostate with mild hyperplasia and were immortalised with an adenovirus 12-SV40 hybrid virus (Ad12-SV40) from a 67 year Caucasian male. The cell line was derived by single cell cloning of a non-producer cell in 3-dimensional Matrigel cultures. The cell line was kindly obtained from Professor Mark Lawler's Lab TCD. Cells were grown to 70% confluent in an atmosphere containing 5% $CO_2$ at 37°C in the following

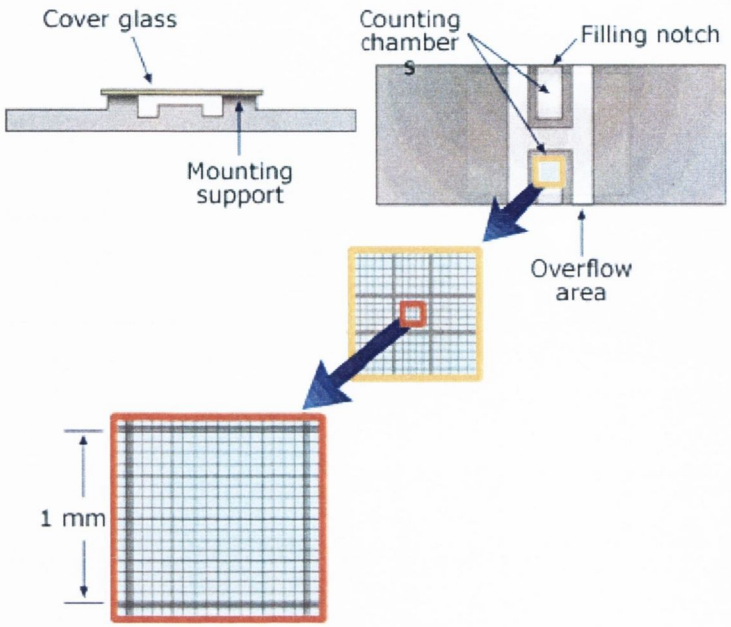
medium: Keratinocyte serum free medium containing 0.05mg/ml BPE and 5ng/ml EGF (Gibco at Invitrogen Life technologies, Maryland, USA).

### 2.2.1 Cell counting and viability

Number of cells per unit volume in suspension was determined by a haemocytometer counting chamber. The counting chamber was cleaned with lens paper and a clean cover slip was carefully placed on the surface. Suspension containing cells and trypan blue vital stain was pipetted into the v-shaped well and the area under the coverslip was filled (by capillary action). A haemocytometer was placed on the microscope stage and the grid was focused at low power. The haemocytometer grid with Neubauer rulings, was viewed at 40X (4X objective). Main divisions separate the grid into 9 large squares.

Trypan blue stains dead cells blue in colour and these cells were excluded as only live viable cells were included in the count. Only half the cells that settled on the border gridline were counted. Cells that touched the bottom and right border were counted and the cells that touched the top and left border were not counted. The number of cells per 1ml was calculated by multiplying the number of live viable cells by 4 (the dilution factor) and then by $10^4$ (the conversion factor). Therefore the following formula was used;

**Total number of live cells/1ml** = number of live viable cells/1mm$^2$ x 4 x $10^4$



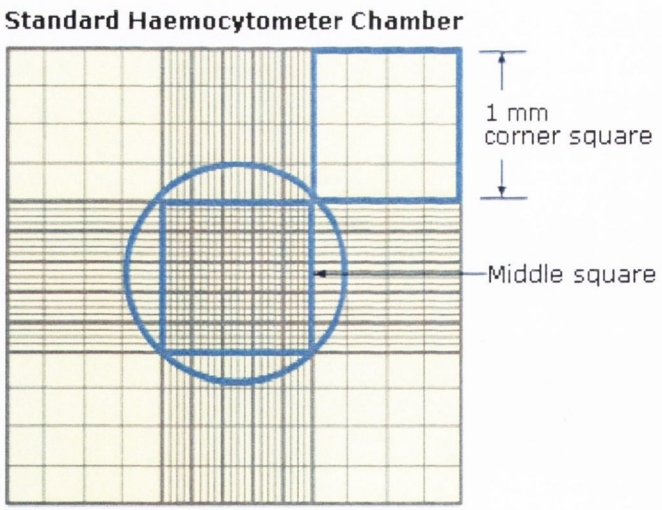




**Figure 2.1: Schematic representation of a haemocytometer with Neubauer ruling.** (http://toolboxes.flexiblelearning.net.au)

### 2.2.2 Long term cell culture and passage

Medium was removed and confluent cells were gently washed with PBS (Lonza Group Ltd. Basel, Switzerland), 0.25mM Trypsin/EDTA (Gibco at Invitrogen Life technologies, Maryland, USA) was added and cells were allowed to detach in the incubator at 37°C for 5 mins. When cells were detached, an equal volume of appropriate medium was added to neutralise the Trypsin/EDTA. Cells were collected and centrifuged for 5 mins at 1000rpm, the supernatant was removed and pellet was obtained. Cell viability and concentration was determined using a haematocytometer (Sigma-Aldrich, St. Louis, MO, USA) and trypan blue (Gibco at Invitrogen Life technologies, Maryland, USA). Sterile flat bottomed 75cm$^2$ cell culture flasks (Starsted AG and Co, Nümbrecht, Germany) were seeded with 33% (1:3 split ratio / 1 X $10^6$) of trypsinised cells upon reaching 70% confluency in appropriate media and maintained in a constant atmosphere containing 5% $CO_2$ at 37°C.

### 2.2.3 Cryopreservation of cells for long-term storage

Cells were centrifuged at 1000rpm for 5 mins and pellet was obtained. Supernatant was discarded and pellet was resuspended in 1ml of Cell Freezing Medium (Gibco at Invitrogen Life technologies, Maryland, USA) and placed in 1.5ml cryovials (Nalge Nunc International, Rochester, NY, USA). Cryovials were placed in a styrofoam box and were stored at –80°C for 24 hours and were transferred to liquid nitrogen for long-term storage.

### 2.2.4 Reconstitution of cells from long-term storage

Complete appropriate culture media was pre-heated to 37°C. Cells were removed from long-term storage (-80°C liquid nitrogen) and were immediately immersed in a 37°C water bath. Vials were agitated in the water bath at 37°C (cells were thawed quickly in order to avoid ice shards and prevent cell lysis). Cryovials were sprayed with 70% IMS and were placed in the laminar air flow hood. Cells were pipetted from the cryovial and transferred to 15ml tubes with appropriate preheated medium. Cells were centrifuged at 1000rpm for 5 mins to obtain pellets. Supernatant was removed and pellets were re-suspended in appropriate culture media into a sterile flat bottomed 25cm$^2$ cell culture flask (Starsted AG and Co, Nümbrecht, Germany). Once cells reached 70% confluency they were transferred to a sterile 75cm$^2$ cell culture flask.

### 2.3 Retinoic Acid treatment of prostate cancer cell lines

All-trans Retinoic Acid (Sigma-Aldrich, St. Louis, MO, USA) was removed from long-term storage (-80°C liquid nitrogen) and 100mg was disolved in 33ml of DMSO to obtain a 0.01M stock. 1ml aliquots were stored at -80°C and thawed at room temperature away from light. $10^{-5}$M (1:100 dilution) was required for the appropriate cell culture medium.

PC-3 cells were grown to 70% confluence in a constant atmosphere containing 5% $CO_2$ at 37°C in the following medium: F12-K containing L-Glutamine (Gibco at Invitrogen Life technologies, Maryland, USA), 10% FBS (Lonza Group Ltd. Basel, Switzerland) and 100U/ml Penicillin/Streptomycin (Lonza Group Ltd. Basel, Switzerland) and $10^{-5}$M All-trans Retinoic Acid (Sigma-Aldrich, St. Louis, MO, USA). Cells were harvested at the following time points; 7 days treated and untreated and 14 days treated and untreated.

LNCaP cells were grown to 70% confluence in a constant atmosphere containing 5% $CO_2$ at 37°C in the following medium: RPMI 1640 containing L-Glutamine and HEPES (Lonza Group Ltd. Basel, Switzerland), 10% FBS and 100U/ml Penicillin/Streptomycin and $10^{-5}$M All-trans Retinoic Acid (Sigma-Aldrich, St. Louis, MO, USA). Cells were harvested at the following time points; 7 days treated and untreated with RA and 14 days treated and untreated with RA.

## 2.4 Optimisation of PC-3 and LNCaP Holoclones by Soft-Agar-Assay

Initially cell lines were set up in an agarose only gradient in order to optimise the appropriate percentage of agarose for each cell line and then then followed by a gradient of NaCl appropriate for the selection of holoclones, see Table 2.1.A and 2.1.B.

**Table 2.1.A:** Optimisation of agaorse concentration for generation of PC-3 and LNCaP Holoclones.

| PC-3 and LNCaP Cells | % Concentration | % Concentration | % Concentration |
|---|---|---|---|
| Initial Agarose only | 0.5 | 1 | 1.5 |
| Final Agarose only | 0.5 | 0.8 | 1 |

**Table 2.1.B:** Optimisation of NaCl concentration for generation of PC-3 and LNCaP Holoclones.

| PC-3 and LNCaP Cells | % Concentration | % Concentration | % Concentration |
|---|---|---|---|
| Initial NaCl | 1 | 5 | 10 |

### 2.4.1 Generation of PC-3 Holoclones by Soft-Agar-Assay

High Salt Soft-Agar Assay mix was set up in 100mls final volume containing the following: 0.8% molecular grade agarose (Sigma-Aldrich, St. Louis, MO, USA) and 1% NaCl (Sigma-Aldrich, St. Louis, MO, USA) and 100 mls of $dH_2O$ and subsequently autoclaved. 10mls of the high salt soft-agar assay mix and 100U/ml Penicillin/Streptomycin was added to the petri dish (Corning Incorporated, NY, USA) and was allowed to set. 10 mls of the complete F12-K media and 1 X $10^6$ PC-3 cells was added to the petri dish on top of the soft-agar assay mix and maintained in a constant atmosphere containing 5% $CO_2$ at 37°C.

### 2.4.2 Generation of LNCaP Holoclones by Soft-Agar-Assay

High Salt Soft-Agar Assay mix was set up in 100mls final volume containing the following: 1% molecular grade agarose (Sigma-Aldrich, St. Louis, MO, USA) and 1% NaCl (Sigma-Aldrich, St. Louis, MO, USA) and 100 mls of $dH_2O$ and was autoclaved. 10mls of the high salt soft-agar assay mix and 100U/ml Penicillin/Streptomycin was added to the petri dish (Corning Incorporated, NY, USA) and was allowed to set. 10 mls of the complete RPMI media and 1 X $10^6$ LNCaP cells was added to the petri dish on top of the soft-agar assay mix and maintained in a constant atmosphere containing 5% $CO_2$ at 37°C.

### 2.4.3 Harvesting of PC-3 and LNCaP Holoclones

The upper layer of media and cell debris was pipetted off the layer of agarose and was then gently washed twice with PBS and removed. Each individual holoclone was removed from the agarose with a scalpel and was washed with PBS and centrifuged for 5 mins at 1000rpm; the supernatant was removed and a pellet was obtained and placed on ice. Cell pellets were stored at -80 °C until needed.

## 2.5 RNA isolation

### 2.5.1 RNeasy Mini Protocol for Isolation of Total RNA (Qiagen, Crawley, West Sussex, UK)

Cultured cell lines were lysed with 600µl for LNCaP and 350µl for PC-3 of Buffer RLT containing β-Mercaptoethanol (10µl of β-ME per 1ml of Buffer RLT) by vortexing to mix. The lysate was directly pipetted onto a QIAshredder spin column and placed in a 2ml collection tube and centrifuged for 2 mins at maximum speed. 1 volume of 70% ethanol was added to the homogenised lysate and was mixed by vortexing. Up to 700µl of the sample including any precipitate was applied to an RNeasy mini column placed in a 2ml collection tube and centrifuged for 15 secs at 10,000rpm and the flow-through was discarded. This step was repeated until all flow-through passed through the column. 700µl of Buffer RW1 was added to the spin column and the lid was gently closed and centrifuged for 15 secs at 10,000rpm to wash the spin column. Flow through was discarded. 500µl of Buffer RPE was added to the RNeasy spin column and the lid was gently closed and centrifuged for 2 mins at 10,000rpm in order to wash the spin column membrane (long centrifugation dries the spin column membrane to ensure that no ethanol is carried over during RNA elution). RNeasy spin column was removed carefully from the collection tube to ensure there was no contact with the flow through to prevent ethanol carryover. RNeasy spin column was placed in a new collection tube and the flow-through was discarded. The lid was closed gently and centrifuged for 1 min at full speed in order to prevent Buffer RPE carryover. RNeasy spin column was placed in a new 1.5ml collection tube and 50µl of RNease free water was placed directly on the spin column membrane. The lid was closed gently and was centrifuged for 1 min at 10,000 rpm to elute the RNA. The eluate was collected and stored at –80°C.

### 2.5.2 mirVana™ miRNA Isolation (Ambion at Applied Biosystems, Foster City, CA, USA) Total RNA Isolation procedure

Cultured cell lines were homogenized using 300µl of Lysis/Binding Buffer for PWR-1E and PC-3 and 600µl of Lysis/Binding Buffer for LNCaP and were vortexed vigorously to completely lyse the cells. 1/10 volume of miRNA Homogenate Additive was added to the lysate and incubated on ice for 10 mins. A volume of Acid-Phenol: Chloroform (Ambion at Applied Biosystems, Foster City, CA, USA) equal to the sample lysate volume was added and vortexed for 60 secs to the mix. Lysate was centrifuged for 5 mins at 10,000 rpm at room temperature in order to separate the aqueous and organic phases and the interphase should be visible after centrifugation. The upper aqueous phase was carefully removed and transferred to a fresh tube. 1.25 volumes of room temperature 100% ethanol was added to the aqueous phase and was mixed thoroughly. 700µl of the lysate/ethanol mixture was pipetted onto the Filter Cartridge contained in a collection tube and centrifuged for 15 secs at 10000 rpm. The flow through was discarded until all the lysate/ethanol mixture was through the filter. 700µl of miRNA Wash Solution 1 was applied to the filter cartridge and centrifuged for 10 secs and the flow through was discarded. 500µl of Wash Solution 2/3 was applied to the filter cartridge and centrifuged for 10 secs and the flow through was discarded and was repeated again with a second aliquot of Wash Solution 2/3. The flow through was discarded and centrifuged for a further 1 min to remove residual fluid from the filter. 100µl of RNAse free water at 95°C was applied to the filter cartridge and centrifuged for 30 secs. Eluate was collected and stored at –80°C.

## 2.6 Quantification of RNA

### 2.6.1 NanoDrop 1000 Spectrophotometry

The quantity and purity of Total RNA isolated was also determined by micro-volume quantitation using a Nanodrop 1000 spectrophometry. The NanoDrop 1000 spectrophotometer was calibrated as follows; the machine was blanked using the same solution that the sample was previously eluted in and was loaded onto the pedestal (ensuring there were no air bubbles) and was measured immediately. The pedestal was wiped with a lint-free wipe. 1.5µl of the Total RNA sample was loaded onto the pedestal and sample using: Sample type RNA-40. When the measurement was completed, the surface was wiped again with a lint-free wipe before going on to the next sample.

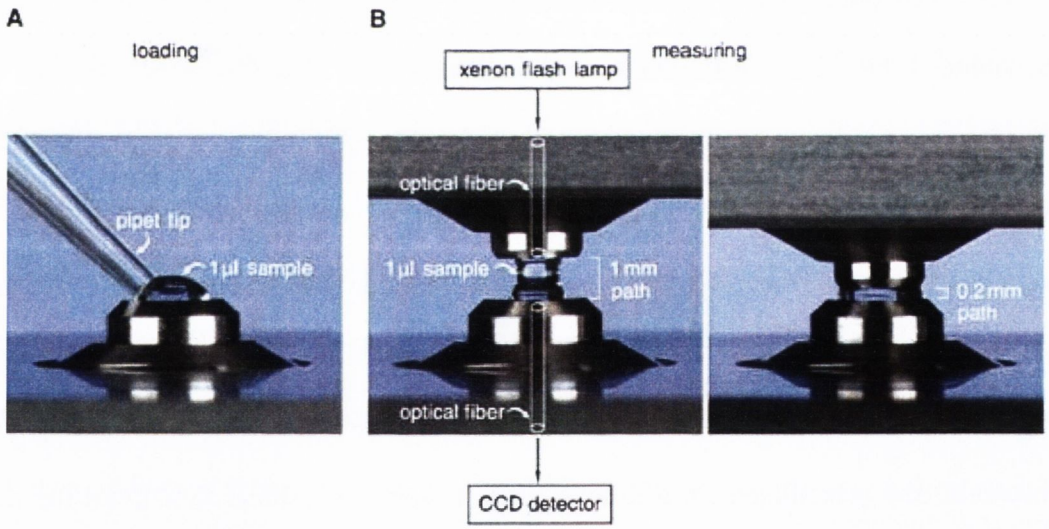



**Figure 2.2: Nanodrop spectrophotometer loading pedestal.** (Gallagher and Desjardins. 2008) **(A)** Loading of sample with pipette and tip onto pedestal **(B)** Measuring of sample placed onto pedestal with CCD detector and xenon flash lamp.

## 2.7 TaqMan® Two-Step RT-PCR

TaqMan® PCR is a quantitative real-time PCR method and was chosen for the quantification of mRNA expression levels in this study. RT-PCR was performed in two individual reactions. Total RNA was reverse transcribed into cDNA and was amplified by PCR.

### 2.7.1 TaqMan® Probe-based chemistry

TaqMan® Probe-based chemistry uses a fluorogenic probe to allow for the detection of specific PCR product during the actual PCR as it accumulates. An oligonucleotide probe is constructed with a fluorescent reporter dye which is bound to the 5' end and a 3' quencher end. As the probe is intact, the proximity of the quencher greatly reduces the fluorescence emitted by the reporter dye. This occurs by fluorescence resonance energy transfer through space. When the target sequence is present, the probe anneals between the primer sites and is cleaved by the 5' nuclease activity of the Taq DNA polymerase during the extension process. Cleavage of the probe allows for the separation of the reporter dye from the quencher and therefore this increases the signal of the reporter dye. Cleavage also allows for the removal of the probe from the target strand. This allows the primer extension to continue to the end of the template strand. The inclusion of the probe does not inhibit the PCR process. Additional molecules of the reporter dye are cleaved from the relevant probes with each cycle. This results in an increase in fluorescence intensity proportional to the amount of produced amplicons. As the higher the starting copy number of the nucleic acid target the sooner there is a significant increase in fluorescence (Figure 2.3).

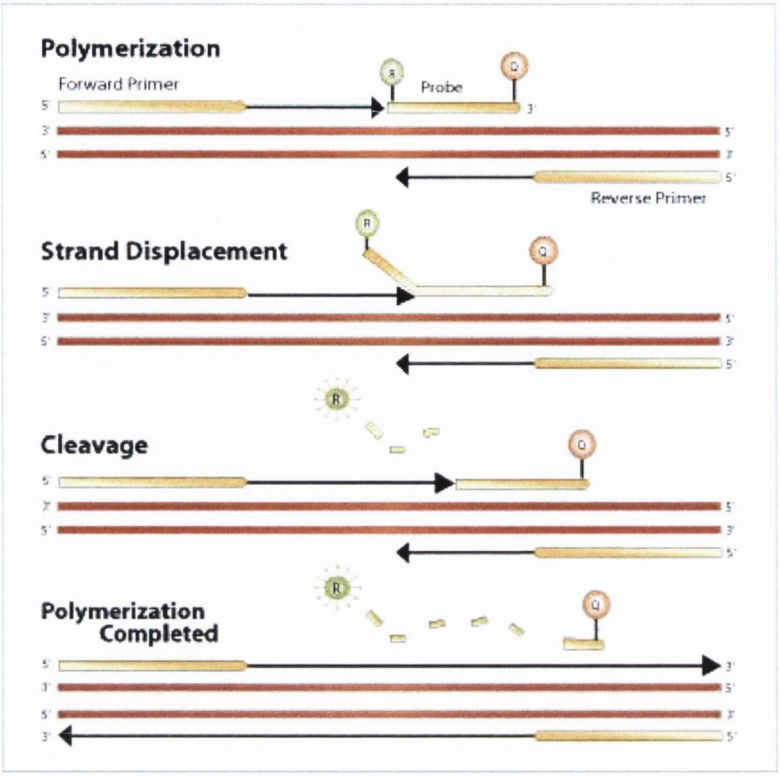



**Figure 2.3:** Schematic representation of TaqMan® PCR probe chemistry mechanism (www.appliedbiosystems.com)

### 2.7.2 TaqMan® gene expression assays

Individual TaqMan® gene expression assays are pre-designed gene-specific primer and probe sets for quantitative gene expression studies on human genes. TaqMan® gene expression assays are built on Applied Biosystems 5' nuclease chemistry. Assays are made up of two unlabeled PCR primers and one FAM dye-labeled probe. TaqMan® endogenous controls are also FAM labelled.

### 2.7.3 TaqMan® RT-PCR reactions

All TaqMan® RT-PCR reactions were set up in a specifically dedicated class 2 laminar air flow hood using dedicated pipetts and aerosol resistant pipette tips. Template RNA and cDNA was added to the RT-PCR reaction plates in a separate dedicated area. RT-PCRs were performed using a two step method. mRNA was converted into cDNA and this template was used in the TaqMan® RT-PCR reaction.

#### 2.7.3.1 cDNA synthesis for TaqMan® RT-PCR

cDNA was generated from mRNA and used in TaqMan® RT-PCR reactions. cDNA was synthesised using the High capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Reverse transcription reactions were set up in 100µl volume reactions containing the following: 10X RT Buffer, 25X dNTPs, 10X Random Primers, Multiscribe enzyme, Nuclease-free water and 500ng of total RNA. Reverse transcription reaction mix was incubated on ice for 5 mins. Reverse transcription reactions were performed on a thermal cycler under the following conditions: 25°C for 10 mins, 37°C for 2 hours, 85°C for 5 sec and 4°C for infinity.

#### 2.7.3.2 TaqMan® RT-PCR Primer and Probes and controls

Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) (Applied Biosystems, Foster City, CA, USA) was used as the endogenous control. A no template control was included on every plate for each primer and probe and endogenous control used.

### 2.7.4 TaqMan® RT-PCR

TaqMan® RT-PCR reactions were set up in volume reactions containing the following: Universal Master Mix, No AmpErase® UNG (Applied Biosystems, Foster City, CA, USA), relevant primer and probe and cDNA template. This master mix contains components needed for 5' nuclease assays, without AmpErase® UNG and also employs AmpliTaq Gold® DNA polymerase to allow for a better yield and a more robust 5' nuclease assay than AmpliTaq Gold® DNA polymerase. It also includes Passive Reference 1 required for signal normalisation in all 5' nuclease assays. Universal Master Mix, No AmpErase® UNG also contains dUTP, so as AmpErase® UNG can protect against carry over contamination.

20µl of RT-PCR reactions were plated into either 96 or 384 well plates (Applied Biosystems, Foster City, CA, USA). Plates were sealed with MicroAmp™ Optical Adhesive Film and were spun briefly at room temperature. RT-PCR reactions were performed on 7900HT or 7600 (Applied Biosystems, Foster City, CA, USA) under the following thermal cycling conditions: 50°C for 1 min to allow for UNG activation, 95°C for 10 mins for AmpliTaq Gold® activation, 50 cycles of 95°C for 15 secs and 60°C for 1 min to allow for melting and annealing.

### 2.7.4.1 Biological and technical replicates

In this study, biological replicates were defined as RNA isolated from cell lines of different passages. Technical replicates were defined as RNA isolated from the same pool of one biological replicate. All reactions were carried out in both biological and technical triplicates in parallel with non-cDNA and non-template controls. TaqMan RT-PCR expression data was consistent. Gene alterations consistently occurred at the same time points and the qualitative directional expression (up/down regulated) was also consistent across multiple biological replicates. However the precise levels of expression varied therefore each data set is presented as a representative median data set of a minimum of three biological replicates.

### 2.7.5 TaqMan® RT-PCR: general data analysis steps

TaqMan® RT-PCR raw data was analysed for this study using the following criteria;

- Viewing amplification plots for the 384 / 96 well plates
- Setting the baseline and threshold values
- Comparative $C_T$ method to determine results

TaqMan® RT-PCR is a real time PCR method and allows for data to be collected throughout the PCR run. Therefore the reaction is characterised at the time point of the cycle when the target is initially amplified. A higher starting copy number of the target shows an earlier significant increase in fluorescence. The initial cycles of the PCR, where little change in fluorescent signal occurs is defined as the amplification plot baseline. Fluorescence increases above the baseline shows the detection of the accumulation target and the fluorescence threshold is set above the baseline. The threshold is set at the point where the PCR product is initially detected. The number of cycles in which the target crosses the threshold is determined by the following software; SDS v2.3/RQ Manager v 1.2 (Applied Biosystems, Foster City, CA, USA). This is referred to as the threshold cycle ($C_T$) and calculates the relative expression. $C_T$ values of the target compared to the calibrator allows the expression of the data to be displayed as a fold change of the levels of expression by the standard curve method or the comparative $C_T$ method.

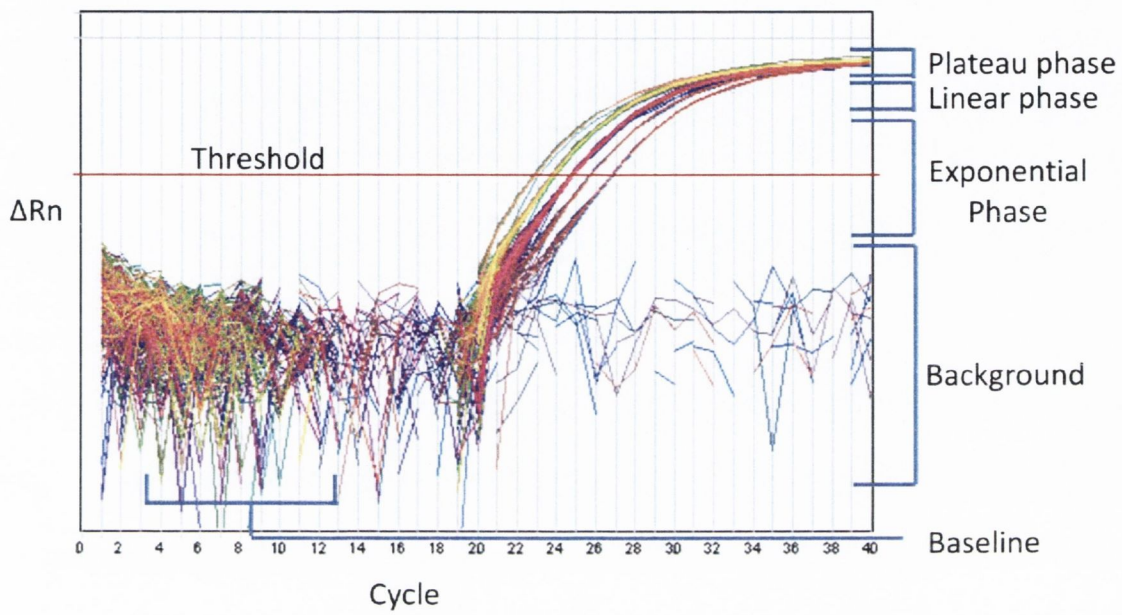



**Figure 2.4:** TaqMan® RT-PCR amplification curve (www.appliedbiosystems).

### 2.7.6 Comparative $C_T$ method

- Setting up and running 384 / 96 well reaction plate
- Analysing the data obtained from the run
- Determining the $\Delta C_T$ value from data (Target – GAPDH endogenous control)
- Calculating the $\Delta\Delta C_T$ to obtain fold change differences in the gene expression ($\Delta C_T$ Target - $\Delta C_T$ calibrator)

#### 2.7.6.1 Comparative $C_T$ Method for relative quantification

Quantitative RT-PCR was performed using sequence specific primers and probes. GAPDH was used as the endogenous control. The comparative $C_T$ method for the relative quantification values, are calculated by the threshold cycle ($C_T$) values. This method calculates relative gene expression fold change values were calculated using the following equation (Livak *et al.*, 2001);

$$\text{Relative Quantity}=2^{-\Delta\Delta CT}$$

The mean Ct value and standard deviation was calculated for each sample. $\Delta C_T$ was generated by normalisation the Ct of the sample with the Ct of the endogenous control GAPDH for example; $C_T$ sample- $C_T$ endogenous control GAPDH. $\Delta\Delta C_T$ was generated by subtracting the $\Delta C_T$ of the calibrator from the $\Delta C_T$ of the target. Relative levels of target gene expression were calculated with the formula mentioned above (Relative Quantity=$2^{-\Delta\Delta CT}$). A non-template control was included in all reactions as a negative control.

### 2.7.7 Predicted gene targets and metabolic pathways

Gene targets were predicted for the top 10 upregulated and downregulated miRNAs in the PC-3 and LNCaP cell lines using the databases; miRGen version 2007(v3) and microrna.org (version September 2008). miRGen is an intergrated database, which the target interface allows access to union and intersections of 4 widely used target prediction programmes and experimentally supports targets from Tarbase. The top 10 upregulated and downregulated miRNAs and the top 10 predicted gene targets were predicted using the databases miRGen and microrna.org.

Pathways were predicted for the top 5 upregulated and downregulated miRNAs in the cell lines PC-3 and LNCaP using the database miRNApath. miRNApath offers a relationship between miRNAs, target genes and metabolic pathways. This database allows for the investigation of miRNAs and shows the pathways affected by these miRNAs. It also allows for the speculative prediction of the disease caused by them.

## 2.8 TaqMan® Low Density Array-Megaplex Pools for miRNA expression

### 2.8.1 Background

TLDAs Megaplex pools are used to detect and quantitate up to 384 human miRNAs per pool (Pool A), Megaplex pools consist of matching primer pools and TaqMan Arrays. Megaplex RT Primers are a set of two pre-defined pools of up to 384 stem-looped RT primers per pool, which allow for the simultaneous synthesis of cDNA for mature miRNA. Each TLDA card contains 8 ports which feed into 48 individual wells that contain pre-loaded dried TaqMan ® miRNA assays. Each TLDA card contains endogenous control assays for data normalisation and an assay for the purpose of a process control.

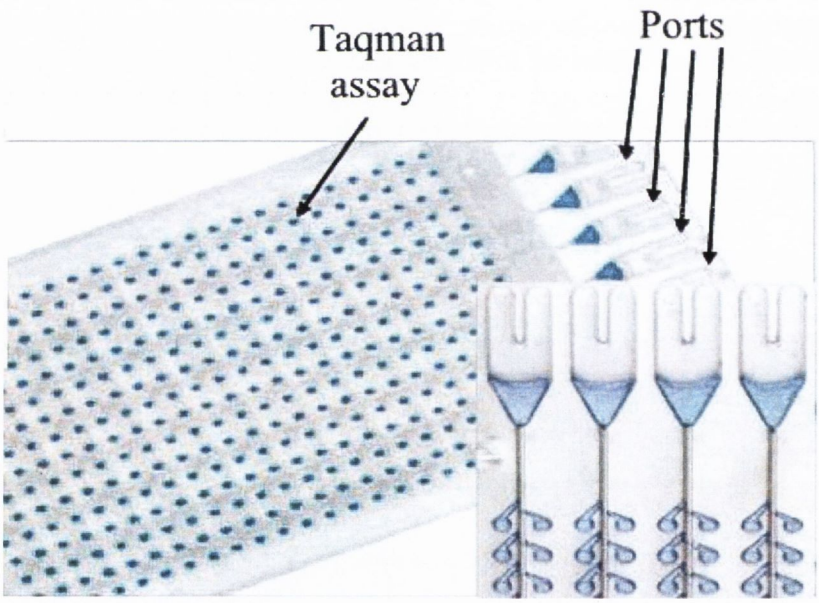



**Figure 2.5:** Representation of 384 miRNA TaqMan® Low Density Arrays containing TaqMan® pre-dried miRNA assays and 8 loading ports to distribute sample into each well (http://mdl.dfci.harvard.edu).

### 2.8.2 Megaplex reverse transcription- cDNA synthesis

Megaplex reverse transcription reactions were set up in 7.5µl volume reactions containing the following: 10X Megaplex RT Primers, 100mM dNTPs with dTTP, 50U/µl Multiscribe Reverse Transcriptase, 10X RT buffer, 25mM $MgCl_2$, 20U/µl Rnase Inhibitor and Nuclease-free water, 1-1000ng of total RNA. Megaplex reverse transcription reaction mix was incubated on ice for 5 mins. Megaplex reverse transcription reactions were performed on a thermal cycler under the following conditions: 16°C for 2 mins, 42°C for 1 min, 50°C for 1 sec, 40 cycles of 85°C for 5 mins and 4°C for infinity.

#### 2.8.2.1 Pre-amplification of RT product

Pre-amplification reactions were set up in 25µl volume reactions containing the following: 2X TaqMan PreAmp Master Mix, 10X Megaplex PreAmp Primers, Nuclease-free water and 2.5µl Megaplex RT product. Pre-amplification reaction mix was incubated on ice for 5 mins. Pre-amplification reactions were performed on a thermal cycler under the following conditions: 95°C for 10 mins, 55°C for 2 mins, 72°C for 2 mins, 12 cycles of 95°C for 15 secs, 60°C for 4 mins, 4°C for infinity. Pre-amplification reactions were diluted with 0.1X Tris-EDTA buffer pH8 (Ambion).

### 2.8.3 Microfludic Cards- Array A

PCR reaction mix per card was set up in 900µl volume reactions containing the following, 2X TaqMan Universal Master Mix No AmpErase UNG, 9µl of diluted PreAmp product and nuclease free water. 100µl of the PCR reaction mix was dispensed into the left arm of each port of the TaqMan miRNA array. Cards were centrifuged twice in a Sorvall Heraeus centrifuge, under the following parameters: up ramp rate 9, down ramp rate 9, rotational speed 1200 rpm, bucket type 15679 for 1 min to distribute

the sample from the loading port into each well. Cards were sealed and the loading port was removed using a scissors. Cards were then run on the ABI 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA), under the following conditions: 50°C for 2 mins 100% ramp, 94.5°C for 10 mins 100% ramp, each of 40 cycles of melt 97°C for 30 secs 50% ramp and anneal/extend 69.7°C for 1 min 1005 ramp.

### 2.8.4 Endogenous and process controls

Each TLDA card contained the assay U6 snRNA (MammU6) repeated in 4 technical replicates on each card and was used for data normalisation. A process control was also used, ath-miR159a, an assay unrelated to the mammalian species.

### 2.8.5 TaqMan® Low Density Array Analysis

TLDA cards were analysed as one relative quantification study using RQ Manager 1.2 software for automated data analysis (Applied Biosystems, Foster City, CA, USA). Expression values were calculated using the comparative threshold cycle ($C_T$) method. This method uses the equation Relative Quantity=$2^{-\Delta\Delta CT}$ (see section 2.7.6.1) to calculate the expression of the target genes normalised to a calibrator (Normal cell line PWR-1E). MammU6 was selected as the endogenous control and ath-miR159a as the negative control. MiRNA clustering analysis was carried out using the Sanger Institute's miRNA registry resource miRBASE. Gene target predictions were obtained using miRGEN and MIRANDA. Predictive pathway associations were obtained using miRNAPATH.

### 2.8.6 Statistical analysis for TaqMan® RT-PCR and TLDAs

Sample significance was determing using a non-parametric t-test was used to generate unadjusted p-values. Sample groups were compared to determine differential expression of the selected gene targets. Only differentially expressed genes with a p-value of $< 0.05$ were deemed to be significant.

## 2.9 Affymetrix microarray

### 2.9.1 Background

Affymetrix whole-transcript analysis allows for the detection of gene expression levels, including alternative isoforms or genomic deletions. This was not previously possible with the classical 3'-based microarrays. Whole-transcript expression profiling tools are currently being used to characterise disease etiology and molecular mechanisms with a new level of resolution and accuracy. GeneChip® Whole Transcript Assay and Affymetrix high-density microarrays have been used to explore diseases such as colon and brain cancer and have revealed new mechanistic pathways and possible treatment targets.

Previously microarrays interrogated many hundreds of bases proximal to the 3' end of every gene and used the expression of the entire gene. This is compatible with the 3' oligo(dT)-based priming and labelling assay. This allows for an important insight into the global gene expression. The 3' end of each gene is clearly defined and each transcript has an intact poly-A tail. The entire length of the gene is also expressed as a single unit.

GeneChip® Human Gene 1.0 ST Array supports whole-transcript coverage. Each gene is represented by approximately 26 probes on the array and is spread across the full length of the gene. This provides for a more complete and accurate picture of gene-expression then the 3'-based expression arrays. This type of array is the most advanced and cost-effective gene expression profiling microarray technology.

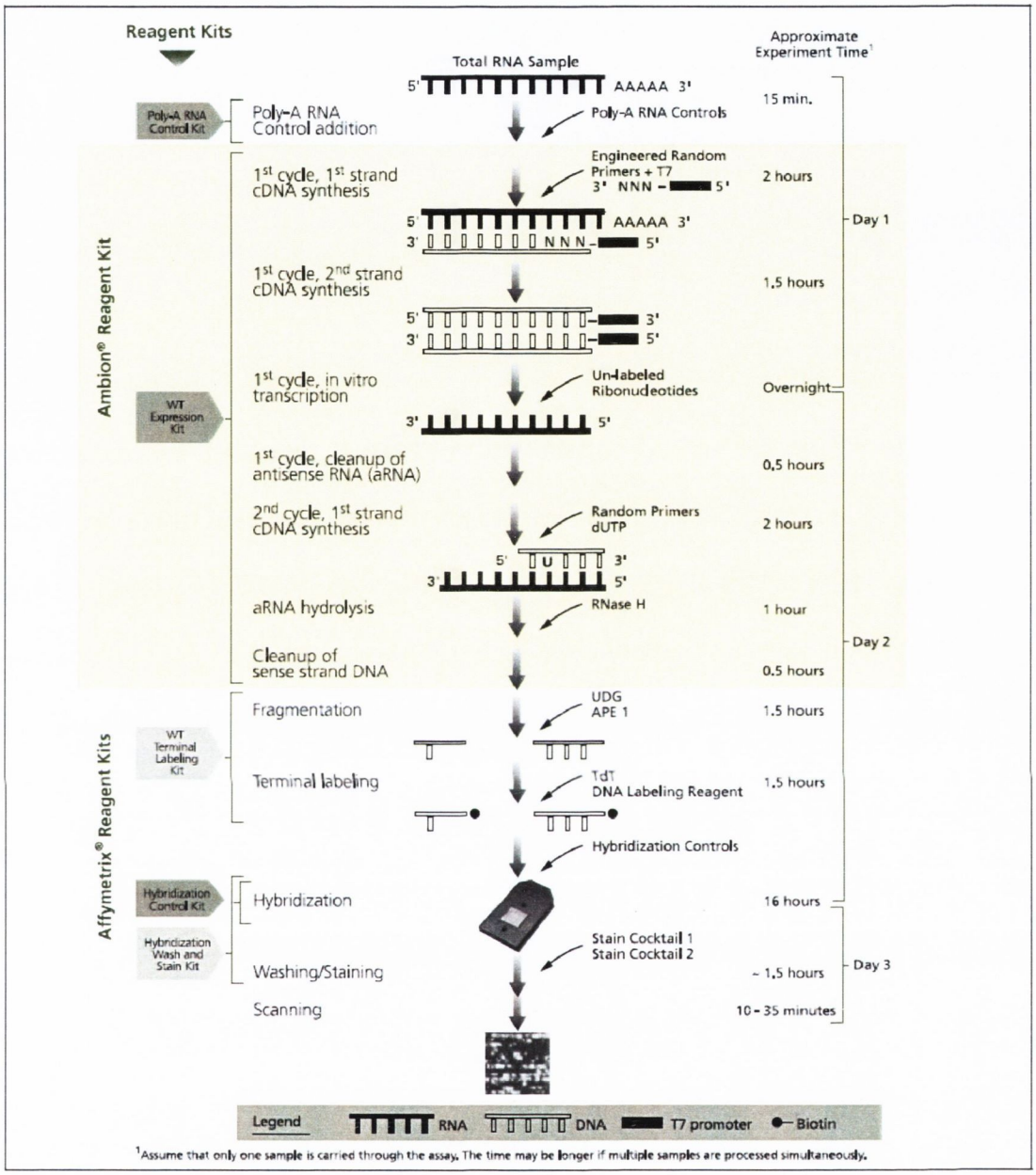



**Figure 2.6:** Schematic of the 3 day whole transcript sense target labelling assay (www.affymetrix.com)

### 2.9.2 GeneChip Whole Transcript (WT) Expression Arrays

#### 2.9.2.1 Preparation of total RNA and Poly-A RNA controls

100ng of total RNA was used as the starting amount of total RNA recommended as per manufacturer's instructions. Poly-A RNA dilutions were prepared for 100ng of total RNA. 2µl of Poly-A RNA control stock was added to 38µl of Poly-A control dilution buffer to make the first dilution (1:20). The first dilution was mixed and spun and the solution was collected at the bottom of the 1.5ml eppendorff tube. 2µl of the first dilution was added to 98µl of the Poly-A control dilution buffer to make the second dilution (1:50). The second dilution was mixed and spun and was collected at the bottom of the tube. 2µl of the second dilution was added to 98µl of the Poly-A control dilution buffer to make the third dilution (1:50). A third dilution was mixed and spun and the solution was collected at the bottom of the tube. 2µl of the third dilution was added to 18µl of the Poly-A control dilution buffer to make the fourth dilution (1:10). A fourth dilution was mixed and spun was collected at the bottom of the tube. 2µl of the fourth dilution was added to 100ng of total RNA to make up the total RNA/Poly-A RNA controls mix (see Table 2.1).

**Table 2.2:** Total RNA imput and serial dilutions.

| **Total RNA input amount** | **Serial Dilutions** | | | | **Volume into Sample** |
|---|---|---|---|---|---|
| | **First** | **Second** | **Third** | **Fourth** | |
| 100ng | 1:20 | 1:50 | 1:50 | 1:10 | 2µl |

### 2.9.2.2 First-strand cDNA synthesis

In this reaction, total RNA was primed with primers containing a T7 promoter sequence and the reaction synthesised single stranded cDNA and contained a T7 promoter sequence.

First-strand synthesis reagents were thawed at room temperature. First-strand master mix contained the following: 4µl of First-Strand Buffer Mix and 1µl of First-Strand Enzyme Mix. 5µl of RNA (2µl of Poly-A Spike controls and 3µl of 100ng total RNA) was added to each tube containing 5µl of First-Strand Master Mix for a final reaction volume of 10µl. Reactions were mixed gently and spun briefly. Reactions were incubated on a thermal cycler under the following conditions: 25°C for 60 mins, 42°C for 60 mins and 4°C for at least 2 mins.

### 2.9.2.3 Second-strand cDNA synthesis

Second-strand master mix was prepared on ice. Second-strand master mix contained the following: 32.5µl nuclease-free water, 12.5µl Second-strand buffer mix, 5µl Second-strand enzyme mix. 50µl of Second-strand master mix was added to 10µl of first-strand synthesis cDNA sample. Reactions were mixed gently and spun briefly. Reactions were incubated on a thermal cycler under the following conditions: 16°C for 60 mins, 65°C for 10 mins and 4°C for at least 2 mins.

#### 2.9.2.4 cRNA synthesis by in vitro transcription

In this reaction, antisense cRNA was synthesised and amplified by *in vitro* transcription of the second-strand cDNA template using the T7 RNA polymerase. The RNA sample preparation was based on the original technology referred to as the Eberwinr or RT-IVT method.

IVT reactions were prepared at room temperature. IVT master mix contained the following: 24µl of IVT buffer mix and 6µl of IVT enzyme mix. 30µl of IVT master mix was added to 60µl of the Second-strand cDNA sample. The reaction mix was mixed gently and spun briefly. Reactions were incubated on a thermal cycler under the following conditions: 40°C for 16 hours and 4°C overnight. (Samples can be stored at -20°C).

#### 2.9.2.5 cRNA purification

In this reaction, enzymes, salts, inorganic phosphates and unincorporated nucleotides were removed to improve the stability of the cRNA.

The elution solution was heated to 54°C for at least 10 mins, 100% ethanol was added to the Nucleic Acid Wash Solution. Nucleic Acid Binding Buffer Concentrate was completely dissolved at 50°C until it was solubilised. Nucleic Acid Binding Beads were vortexed vigorously to ensure they were fully dispersed.

cRNA Binding mix was prepared at room temperature. cRNA Binding Mix contained the following: 10µl of Nucleic Acid Binding Beads and 50µl of Nucleic Acid Binding Buffer Concentrate. 60µl of cRNA Binding Mix was added to each sample and gently

mixed. Each sample was transferred to a well of a U-Bottom plate. 60µl of isopropanol was added to each sample and was gently mixed by pipetting. Samples in the plate were gently shaken for 2 mins. The plate was moved to a magnetic stand to capture the magnetic beads. Capture was complete after 5 mins and the mixture appeared transparent and the beads formed a pellet against the magnets on the stand. Supernatant was carefully aspirated and discarded and beads were not disturbed and the plate was removed from the magnetic stand. 100µl of Nucleic Acid Wash solution was added to each sample and was gently shacked for 1 min at a moderate speed. The plate was moved to a magnetic stand to capture the magnetic beads. Supernatant was carefully aspirated and discarded and beads were not disturbed and the plate was removed from the magnetic stand. The wash step was repeated again (as previously described). The plate was moved to the shaker and was vigorously shaken for 1 min to evaporate residual ethanol from the beads. 40µl of preheated elution solution was added to each sample and was incubated for 2 mins without shaking. The plate was vigorously shaken for 3 mins and pellets were disrupted with manual pipetting. The plate was moved to the magnetic stand and beads were captured. Supernatant containing cRNA was transferred to a nuclease free tube. cRNA yields were determined by UV absorbance using a Nanodrop spectrophotometer. Reactions were stored at -20°C.

#### 2.9.2.6 Second-strand cDNA synthesis

10µg of cRNA was prepared in a volume of 22µl on ice. Second-cycle synthesis reagents were thawed and placed on ice. 22µl of cRNA was combined with 2µl of random primers and was mixed gently and spun briefly. Reactions were incubated on a thermal cycler under the following conditions: 70°C for 5 mins, 25°C for 5 mins and 4°C for at least 2 mins. Second-cycle Master Mix contained the following: 8µl of Second-cycle buffer mix, 8µl of Second-cycle enzyme mix. 16µl of the second-cycle master mix was transferred to 24µl of cRNA/random primer sample and was mixed gently and spun briefly. Reactions were incubated on a thermal cycler under the following conditions: 25°C for 10 mins, 42°C for 90 mins, 70°C for 10 mins and 4°C for at least 2 mins.

#### 2.9.2.7 Hydrolysis using RNase H

In this reaction, RNase H degrades the cRNA template leaving single-stranded cDNA.

On ice, 2µl of RNase H was added to the second-cycle cDNA reaction and was mixed by pipetting and spun briefly. Reactions were incubated on the thermal cycler under the following conditions: 37°C for 45 mins, 95°C for 5 mins and 4°C for at least 2 mins. (Samples can be stored at -20°C overnight).

### 2.9.2.8 Second-cycle cDNA purification

In this reaction after synthesis, the second-strand cDNA was purified in order to remove the enzymes, salts and unincorporated dNTPs, which allowed for the cDNA fragmentation and labelling.

Elution solution was heated to 54°C for at least 10 mins. 100% ethanol was added to the Nucleic Acid Wash Solution. Nucleic Acid Binding Buffer Concentrate was completely dissolved at 50°C until it was solubilised. Nucleic Acid Binding Beads were vortexed vigorously to ensure they were fully dispersed.

cDNA binding mix was prepared at room temperature and contained the following: 10µl of Nucleic Acid Binding Beads, 50µl of Nucleic Acid Binding Buffer Concentrate. 18µl of nuclease free water was added to each sample for a final volume of 60µl. 60µl of cDNA binding mix was added to sample and was pipetted to mix. Each sample was transferred to a well of a U-bottomed plate. 120µl of 100% ethanol was added to each sample and pipetted to mix. (See section 2.9.2.5)

30µl of preheated elution solution was added to each sample and was incubated for 2 mins without shaking. The plate was vigorously shaken for 3 mins and pellets were disrupted with a manual pipetting. The plate was moved to a magnetic stand and beads were captured. Supernatant containing cRNA was transferred to a nuclease free tube. cRNA yields were determined by UV absorbance using a Nanodrop spectrophotometer. Reactions were stored at -20°C.

### 2.9.3 Terminal Labelling and Hybridisation

#### 2.9.3.1 Fragmentation of single-stranded DNA

5.5µg of single-stranded DNA was added to a volume of 31.2µl of RNase-free water. Fragmentation master mix was set up in a 16.8µl volume reaction and contained the following: 10µl of RNAse-free water, 4.8µl of 10X cDNA fragmentation buffer, 1µl of 10 U/µl UDG and 1µl of 1,000 U/µl APE 1. 16.8µl of fragmentation master mix was added to 31.2µl of single-stranded DNA and was gently mixed and spun briefly. Reactions were incubated on a thermal cycler under the following conditions: 37°C for 60 mins, 93°C for 2 mins and 4°C for at least 2 mins. (Samples can be stored at -20°C overnight).

#### 2.9.3.2 Labelling of fragmented single-stranded DNA

Labelling reaction was set up in 60µl volume reactions and contained the following: 45µl of fragmented single-stranded DNA, 12µl of 5X TdT buffer, 2µl of TdT, and 1µl of 5mM DNA labelling reagent and were gently mixed and spun briefly. Reactions were incubated on a thermal cycler under the following conditions: 37°C for 60 mins, 70°C for 10 mins and 4°C for at least 2 mins.

#### 2.9.3.3 Hybridisation

Frozen stocks of 20X GeneChip® Eukaryotic Hybridisation controls were heated to 65°C for 5 mins to completely re-suspend the cRNA before aliquoting. A hybridisation cocktail was prepared as per Table 2.2.

**Table 2.3:** Hybridisation Cocktail: 169 format array

| Component | Volume for one 169 format array | Final Concentration |
|---|---|---|
| Fragmented and labelled DNA target | 27µl | ~25ng/µl |
| Control Oligonucleotide B2 (3nM) | 1.7µl | 50pM |
| 20X Eukaryotic Hybridiasation controls (*bioB, bioC, bioD, cre*) | 5µl | 1.5, 5, 25 and 100pM, respectively |
| 2X Hybridisation mix | 50µl | 1X |
| DMSO | 7µl | 7% |
| Nuclease-free water | Up to 100 | |
| **Total Volume** | **100µl** | |

The reaction mix was gently mixed and spun down. The hybridisation cocktail was heated for 5 mins at 99°C and cooled to 45°C for 5 mins and then centrifuged at max speed for 1 min. The GeneChip ST Array was equilibrated at room temperature. 80µl was injected into the array through one of the septa. Two pipette tips were used to fill the probe array cartridge, one was used to fill and the other was used to allow the air to vent from the hybridisation chamber. Bubbles inside the hyb chamber were allowed to float freely upon rotation in order to ensure that the hybridisation cocktail made contact with all portions of the array.

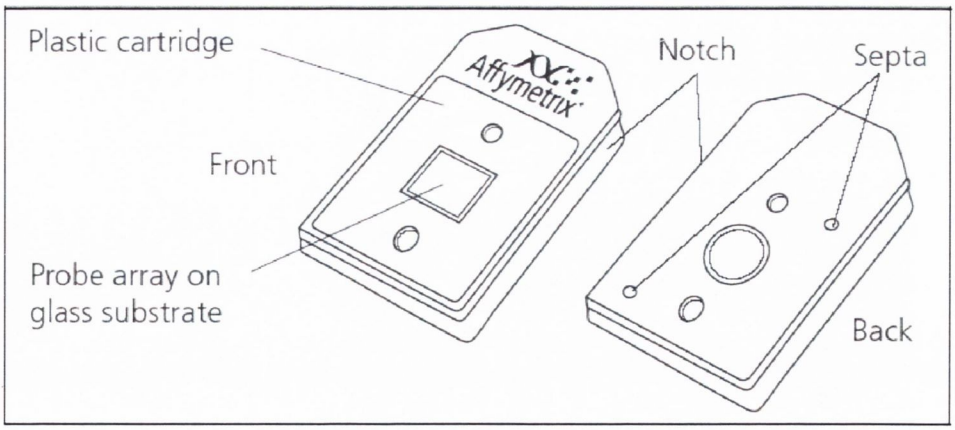



**Figure 2.7:** GeneChip® Probe Array. (www.affymetrix.com).

### 2.9.4 Fluidics station setup

The sample file was registered in the Affymetrix GeneChip Command Console (AGCC) and contained the following information: sample attributes and array information. The fluidics station was prepared and primed as follows: intake buffer reservoirs were filled with the appropriate Wash A and Wash B solutions. The water reservoir was filled with deionised water and the waste bottle was emptied. 3 empty 1.5ml eppendorf tubes were placed in stain holder positions 1, 2, 3 and 4. The washblock lever was placed into the closed position and the needle lever was pushed into the down position. Prime_145 maintenance protocol was run from the AGCC Fluidics Control.

#### 2.9.4.1 Probe array wash and stain

After +/- 16 hours incubation the arrays were moved from the hybridisation oven. Arrays were vented by inserting a clean pipette tip into the septa and the hybridisation cocktail was extracted with a pipette through the remaining septum. (Used hybridisation cocktail can be rehybed on another array and stored at -20°C). Probe array was completely refilled with 100µl of Wash Buffer A. (Probe array can be stored at 4°C for up to 3 hours before washing and staining). Probe array was equilibrated at room temperature before washed and stained.

#### 2.9.4.2 Stain reagent preparation

Reagents were mixed well before alliquoting as follows: 600µl of stain cocktail 1 into an amber 1.5ml eppendorf tube (Stain cocktail 1 is light sensitive), 600µl of stain cocktail 2 into a 1.5ml clear eppendorf tube and 800µl of array holding buffer into a clear 1.5ml eppendorf tube. Reagents were spun briefly to remove any air bubbles present.

### 2.9.4.3 Fluidics station 450/250; wash and stain the probe array

Instructions on the LCD window of the fluidics station were followed by placing the three 1.5ml eppendorfs into the sample holders 1, 2 and 3 on the fluidics station. 600µl of stain cocktail 1 was placed in sample holder 1, 600µl of stain cocktail 2 was placed in sample holder 2 and 800µl of array holding buffer was placed in sample holder 3. Needle levers were pressed down to snap needles intp position and the run was started. Upon completion of the run, the cartridges were ejected and inspected for bubbles and air pockets. If bubbles were not present the probe array was ready to scan on the GeneChip Scanner. If bubbles were present, the probe array was drained, and carefully refilled with 100µl of array holding buffer manually with a pipette to ensure there were no air bubbles or air pockets (excessive washing will result in a loss of signal intensity).

### 2.9.4.4 Probe array scan

Probe arrays were scanned after the wash and stain protocols were completed. Scanner laser required 10 mins to warm up and stabalise. A green solid light indicated that the scanner was ready to scan the arrays. Glass surfaces of the arrays were inspected and gently cleaned if necessary with a non-abrasive tissue before the probe arrays were scanned. Tough-Spots® were applied to the two septa. Cartridges were inserted into the scanner and after the scan was complete the image data was saved by the AGCC. A grid was aligned on the image to identify the probe cells and computed the probe cell intensity.

## 2.10 Affymetrix GeneChip® Gene Array: quality assessment of human gene 1.0 ST arrays

Quality assessment procedures were carried out on Whole Transcript (WT) based arrays. Many of the quality assessment procedures involve computing summary statistics for each individual array in a set of arrays and then allows for the comparison levels of the summary statistics across all of the arrays. The set of arrays include 4 biological replicates for each sample of PC-3 and LNCaP cell lines and PC-3 and LNCaP holoclones (n=4) and these set of arrays are analysed together to address substantive biological questions. Three biological replicates are the gold standard recommended by the manufacture however due to the *de novo* nature of the holoclone study four biological replicated were selected in order to increase accuracy of the study. Quality assessment procedure deals with various metrics in order to identify outlier arrays within the data set.

### 2.10.1 Quality assessment software

**Probe level metrics:** is the quality assessment metrics based on the probe level data generated.

- **pm_mean:** is the mean of the raw intensity for all of the perfect match (PM) probes on the array before any intensity transformations such as; quantile normalization and Robust Multi-array Analysis (RMA) background correction. This value indicates if arrays are abnormally dim or bright. Overly dim or bright arrays are not a problem; however they can indicate probe set based metrics irregularities may be present. For example; an unusually high median absolute derivation of residuals or an unusually high mean absolute relative log expression values within replicates.

### 2.10.2 Probe set summarization based metrics

These metrics are based on summarised probe set intensity results. Most of these metrics are performed for different categories of probe sets.

- **pos_vs_neg_auc:** is the area under the curve (AUC) for a receiver operating characteristic plot comparing signal values for both the positive and negative controls. The receiver operating characteristic curve is generated by evaluating the effectiveness of the probe set signals separating the positive controls from the negative controls, assuming the negative controls are a measure of false positives and the positives measure true positives. An AUC of 1 displays perfect separation, however an AUC of 0.5 displays no separation. The expected value of this metric is tissue specific and may be sensitive to the quantity of the RNA sample. Values between 0.8 and 0.9 are typically obtained.

- **X_mean:** is the mean signal value for all the probe sets analysed from category X.

- **X_mad_residual_mean:** is the mean of the absolute deviation of the residuals from the median, from all probe sets analysed from category X. Different probes that feature on the chip return to different intensities when hybridised to a common target. In order to account for relative intensity differences the RMA algorithm creates a model for individual probe responses. The difference between the signal intensity value and the predicted value is the residual. In cases where the residual for a probe on an array is very different from the median, this means that it is a poorer fit to the model. Therefore calculating the mean of the absolute value of all deviations generates a measure of how well or poor all the probes on the array fit the model. An unusual high mean absolute deviation of the residuals from the median indicates problematic data for that particular array.

- **X_rle_mean:** is the mean absolute relative log expression (RLE) for all the probe sets analysed from category X. This metric is produced by taking the signal estimate for a probe set on a chip and calculating the difference in log base 2 from the median signal value of that probe set over all the chips. The mean is calculated from the absolute RLE for all the analysed from category X. When only replicates are analysed together the mean absolute RLE should be consistently. This indicates the low biological variability of the replicates.

### 2.10.3 Probe set signals as quality metrics

These metrics are individual probe set signal values for various controls. On the gene arrays these include bacterial and poly-A spike probe sets. The probe sets are automatically extracted from the CHP file and are included in the CHP headers by the EC. The main function in assessing the specific probe set values is to determine if expected behaviors' for these probe sets are present. For example; if there is constant expression levels for housekeeping genes, there is rank order of signal values between spike probe sets.

### 2.10.4 Probe set categories

Many of the quality assessment metrics values are reported for particular subsets of probes sets and also all the probe sets analysed. This is necessary when troubleshooting a poorer performing sample.

- **all_probe set:** is all probe sets analysed. In most cases, this category is the bulk of probe sets that will be carried into downstream statistical analysis. Therefore the metrics reported in this category are the most representative of the quality of the data being used downstream.

- **bac_spike:** is the set of probe sets which hybridize to the pre-labeled bacterial spike controls (BioB, BioC, BioD and Cre). This category identifies problems with the hybridization and/or the chip. This category has more metric variability than other categories due to the limited number of spikes and probe sets present.

- **polya_spike:** is the set of polyadenylated RNA spikes (Lys, Phe, Thr and Dap). This category identifies problems with the target prep. This category has more metric variability than other categories due to the limited number of spikes and probe sets present.

- **neg_control:** is the set of putative intron based probe sets from putative housekeeping genes. In many samples, these putative intronic regions may be transcribed and retained. Some of these genes may not be constitutive within certain data sets. Aside from that, these probe sets form a moderately large collection that generally has very low signal values. These probe sets are also used to estimate the false positive rate for the pos_vs_neg_auc metric.

- **pos_control:** is the set of putative exon based probe sets from putative housekeeping genes. Multiple species specific probe sets are selected against the putative exonic regions in these genes as they were shown to have constitutive expression over a large number of samples. These probe sets form a large number of probe sets with target s present that generally have moderate to high signal values. These probe sets are used to estimate the true positive rate for the pos_vs_neg_auc metric.

## 2.11 Statistical analysis used to identify differentially expressed genes

### 2.11.1 Microarray data analysis

All data generated by the microarrays was analysed using XRAY version 3.99 software (Biotique Systems Inc. Reno, NV, USA). Obtained images from scanning were analysed using Affymetrix Expression Console software. Arrays were analysed with XRAY-Excel® Array Analysis, specifically at the significant gene expression level using the following criteria FDR $\leq 0.05$ and FC $\pm 2$.

### 2.11.2 Probe transformation and summarisation

Expression scores for every probeset were generated by standard Robust Multi-array Analysis (RMA) normalisation and probe summarisation methods. The RMA algorithm consists of three key steps; normalisation, transformation and background correction of the probe scores. This approach allows for greater sensitivity to small changes between experiments and sample controls and also minimises variance across the dynamic range. However it does compress calculated fold change values (Irizaray *et al.*, 2003). Each probe score was normalised with quantile normalisation. Normalised probes scores were then filtered and probes with a GC content less than 6 and greater than 17 were not included in any further analysis. All remaining probes were transformed by taking the natural logarithm of 1 plus their scores. Normalisation was necessary so as multiple chips could be compared to each other and analysed together. Background correction was performed by subtracting from the individual probe scores the median probe expression score of the anti-genomic background probes of similar GC content. The summarisation of probes into probe sets (represents exons) was achieved by Turkey's Median Polish method (Choe *et al.*, 2005, *Gautire et al.*, 2003).

### 2.11.3 Identification of group specific gene expression

In order to identify group specific gene expression a Mixed Model, Nested Analysis of Variance known as ANOVA (Montgomery., 2006) was used to identify genes that were differentially expressed between the different groups (holoclones versus the resting founder cell lines). The nested model is appropriate, as data is not sampled in a truly randomized fashion because the expression points are harvested in batches defined by hybridizations known as CEL files. The mixed model is used because the CEL files are random factors.

### 2.11.4 Gene target analysis

PANTHER and DAVID (Database for Annotation, Visualisation and Integrated Discovery) version 6.7 (http://david.abcc.ncifcrf.gov/); both online databases were used to classify genes according to their function and pathways. Both databases generate large lists of relevant genes that have associated biological annotation such as, gene ontology. They statistically highlight the most enriched or over-represented biological annotation from thousands of linked terms and contents.

### 2.11.5 Validation by TaqMan® Low Density Arrays

Validation of selected genes was performed by TaqMan® RT-PCR using custom designed TLDAs (Applied Biosystems, Foster City, CA, USA). RNA was reverse transcribed into cDNA as described in section and TLDA RT-PCRs were performed as described in section 2. Relative quantity of gene expression was calculated by the $2^{-\Delta\Delta CT}$ method as described in section 2 and GAPDH was used as the endogenous control.

# Development of cancer stem cell progenies

Chapter 3

## 3.0 Introduction

### 3.1.1 Prostate cancer stemness

Prostate cancer is one of the five most prevalent malignancies in all male age groups worldwide. Prostate cancer is a heterogeneous disease and is the second most common cause of cancer resulting in male deaths caused by cancer. There are over one thousand, eight hundred new cases diagnosed every year in Ireland, the second leading cause of fatal cancer in Irish men (National Cancer Registry, Ireland 2010). Stem-like cells have been identified in several malignancies including prostate cancer (Richardson *et al.*, 2004) and are thought to drive primary tumourigenesis through self-renewal and differentiation. Additionally, the persistence of stem cells post-therapeutic intervention has been proposed as an explanation for metastasis and recurrence.

Recently it has emerged that solid tumours, tissues and cancer cell lines contain small populations of cancer stem cells. Stem cells can divide asymmetrically and produce two daughter cells; one identical cell and one programmed to differentiate into different mature cell types. Both normal stem cells and cancer stem cells can divide asymmetrically and they use this system in order to self-renew and differentiate (Clarke *et al.*, 2006). Similarities between cancer stem cells and normal stem cells during self-renewal and differentiation are striking and have facilitated identification of key stemness processes (Schoenhals *et al.*, 2009).

Several studies have demonstrated that cancer stem cells occur in various malignancies with self-renewal and differentiation; this is known as 'stemness' and can drive tumour growth (Li *et al.*, 2007 and Prince *et al.*, 2007). A single cancer stem cell is adequate to generate a *de novo* tumour (Al-Hajj *et al.*, 2003). The cancer stem cell paradigm indicates that these undifferentiated cells will not allow for successful treatment of the

tumour and allows for reccurrence and metastasis as they are resistance to current conventional cancer treatments and therapies (see Figure 3.1). Thus cancer stem cells that remain after cancer treatments may lead to metastasis and recurrence of the tumour. Thus the cancer stem cell phenotype needs to be targeted and treated as these cells are both key component in malignancy and recurrence (see Figure 3.1).

Cancer stem cells are thought to give rise to a clone or clones of cells that are differentiated and maintain a high survival level compared to the other cells contained within the tumour. Holoclones are described as a tightly packed clone of small cells generally thought to be stem and progenitor cells (Li *et al.*, 2008). These are tightly packed clones of cells generally contain both stem cells and progenitors (Li *et al.*, 2008). The holoclone is the only clonal type that has the ability to self-renew (Barrandon & Green, 1987). However Paraclones (described in section 1.6) are a loosely packed clone of large cells with a very short replicative life span (Li *et al.*, 2008). Meroclones contain a combination of cells of different proliferative potential and is a transitional stage between a holoclone and paraclone (Li *et al.*, 2008).

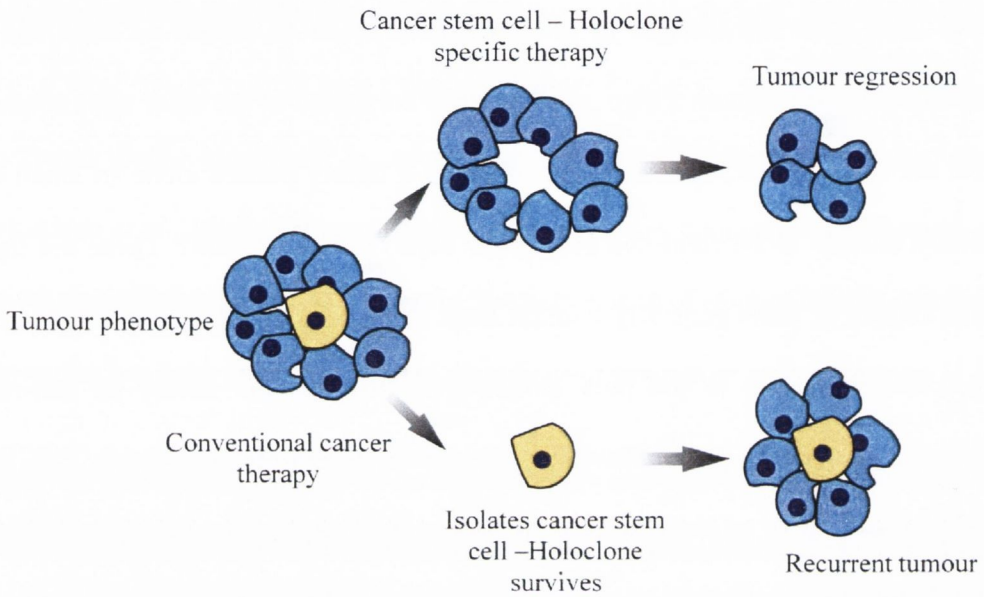



**Figure 3.1: Cancer stem cell Holoclone and recurrence.** The cancer stem cell theory suggests that tumours contain a small population of stem-like cells often called cancer stem cells/ tumour initiating cells/ Holoclones. These cells have been implicated in solid tumours also, such as; breast, brain and prostate and are resistant to conventional chemotherapy and radiation therapy.

### 3.1.2 All trans-Retinoic Acid treatment

Retinoic Acid is a retinoid and a lipophilic molecule and also a metabolite of Vitamin-A (all-trans-Retinol). It affects gene transcription and modulates a wide range of biological processes such as; cellular proliferation, differentiation, including apoptosis in normal and transformed cells (Lokshin *et al.,* 1999). Retinoic Acid mediated gene transcription depends on the rate of transport of Retinoic Acid to target cells and also the timing of exposure of Retinoic Acid to Retinoic Acid Receptors in the target tissues. All-trans-Retinoic Acid, the Carboxylic Acid form of Vitamin-A is of biological significance as it contains high circulating levels than various other isomers of Retinoic Acid. The targets of all-trans-RA and RA receptors include a large number of structural genes, oncogenes, transcription factors and cytokines.

RA functions have major importance in regulatory signaling of molecules for cell growth, differentiation and neurodegeneration both during embryogenesis and in adult tissues (Smith *et al.,* 1998). RA receptors mediate transcription of various different groups of genes that control differentiation of a variety of different types of cells; therefore the target genes regulated depend on the target cells. RA induced apoptosis through death receptors is a potentially promising approach for treatments of diseases like Schizophrenia, Alzheimer disease and also as a cancer therapy (Degos and Wang., 2001, Huang *et al.,* 1988, Castaigne *et al.,* 1990).

In this chapter, cancer cell lines representing a grade four adenocarcinoma and a metastatic carcinoma were treated with RA in order to further differentiate these cells. In terms of malignancy, differentiation levels are linked to the grade of the tumour. Tumours that contain high numbers of differentiated tissues such as; tumours that

develop from pluripotent cancer stem cells are considered to be malignant however they are low grade tumours. In contrast, tumours containing high numbers of undifferentiated cells are considered to be high grade malignancies.

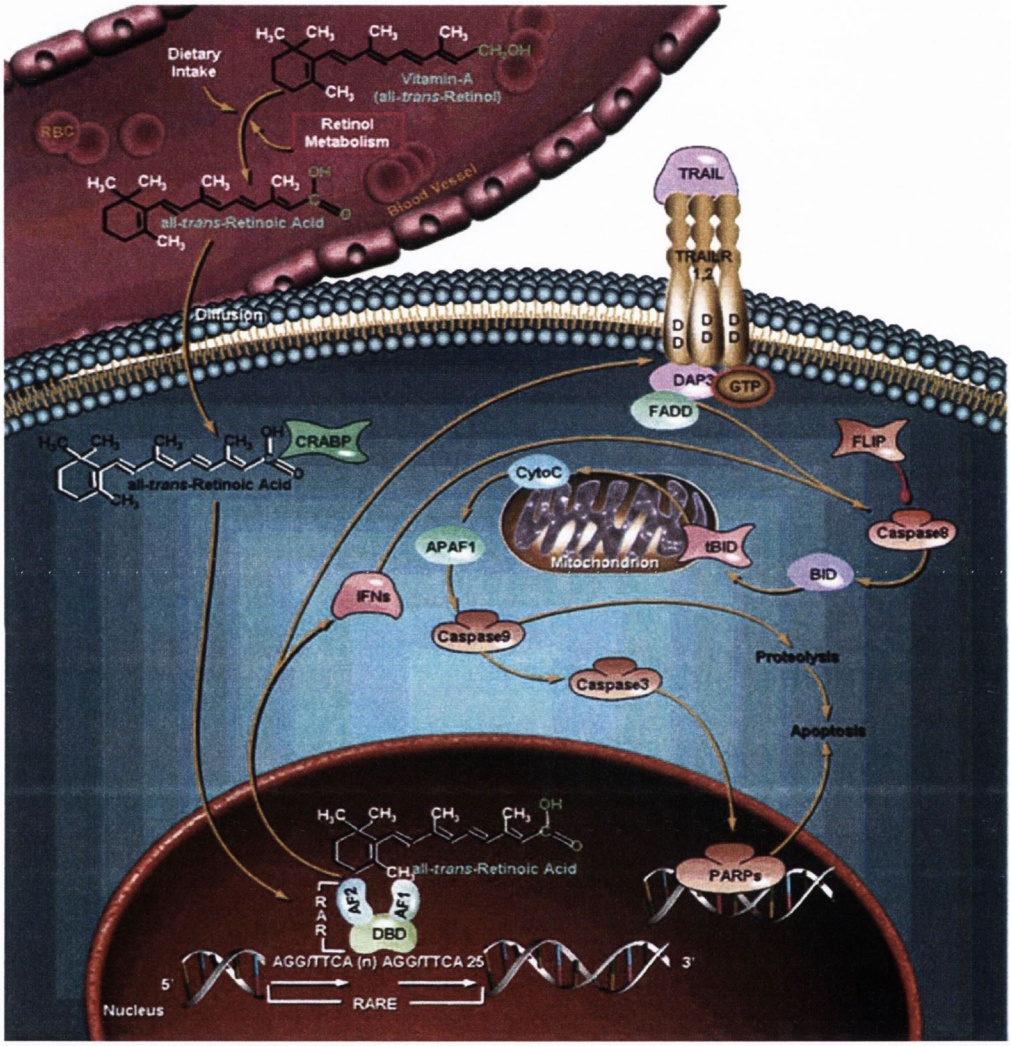



**Figure 3.2: All trans-Retinoic Acid signaling pathway.** Retinoic acid acts by binding to the retinoic acid receptor which is bound to DNA as a heterodimer with the retinoid X receptor in regions referred to as retinoic acid response elements. Binding of the retinoic acid ligand to RA receptors allows for the alteration of the conformation of the RA receptor which in turn affects the binding of other proteins. These proteins can either induce or repress transcription of a gene in close proximity such as the Hox genes and many other target genes. The control of RA levels is maintained by a collection of proteins which control synthesis and degradation. (www.sabiosciences.com).

### 3.1.3 High salt soft-agar assay

In this study, holoclones were generated using a high salt-soft agar assay (Hamburger and Salmon, 1977, Kern *et al.*, 2006, Papini *et al.*, 2003, Morel.*et al.*, 2008, Olszewski *et al.*, 2005). Cells were grown *in vitro* in soft agar also referred to as semi-solid agar. The semi-solid agar assay reduces the cell movement and allows individual cells to develop into clones that are identified as single colonies. This culture method is a 3-dimensional format rather than the traditional monolayer method and without any additional growth factors other than complete growth media the founder cell lines are cultured in normally. This colony forming assay selectively promotes the growth of cancer stem cells however it does not contain any colony stimulating growth factors and does not induce cancer stem cells or progenitors artificially.

This culture technique starts with single malignant cells in suspension which are incubated and cultured for a period of time until they reach appropriate confluence and then cultured in a bi-layered Petri dish. The cells are plated in the top layer containing complete growth media while the bottom layer contains the appropriate agarose and sodium mix which is semi-solid and solidified before the top layer is added.

Agarose is a marine polysaccharide, a hydrogel and is a model biopolymer in gelation. Agarose forms thermo reversible gels that are made up of thick bundles of agarose chains linked by hydrogen bonds and has large pores holding water. The mechanical properties of agarose gel are that of a network of mesh size pores that depends on the type of agarose, concentration and the setting/ solidifying temperatures.

Based upon these properties, this method is designed to assay the cell's ability to grow unattached to a surface and is therefore suspended in the pores of the agarose while the high sodium content selects for an isolated population of holoclone cancer stem cells. In the literature, colonies are defined as a group of thirty or more cells which are regarded as having originated from a single malignant cell after five or more cell divisions (Slee *et al.,* 1986).

### 3.1.4 Markers of miRNA processing machinery, differentiation, stemness genes and pathways

In this study, derived Holoclones and Retinoic Acid treated cell lines were characterized by the expression levels of markers of differentiation and key stemness genes and pathways and also miRNA processing machinery (Dicer, Drosha and eIF6). Expression patterns of these markers were assessed over time in order to determine the gene expression patterns characteristic to the Holoclones derived from the cell lines PC-3 and LNCaP and also the RA cell lines that were stimulated to further differentiate or "super-differentiate".

All stem cells are defined by their ability to self-renew and differentiate (Nussei., 2008). The self-renewal state is characterised by low expression levels of differentiation markers such as NCAM1 (ectoderm), ENO3 (mesoderm) and AFP (endoderm) and high expression levels of the genes Oct4 and Nanog and stemness pathway markers such as Wnt, Notch, Snail and Sonic Hedgehog (Chamber *et al.,* 2005, Gaiano *et al.,* 2002, Li *et al.,* 2006, Looijenga *et al.,* 2003, Logan and Nussi *et al.,* 2004, Pereiva *et al.*, 2006). However many of these genes are expressed in the reverse upon differentiation and demonstrate significant altered expression of these differentiation markers.

Recent studies have shown that a group of key signaling pathways are involved in the regulation of stemness. Sonic hedgehog is a secreted protein which regulates the cascading Hedgehog pathway and has been found to act as a regulator of cell fate determination (Hyslop *et al.*, 2005, Fong *et al.*, 2008). The Transforming Growth Factor signaling pathway has a major involvement in the cellular processes such as, cell growth differentiation, apoptosis and homeostasis. Analysis of these stemness pathways involvements in Holoclones and Retinoic Acid differentiated cell lines at the mRNA level is presented in the results section of this chapter. Real-time TaqMan® RT-PCR was the method employed to assess these markers at the mRNA level.

### 3.2 Chapter aims

The overall aim of this chapter was to generate a cancer stem cell model from the cell lines PC-3 and LNCaP and characterise their stemness characteristics. The first aim of this chapter was to treat cell lines PC-3 and LNCaP with Retinoic Acid in order to generate "super-differentiated" cells. The second aim of this chapter was to optimise and generate a prostate cancer holoclone cell model for both cell lines PC-3 and LNCaP. This was achieved by using a 3D culture method known as a high-salt soft-agar assa. The final aim was to then identify key stemness genes and pathways present in the "super-differentiated" and holoclone cell models when compared to that of the resting cell lines, achieved by Real-Time TaqMan® PCR.

### 3.3 Methods

Full details of methods employed are described in detail in chapter 2.

### 3.4 Results

#### 3.4.1 Retinoic Acid treated cell lines- induced differentiation

Both cell lines PC-3 which represents a Grade 4 non-metastatic adenocarcinoma and LNCaP which represents a metastatic carcinoma were cultured in their resting states and also treated with Retinoic Acid in order to induce the cells to further differentiate or 'super-differentiate'. The cells were harvested at the following time points; 0 days, 7 days and 14 days. Both PC-3 and LNCaP cells were treated with RA displayed a visible change in morphology in the presence of RA, Figure 3.3.

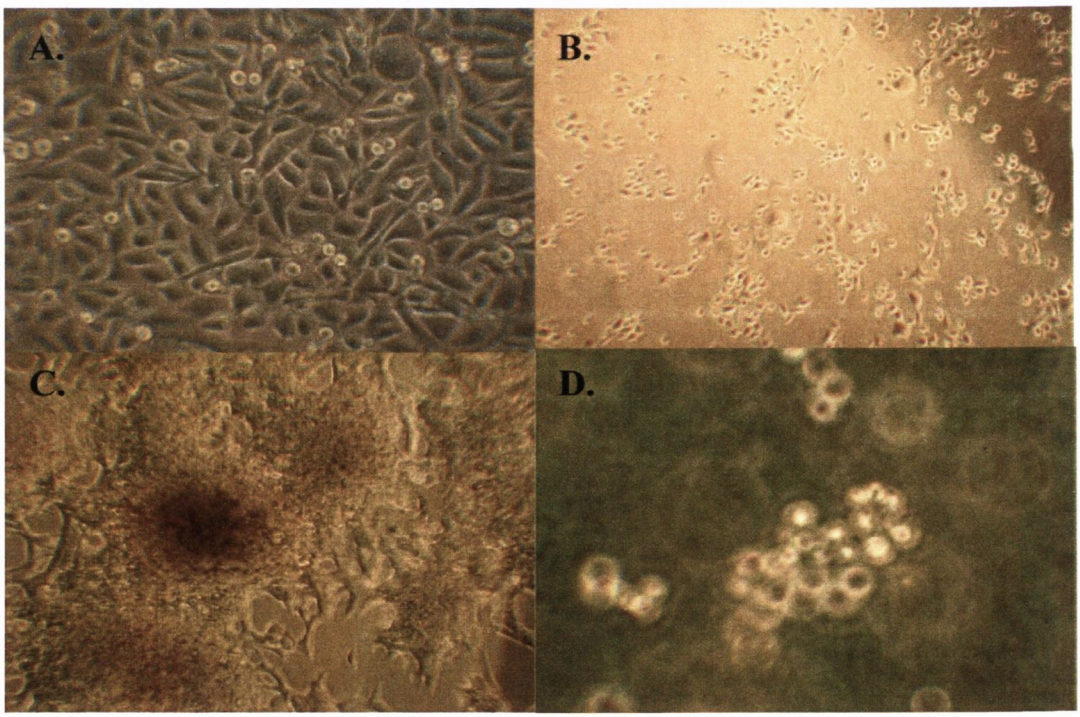



**Figure 3.3: Cell lines PC-3 and LNCaP morphology in the absence and presence of Retinoic Acid. A.** PC-3 cells cultured to confluence in their resting state in the absence of RA at time point Day 14 (60x). **B.** PC-3 cells treated with RA at time point Day 14 (10x). **C.** LNCaP cells cultured to confluence in resting state in the absence of RA at time point Day 14 (60x). **D.** PC-3 cells treated with RA at time point Day 14 (120x).

### 3.4.2 Assessment of RNA quantity and purity

Quantity and purity of total RNA isolated and purified from both cell lines in the presence and absence of RA was examined prior to quantative TaqMan® PCR analysis. In all cases, total RNA was determined by micro-volume quantitation using a Nanodrop 1000 spectrophometry. As recommended by manufactures instructions (Qiagen, Crawley, West Sussex, UK) only highly pure RNA with ration 1.8 to 2.1 measured at the absorbance 260nm to 280nm was included in the analysis, see Table 3.1.

**Table 3.1:** Total RNA quantity and purity of cell lines PC-3 and LNCaP ± RA.

| Cell lines | Quantity (ng/µl) | | | 260nm-280nm Ratio | | |
|---|---|---|---|---|---|---|
| | D0 | D7 | D14 | D0 | D7 | D14 |
| PC-3 Rep1 | 31.6 | 28.2 | 40.3 | 1.8 | 1.8 | 1.8 |
| PC-3 Rep2 | 47.9 | 41.6 | 40.8 | 1.8 | 1.8 | 1.9 |
| PC-3 Rep3 | 34.2 | 36.1 | 40.5 | 1.8 | 1.8 | 1.8 |
| PC-3+RA Rep1 | - | 26.2 | 36.9 | - | 1.8 | 1.9 |
| PC-3+RA Rep2 | - | 43.3 | 37.7 | - | 1.8 | 1.8 |
| PC-3+RA Rep3 | - | 27.2 | 31.8 | - | 1.8 | 1.8 |
| LNCaP Rep1 | 52.9 | 62.5 | 66.5 | 1.8 | 1.8 | 1.8 |
| LNCaP Rep2 | 64.5 | 13.9 | 68.0 | 1.8 | 1.8 | 1.8 |
| LNCaP Rep3 | 11.4 | 11.4 | 14.2 | 1.8 | 1.8 | 1.9 |
| LNCaP+RA Rep1 | - | 47.8 | 19.6 | - | 1.8 | 1.8 |
| LNCaP+RA Rep2 | - | 71.1 | 33.9 | - | 1.8 | 1.8 |
| LNCaP+RA Rep3 | - | 13.1 | 26.0 | - | 1.9 | 1.8 |

### 3.4.3 Evaluation of GAPDH gene as an endogenous control

Both PC-3 and LNCaP cells were cultured in their resting state and in the Retinoic Acid induced differentiated states and assessed at the following time points; 0 days (T0), 7 days and 14 days. GAPDH along with the non-template control were tested by quantative TaqMan® PCR to ensure that they were not affected by RA induced differentiation as GAPDH gene expression was successfully amplified and analysed and was shown not to be affected by differentiation and the NTC did not amplify in either states. Thus GAPDH proved to be a successful gene suitable for the use as an endogenous control and as the normalisation gene, see figure 3.4. Differentiation did not affect the NTC control which functioned as a negative control. GAPDH and NTC were included in all quantative TaqMan® PCR reactions employed in this chapter. GAPDH has been used as a reliable endogenous control in differentiating cells (Gallagher *et al.*, 2009, Willems *et al.*, 2006).

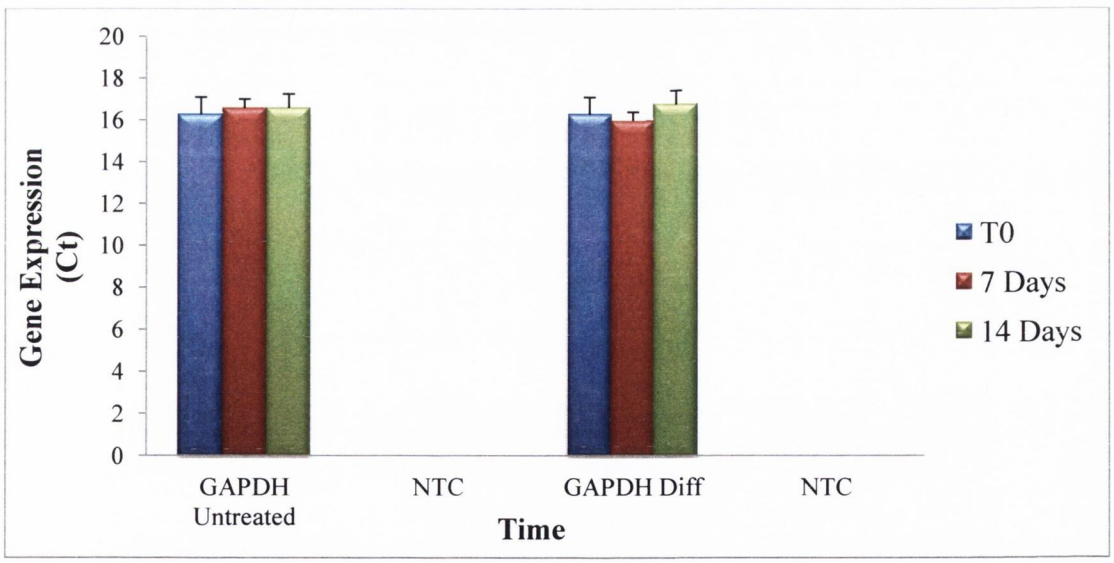
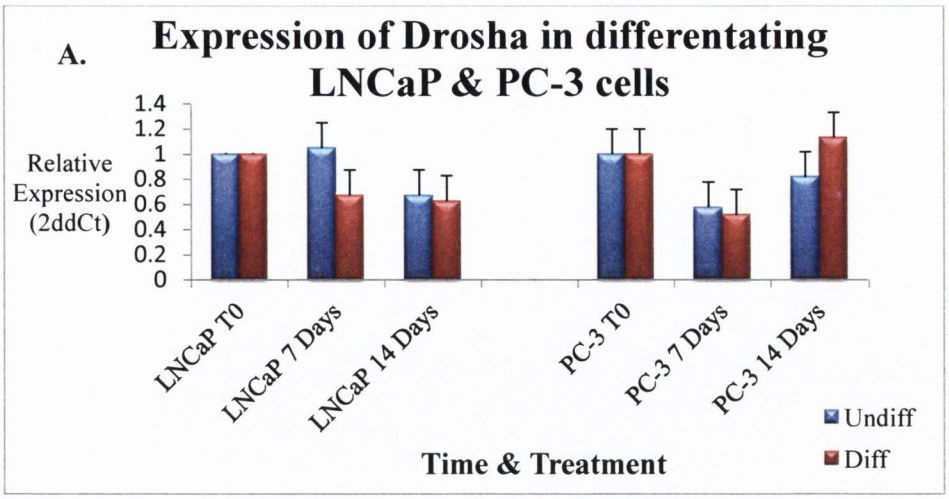
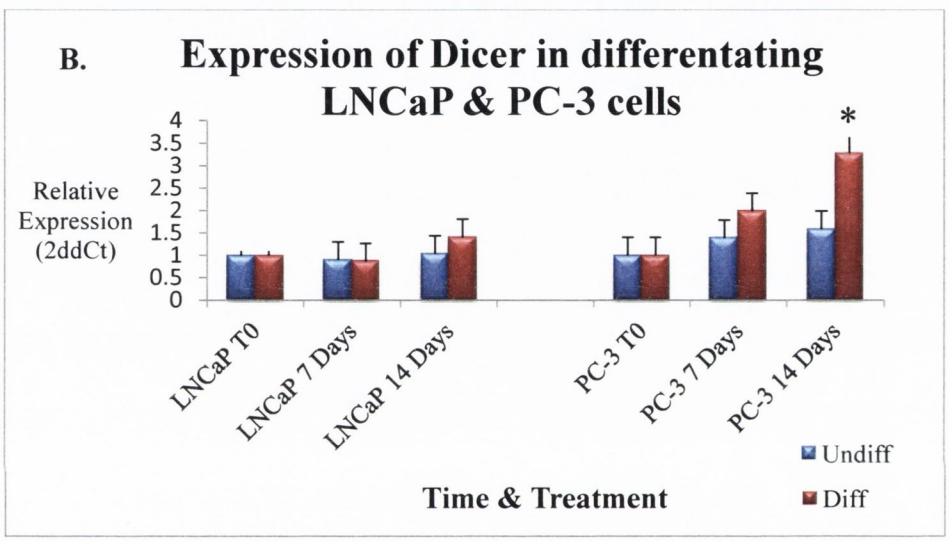



**Figure 3.4. Gene expression of GAPDH as an endogenous control.** Expression of internal endogenous control GAPDH and Non-template controls used in the PC-3 cell line in both states: untreated and treated (Diff) over a time period of time 0 (T0), 7 days and 14 days (n=3). Endogenous control gene GAPDH was detected and unaffected by induced differentiation confirming suitability as an internal endogenous control.

### 3.4.4 MiRNA processing machinery in PC-3 and LNCaP cells induced to differentiate

The expression of miRNA processing machinery was assessed in RA treated cells PC-3 and LNCaP stimulated to differentiate over time at the following time points; 0 days (T0), 7 days and 14 days. TaqMan® Real Time PCR was performed on cell lines treated with retinoic acid in order to induce differentiate and the cells were assessed for the gene expression of miRNA processing machinery of Drosha, Dicer and eIF6 at the mRNA level. A 2 fold increase or decrease is considered significant. Expression of Drosha and eIF6 did not display significant change in either the LNCaP or PC-3 cell induced to differentiate over time. The expression of Dicer was significantly ($P<0.0038$) increased in PC-3 cells over time after being stimulated to differentiate, see Figure 3.5 A & C. PC-3 RA treated cells increased 1 fold after 7 days and increased 2.4 fold after 14 days however Dicer expression in LNCaP RA treated cells stimulated to differentiate did not display significant change over time, see Figure 3.5 B.



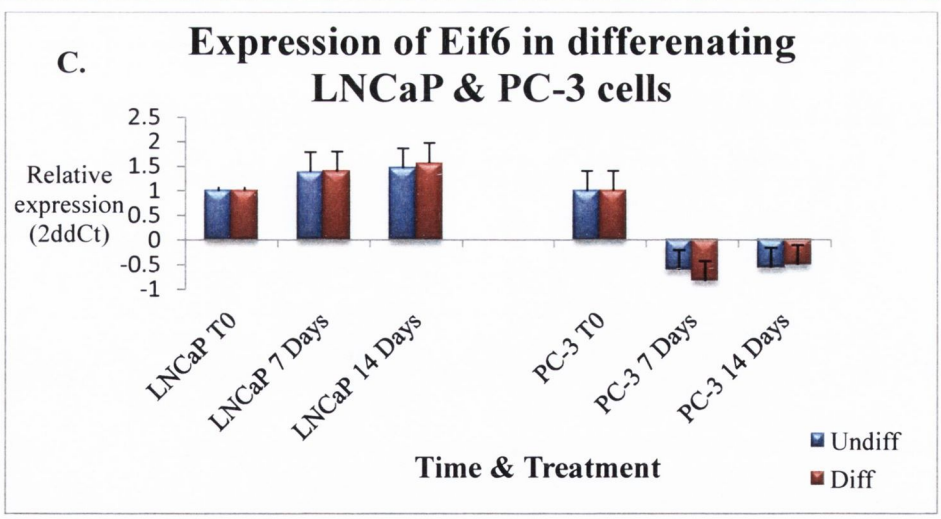




**Figure 3.5: miRNA processing machinery in PC-3 and LNCaP cell lines. A.** Expression of the Drosha enzyme in both PC-3 and LNCaP cells and in Retinoic Acid treated differentiating cells (n=3). **B.** Expression of the Dicer enzyme in both PC-3 and LNCaP cells and in Retinoic Acid treated differentiating cells * $P<0.0038$ (n=3). **C.** Expression of the Eif-6 in both PC-3 and LNCaP cells and in Retinoic Acid treated differentiating cells (n=3).

### 3.4.5 Expression of key stemness genes and pathways

The expression of key stemness pathways were examined in cells during RA induced differentiation of the LNCaP cell line over time at the following time points; 0 days, 7 days and 14 days. TaqMan® Real Time PCR was performed on LNCaP cell lines treated with retinoic acid in order to induce differentiate and the cells were assessed for the expression makers of key stemness genes and pathways as follows; TGF-β2, Wnt5a, Snail2, Notch2 and Shh at the mRNA level. A 2 fold increase or decrease is considered significant.

Notch2 and Wnt5a were significantly downregulated in the LNCaP cells over time after being stimulated to differentiate and were decreased to -3.4 fold after 14 days, see Figure 3.6 A. TGF-β2 was significantly upregulated in LNCaP RA treated cells over time. At day 7 LNCaP cells over time after being stimulated to differetiate increased 1 fold and increased further by 3.5 fold after 14 days. Expression of Snail2 did not display significant change in LNCaP cells induced to differentiate over time.

PC-3 cells induced to differentiate were also assessed for the expression markers TGF-β2, Wnt5a, Snail2, Notch2 and Shh at the mRNA level however only Shh displayed significant upregulation. Sonic Hedgehog was significantly upregulated in both PC-3 and LNCaP cells over time after being stimulated to differentiate. PC-3 RA treated cells demonstrated a 4 fold increase after 7 days and increased further from 4 to 16 fold after 14 days, see Figure 3.6 B. LNCaP RA treated cells demonstrated an 8 fold increase after 7 days and increased further from 8 to 22 fold after 14 days, see Figure 3.6 B. Four out of the five key stemness pathways demonstrated a fluctuation in expression in LNCaP cells over time stimulated to differentiate. Therefore stemness marker genes were both

significantly downregulated and upregulated in treated differentiating cells but not in the untreated founder LNCaP cells.

Both PC-3 and LNCaP cell lines showed a high level of increased expression of Sonic Hedgehog in treated differentiating cells when compared to untreated cells at the time points of 7 days and more significantly at 14 days. However, the LNCaP cell lines showed the most significant level of expression changes including the stemness markers Notch2 and Wnt5a which were significantly downregulated in the RA treated LNCaP cells stimulated to differentiate. However this was not shown in RA treated induced to differentiate PC-3 cell line. TGF-β2 was significantly upregulated in the LNCaP RA treated cells when compared to the untreated founder cells also and once again this pattern of expression was not displayed in the PC-3 RA treated or founder cells. The metastatic cell line LNCaP demonstrates that it retains the potential/ability to be further differentiated indicating stemness properties within this cell line. This is further supported by the increased expression levels of stemness markers versus the untreated cell line and the PC-3 cells.

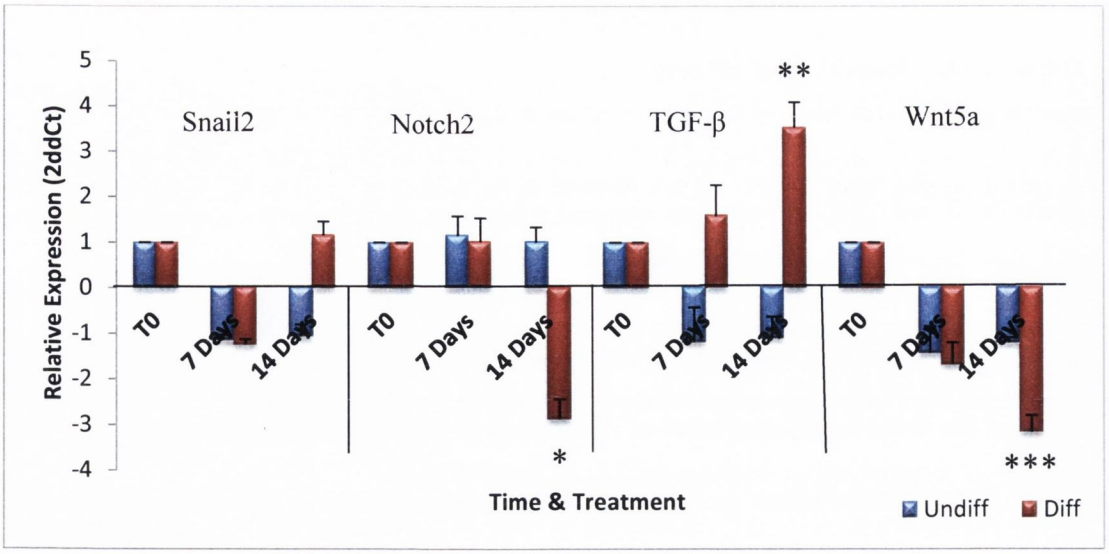



**Figure 3.6 A: Expression of key stemness and pathway markers in differentiating LNCaP cells.** Relative expression of Snail2, Notch2, TGF-β and Wnt5a in differentiating LNCaP cells compared to untreated LNCaP cells at corresponding time points * P< 0.005 ** P< 0.0018 *** P<0.004 (n=3).

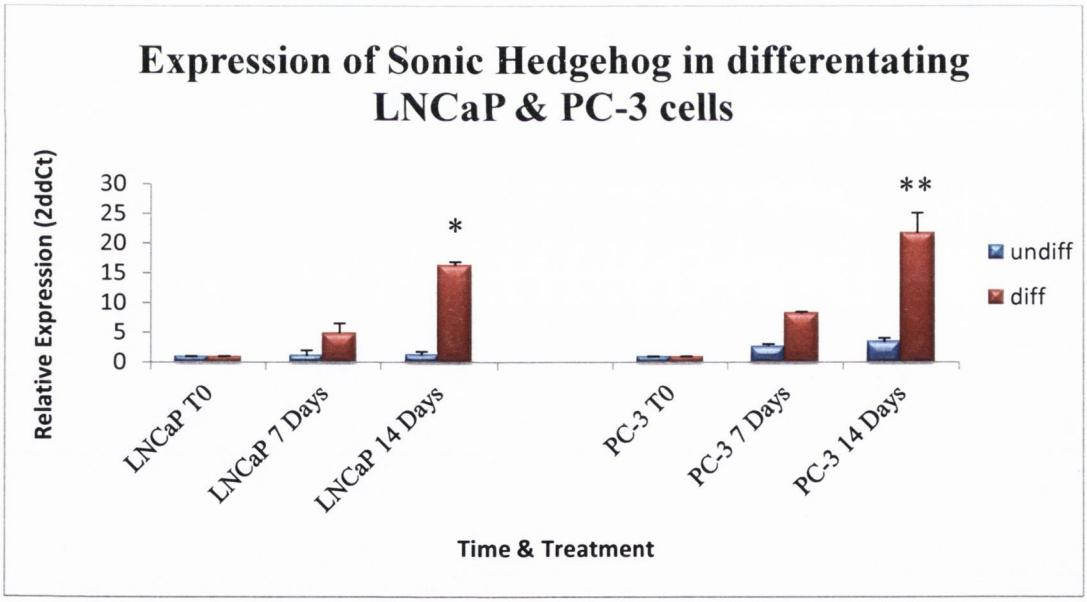
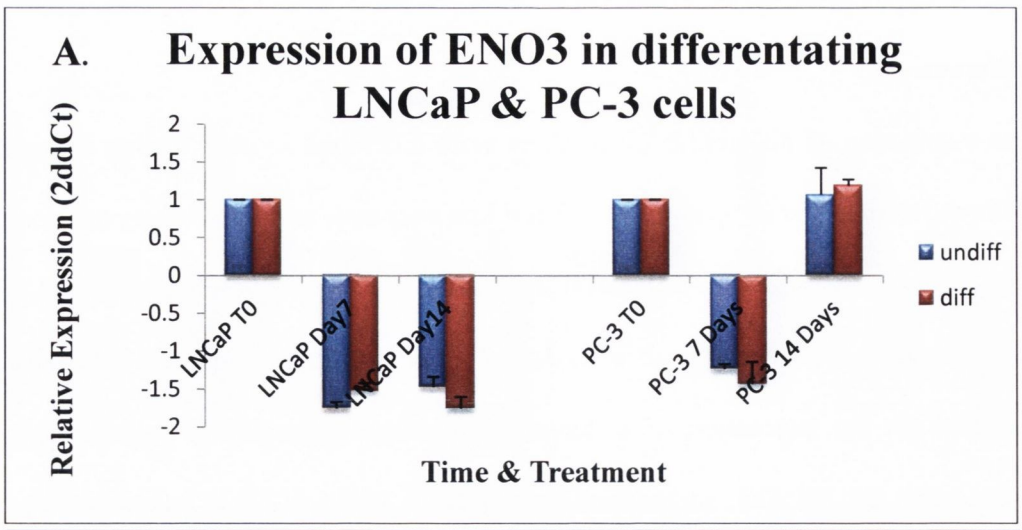


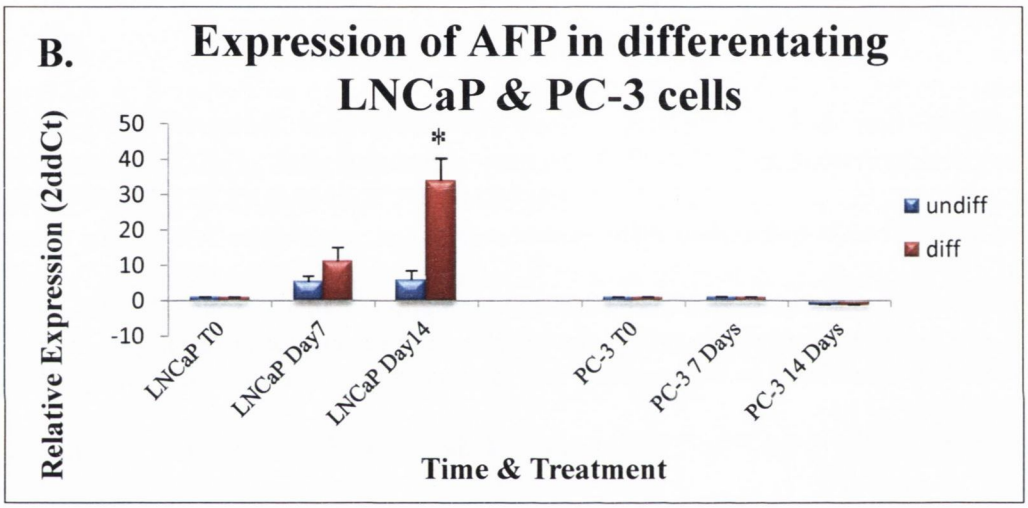

**Figure 3.6 B: Expression of key stemness and pathway marker Sonic Hedgehog in differentiating LNCaP cells.** Relative expression of Shh in differentiating LNCaP and PC-3 cells compared to untreated LNCaP and PC-3 cells at corresponding time points * P<0.007, ** P<0.001, (n=3).

### 3.4.5.1 Expression of differentiation markers PC-3 and LNCaP cells induced to differentiate

The expression of differentiation markers were examined in cells during RA induced differentiation of the PC-3 and LNCaP cell line over time at the following time points; 0 days, 7 days and 14 days. TaqMan® Real Time PCR was performed on LNCaP cell lines treated with retinoic acid in order to induce differentiation and the cells were assessed for the expression of differentiation maker genes ENO3 (mesoderm), AFP (endoderm) and NCAM1 (ectoderm) at the mRNA level. A 2 fold increase or decrease is considered significant.

The mesoderm marker ENO3 did not display significant gene expression change in PC-3 or LNCaP cells induced to differentiate over time, see Figure 3.7 A. The endoderm marker AFP was significantly ($P<0.0016$) upregulated in the LNCaP cells over time after being stimulated to differentiate and displayed a 9 fold increased after 7 days and increased further from 9 to 33 fold after 14 days however expression of AFP did not display significant change in PC-3 cells induced to differentiate over time, see Figure 3.7 B. The ectoderm marker NCAM1 was also significantly ($P<0.006$) upregulated in the LNCaP cells over time after being stimulated to differentiate and displayed a 1.2 fold increased after 7 days and increased further from 1.2 to 3 fold after 14 days however expression of AFP did not display significant change in PC-3 cells induced to differentiate over time, see Figure 3.7 C.



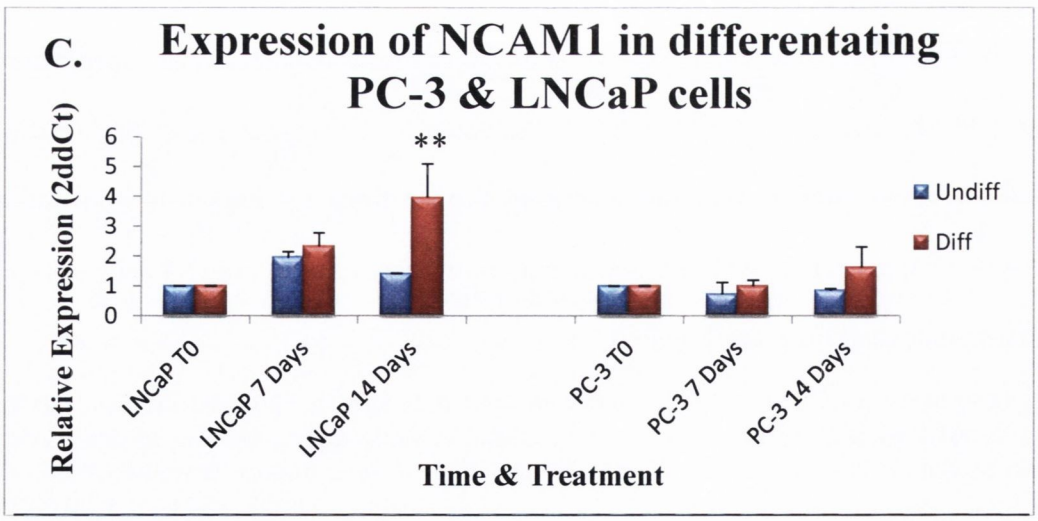




**Figure 3.7: Differentation phenotypes in LNCaP and PC-3 cell lines**. Relative expression of ENO3, AFP and NCAM1 in LNCaP and PC-3 cells treated with RA stimulated to differentiate over time, day 0, day7 and day 14 in comparison to founder untreated cells. T0 is set at 1, * $P<0.0016$, ** $P<0.006$, (n=3).

### 3.4.6 Creation of prostate cancer stem cell progenies: Holoclones

Holoclones were cultured using a high-salt soft-agar assay for metastatic LNCaP (and non-metastatic PC-3 cell lines. LNCaP holoclones were maintained for 26 days as this was when cells reached optimal growth. PC-3 holoclones were maintained for 6 days as this was when their optimal growth peaked, after this time point the holoclones growth declined (see Figure 3.6). Holoclones were generated in NaCl as the high salt content allows for the selective growth of holoclones. The paraclones and meroclones cannot survive in this environment therefore selecting for a pure population of holoclones.

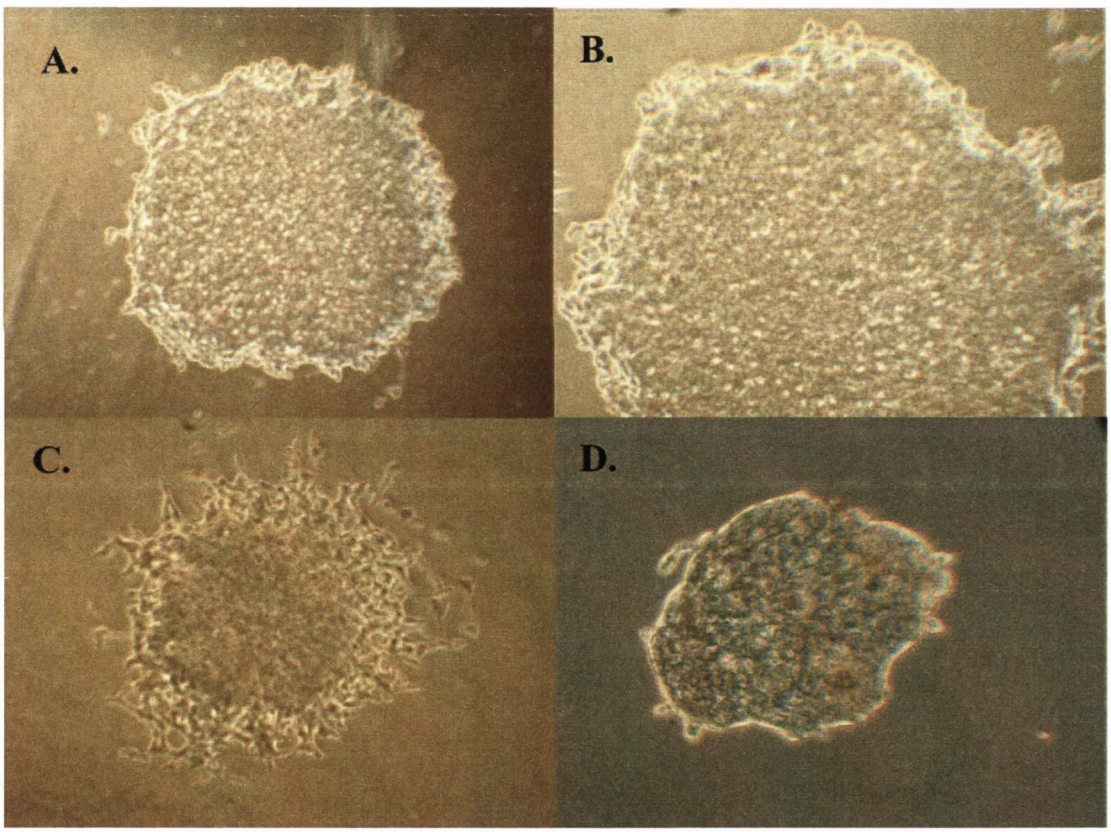



**Figure 3.8: Holoclones derived from cell lines PC-3 and LNCaP. A.** LNCaP Holoclone derived from LNCaP cell line embedded in high salt soft agar assay at day 24 (20X). **B.** LNCaP Holoclone derived from LNCaP cell line embedded in high salt soft agar assay at day 24 (40X). **C.** Formation of LNCaP Holoclones at day 20. **D.** PC-3 Holoclone derived from PC-3 cell line embedded in high salt soft agar assay at day 6 (20X).

### 3.4.7 Optimisation of total RNA in PC-3 and LNCaP Holoclones

Optimisation of total RNA isolation was performed in order to isolate total RNA as holoclone cell pellets contained small amounts of semi-solid agarose. Quantity and purity of total RNA from both cell lines and derived holoclones were examined, as holoclones were embedded and extracted from semi-solid agarose. The Trizol® kit (Invitrogen Life technologies, Maryland, USA) isolated high quality RNA however a very low yield (n=3) was obtained, see Table 3.2 A. The mirVana™ miRNA Isolation kit (Ambion at Applied Biosystems, Foster City, CA, USA) generated high quantity and pure total RNA from the holoclones thus this kit was used effectively to isolate total RNA, see Table 3.2 B. Founder cell lines total RNA was also isolated using this kit, see Table 3.2 C. In all cases the total RNA was determined by micro-volume quantitation using a Nanodrop 1000 spectrophometry. As recommended by manufactures instructions (Ambion at Applied Biosystems, Foster City, CA, USA) only highly pure RNA with ratio of 1.8 to 2.1 measured at the absorbance 260nm to 280nm was included in the analysis.

**Table 3.2 A:** Total RNA quantity and purity of LNCaP Holoclones isolated using the Trizol® kit.

| Holoclones | Quantity (ng/µl) | 260-280nm Ratio |
|---|---|---|
| LNCaP Holoclone Rep1 | 4.4 | 2.0 |
| LNCaP Holoclone Rep2 | 5.6 | 2.2 |
| LNCaP Holoclone Rep3 | 7.9 | 1.9 |

**Table 3.2B:** Total RNA quantity and purity of PC-3 and LNCaP Holoclones from the mirVana™ miRNA Isolation kit.

| Holoclones | Quantity (ng/µl) | 260-280nm Ratio |
|---|---|---|
| PC-3 Holoclone Rep1 | 28.8 | 1.9 |
| PC-3 Holoclone Rep2 | 29.3 | 1.8 |
| PC-3 Holoclone Rep3* | 278.4 | 1.9 |
| LNCaP Holoclone Rep1 | 21.6 | 1.8 |
| LNCaP Holoclone Rep2 | 24.8 | 1.8 |
| LNCaP Holoclone Rep3 | 33.6 | 1.8 |

*PC-3 Holoclone Rep3 yielded approximately 10 fold more Total RNA due to improved isolation technique of Holoclones from the agarose.

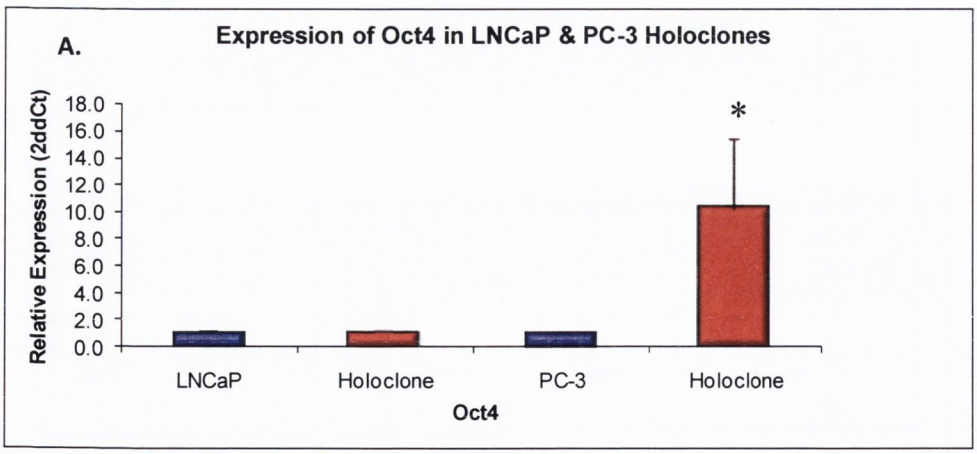
**Table 3.2 C:** Total RNA quantity and purity of cell lines PC-3 and LNCaP Holoclones from the mirVana™ miRNA Isolation kit.

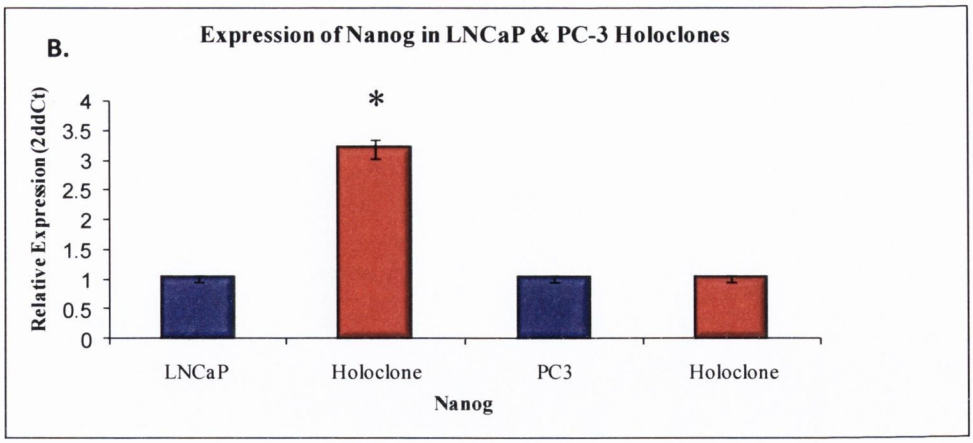
| Cell lines | Quantity (ng/µl) | 260-280nm Ratio |
|---|---|---|
| PC-3 Rep1 | 744.0 | 2.1 |
| PC-3 Rep2 | 913.9 | 2.1 |
| PC-3 Rep3 | 781.9 | 2.1 |
| LNCaP Rep1 | 1791.1 | 2.0 |
| LNCaP Rep2 | 1692.1 | 2.0 |
| LNCaP Rep3 | 2175.7 | 2.0 |

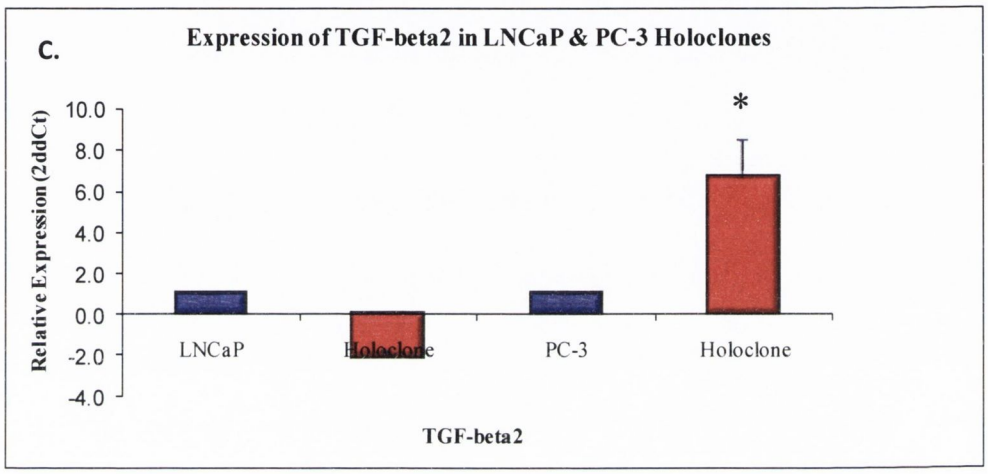
### 3.4.7.1 Characterisation of stemness potential in LNCaP and PC-3 holoclones

The expression of key stemness genes and pathway markers were examined in PC-3 and LNCaP holoclones. TaqMan® Real Time PCR was performed on holoclones and was assessed for the expression of key stemness genes and pathways: TGF- β2, Oct4, Sonic Hedgehog and Nanog at the mRNA level. A 2 fold increase or decrease is considered significant. Both LNCaP and PC-3 holoclones showed significant increased levels of stemness markers compare to the resting cell lines.

Oct4 was found to be significantly upregulated ($P<0.044$) in the PC-3 holoclones and displayed a 9.5 fold increase when compared to the founder cell line. Nanog was significantly upregulated ($P<0.017$) in the LNCaP holoclones and displayed a 2.3 fold increase when compared to the founder cell line. TGF- β2 demonstrated a significant ($P<0.00038$) 5.8 fold increase in PC-3 holoclones when compared to the founder cells. Sonic Hedgehog was found to be significantly upregulated ($P<0.0003$) in the LNCaP holoclones and displayed a 1250 fold increase when compared to the founder cells, see Figure 3.7.





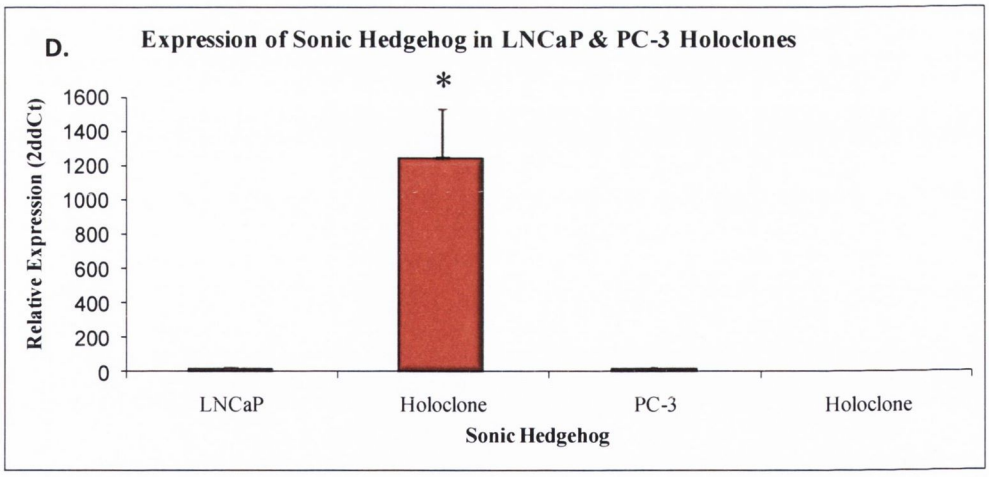




**Figure 3.9: Expression analysis of stemness pathway in PC-3 and LNCaP cells and holoclones when compared to founder cells. A.** Expression of Oct4 in LNCaP and PC-3 holoclones *P<0.044, n=3. **B.** Expression of Nanog in LNCaP and PC-3 holoclones *P<0.017, n=3. **C.** Expression of TGF-β2 in LNCaP and PC-3 holoclones *P<0.00038, n=3. **D.** Expression of Sonic Hedgehog in LNCaP and PC-3 holoclones *P<0.0003, n=3.

### 3.5 Discussion

Cancer stem cells undeniably are present in various tumour types including the prostate. This hypothesis could possibly clarify both the heterogeneity of these tumours and also the variable responses of prostate tumours to several conventional therapies such as radiotherapy and chemotherapy. Long term therapies directed against the cancer stem cell rather than the actual tumour itself could offer a real potential to the long term successful treatment and therapies and also generate useful biomarkers for the precise and successful and early detection of prostate cancer. Identifying specific tumour stem cell markers could potentially enhance this process, such as identifying unique cancer stem cell specific patterns of gene expression. Since one of the main characteristics of a stem cell is pluripotency, the ability of the cells to differentiate was assessed. This was achieved by examining and characterising the miRNA processing machinery, differentiation and stemness potential by analysing the following generated cell types;

- Retinoic Acid treated cells induced to differentiate
- A pure population of cancer stem cell Holoclones

Broadly, two main questions were addressed in the super-differentiated cells, as follows; is each gene or pathway representing stemness and differentiation regulated in both cell lines in the presence or absence of Retinoic Acid. The comparisons addressed in this section included PC-3 and LNCaP resting cells in their founder state and cells treated with RA to induce differentiation. As expected key processes were detectably regulated in RA treated differention induced cells. PC-3 and LNCaP in the presence and absence of RA were analysed as follows;

- Effects on differentiation marker genes: NCAM1, AFP and Eno3
- Key stemness pathways and modulators: TGF-β, Shh, Wnt, Notch and Snail
- miRNA processing machinery: Drosha, Dicer and Eif6

Upon RA treatment of the both cells PC-3 and LNCaP stimulated to differentiate, the morphological appearance of both cells changed considerably over time when compared to the founder untreated cells over the time points of day 7 and more noticeable at the time period of day 14.

These markers clearly show that the LNCaP cells treated with Retinoinc Acid showed increased expression levels of stemness genes when compared to that of the founder, untreated cell lines. The PC-3 cell line displayed an increased expression level in Sonic Hedgehog only. However, the other markers were tested but did not display any significance expression levels in TGF-β, Oct4a, Snail2 or Wnt5a. Therefore this data suggests that the LNCaP cell line induced to differentiaite displays more stemness potential than the PC-3 cell line treated with RA. Analysis of these cell lines strongly indicates that the metastatic phenotype has greater stemness potential. Currently there is no published literature available in relation to this stemness expression at the mRNA level in either PC-3 or LNCaP cells in the presence of RA.

The Dicer, Drosha and eIF6 expression at the mRNA level was analysed in both PC-3 and LNCaP cells in the presence and absence of RA to induce differentiation. Drosha and eIF6 did not display a significant change in expression associated with either cell lines in either states over time. However Dicer showed significant altered upregulated expression in PC-3 RA treated differentiating cells when compared to untreated cells at

the time points of 7 days and more significantly at 14 days. MiRNA processing machinery enzyme Dicer displayed altered upregulated expression is associated with the non-metastatic PC-3 cell lines stimulated to differentiate but not in the untreated founder cells. Currently there is no published literature available in relation to Dicer, Drosha and eIF6 expression at the mRNA level in either PC-3 or LNCaP cells in the presence of RA.

The RA treated cells differentiation responses of differentiation responses of differentiation markers NCAM1, AFP and Eno3 were assessed. The expression of two out of the three differentiation markers were detectable in the cell lines treated with RA. The expression of AFP was detectable in LNCaP RA treated cells with a significant increase in gene expression when compared to the LNCaP founder cells not treated with RA. AFP was found to be regulated over time in LNCaP RA treated cells therefore displaying an increase in endodermal differentiation in this phenotype. The data demonstrates that mesodermal differentiation is not functional in either the PC-3 or LNCaP resting cells or the RA treated cells as Eno3 was not significantly regulated over time. This data suggests that ectodermal and ectodermal differentiation is important in the LNCaP RA treated differentiating cells over time. Currently there is no published literature available in relation to AFP, NCAM1 and Eno3 expression at the mRNA level in either PC-3 or LNCaP cells in the presence of RA.

When cells are cultured by the regular monolayer method, they soon lose their more differentiated phenotypes. This is usually due to differentiating cells being shed into the medium and a failure to develop inter-cellular interactions between the cells that are present in three dimensional tissues (Hudson. 2003). The culture method that allows for

close similarity to this *in vivo* environment is the growth of cells suspended in a semi-solid gel such as agarose. Only a very small percentage of cells contained within the cell lines have the ability to establish holoclones, however the majority of the cells can form paraclones and meroclones (Li *et al.*, 2008). The high salt soft-agar culture method allowed for the generation of three dimensional spherical colonies that maintained a steady and constant growth pattern while the sodium selected for the generation of a pure holoclone population contained within the agarose gel. In contrast, paraclones and meroclones cannot be cultured or propagated over time as they have a short life-span, uniformly abort and terminally differentiate.

Holoclones were generated from cell lines (LNCaP, PC-3) using a high salt-soft agar assay. LNCaP holoclones were maintained for 24 days and PC-3 holoclones were maintained for 6 days. The differential duration of the time points denotes that the LNCaP cell line represents a metastatic prostatic carcinoma and should potentially contain a higher number of cancer stem cells (Dalerba and Clarke., 2007, Diehn and Majeti., 2010 Wicha., 2006). In combination, stemness genes were used to exemplify the stem cell potential and characteristics of these hololcones. Expression profiles were generated for genes and pathways representing stemness. LNCaP and PC-3 holoclones both indicated stemness characteristics. However both LNCaP and PC-3 holoclones expressed different stemness markers and this disparity was also further represented by the duration differences in time points.

Stemness potential of the LNCaP and PC-3 holoclones were assessed at the mRNA expression level by TaqMan RT-PCR using the following key stemness genes and pathways; Sonic Hedgehog, TGF-β, Oct4a and Nanog. These markers clearly show that

the LNCaP holoclones showed increased expression levels of stemness genes when compared to that of the founder, untreated cell lines; these include Nanog and Sonic Hedgehog. However the PC-3 holoclones also displayed increased levels of stemness expression but in Oct4 and TGF-beta and not Nanog or Sonic Hedgehog. This indicates that both the generated holoclones from LNCaP and PC-3 are two very different cancer stemness phenotypes.

The PC-3 Holoclone displayed a significant increase in TGF-β2 expression when compared to the founder cell line. The TGF-β2 pathway has been shown in cancer cells to lose the ability to control the cell as it cannot control the cell normally and allows the cancer cell to proliferate; various parts of the pathway are mutated as the cell transforms from a normal cell to a cancer cell. It is also known to induce differentiation, an important stem cell property. As the gene expression of this pathway is significantly increased in the PC-3 Hololcones this data indicates that the Holoclone is a more potent cancer cell than the founder cell and also displays stem cell properties.

Regulation of Oct4 was significantly ($P<0.044$) increased in the PC-3 holoclones also when compared to the founder cells. Oct4 has been shown to play an essential role in the maintance of pluripotency of cells (Kehler *et al.*, 2004). Oct4 is also involved in the undifferentiated phenotype of tumours. Therefore this data has identified that both TGF-β2 and Oct4 expression is a key difference between the founder PC-3 cell and the derived cancer stem cell holoclones and both are extremely important to stemness.

Sonic Hedgehog was significantly upregulated in the LNCaP holoclones when compared to the founder LNCaP cells. Shh plays a crucial role in development and is

involved in processes such as, controlling cell division of the adult stem cells and also in the development of various cancers in organs including the prostate. Nanog too was significantly ($P<0.017$) increased in the LNCaP holoclone. Nanog is one of three genes involved in maintaining pluripotent stem cells, is expressed in embryonic stem cells and also functions in establishing embryonic stem cell identity. The data generated displays the differences between the holoclones and the founder cells and suggests that the holoclones are a much more potent cancer cell than the founder cells. This data further demonstrates the stem cell characteristics present in the LNCaP holoclones is more significant and highly expressed than in the founder LNCaP and PC-3 cells, further supporting the cancer stem cell hypothesis.

Currently in the literature it is known that the epidermal growth factor receptor (EGFR) and the hedgehog cascades provide a crucial role in the progression of prostate cancer and is also known to contribute to the resistance of current clinical therapies and future disease relapse (Mimeault *et al.,* 2007). The LNCaP holoclone displays an increase in SHH expression indicating that the holoclone cancer stem cell could potentially be the driver of prostate cancer progression and could also cause or contribute to disease recurrence.

As Hedgehog signaling is thought to play a role in prostate cancer and although prostate cancer cells express many of the gene products involved in hedgehog signaling, these cells are known to be refractory to the canonical signaling effects of exogenous hedgehog ligands. It has been shown that the expression of hedgehog ligands and some hedgehog target genes are regulated by androgen in the LNCaP cells (Chen *et al.*, 2009). This group showed that androgen strongly suppressed the expression of

hedgehog ligands and further prolonged their maintenance in androgen-deficient medium upregulated Sonic and Indian hedgehog mRNA levels by up to 30,000-fold. The data from this group correlates with the SHH data generated from the LNCaP holoclones and also the Retinoic Acid treated cell line in this chapter. Androgen is known to have the ability to modulate the expression and release of hedgehog ligands and activity of the autocrine hedgehog signaling pathway in prostate cancer cells, the results from Chen *et al.,* 2009 suggest that chronic androgen deprivation therapy for prostate cancer might create a hedgehog signaling environment in the region of the tumor that could ultimately impact on the long term effectiveness of treatments in patients with advance prostate cancer. The data from this group supports the data generated here from the LNCaP holoclones. However, currently there is no literature on the mRNA stemness expression in LNCaP cancer stem cells or holoclones. As previously mentioned the LNCaP holoclone displayed and increase expression of Nanog and this data correlates with the current literature from Jeter *et al.,* 2011. This group has shown that Nanog promotes the characteristics of cancer stem cells and importantly prostate cancer resistance to androgen deprivation. Within the context of the current literature this indicates that the LNCaP holoclone could potentially enhance the cancer cells ability to proliferate and self-renew.

In relation to the PC-3 holoclones, Jeter *et al.*, 2011 have shown that the knock-down of Nanog in the cells significantly decreased long-term clonogenic growth and also substantially inhibited tumor growth. Oct4 too has been shown to play important roles in the progression of prostate cancer (Sotomayor *et al.,* 2009) which the data in this chapter indicates that the PC-3 holoclones display an increase expression of Oct4

indicating that these cancer stem cell phenotypes may greatly aid prostate cancer progression.

LNCaP cell lines can be further differentiated upon Retinoic Acid treatment. Further analysis displayed an increased unregulated expression of genes representing differentiation and stemness. Cancer stem cell progenies; holoclones were generated and an isolated population was obtained with the high salt soft-agar assay and these cells were shown to display stemness characteristics as predicted. However both the PC-3 and LNCaP cell lines generated different cancer stem cell phenotypes.

# Genome expression profiling of Holoclones

Chapter 4

## 4.1 Introduction

Investigations into molecular signatures rather than specific individual molecules allow for a greater understanding of the cell or tumour cell characteristics enabling a more complete evaluation of the pathogenesis of tumour development, initiation and progression. No one specific gene or pathway completely defines the phenotype of any tumour cell. The majority of malignant cells display deregulation of many cellular pathways, and it is this combined disarray that defines a tumour cells' specific characteristics. Significant gene expression levels in various pathways constitute the molecular signature of the cell.

Currently there are many microarray studies of prostate cancer (Ho *et al.*, 2002, Milton *et al.*, 2007, Sørensen *et al.*, 2010). Many of these microarray studies published to date have identified specific genes that show differential gene expression between the various types of prostate cancer and benign prostate tissue correlates with multiple clinical outcomes. However there are no microarray studies of prostate cancer stem cell or stem-like holoclone populations.

DNA microarray analysis is a high throughput technique that allows for large scale gene expression analysis. With high density microarrays, it is possible to detect and monitor sequence and expression changes simultaneously and rapidly in thousands of genes involved in numerous cellular pathways (Ho *et al.*, 2002). Thus, multiple mRNA patterns can be analysed by these microarrays. The microarray technology base allows for the analysis of many mRNA expression patterns and ensures for an unbiased approach to study downstream effects on gene expression alterations and allows for gene function to be determined. Without this type of large scale gene expression

analysis, only a small number of genes could be investigated at any one given time and the interactions between many different genes, their function and genetic pathways would not be feasible.

In this chapter, the Affymetrix Human GeneChip® Gene 1.0 ST Array System was used to generate a whole genome profile of the founder cell lines and derived holoclones. High throughput DNA microarrays generate extensive quantities of data that require a great deal of analysis in order to generate relevant results such as, identifying key genes and pathways of relevance from the thousands of genes that are significantly upregulated and downregulated in each holoclone and cell line sample. Expression microarray analysis of the cell lines PC-3, LNCaP and their derived holoclones allowed for the following analysis to be assessed;

- Generation of a genome wide expression profile in the holoclones and founder cell lines.
- Determining the differences between holoclones and the founder cell lines.
- Identification of genes and pathways implicated in tumour formation, progression and possibly recurrence: including genes involved in stemness, differentiation and EMT present in the holoclones.

## 4.2 Chapter aims

The aim of this chapter was to genome profile PC-3 and LNCaP cell lines and PC-3 and LNCaP holoclones and to identify specific cellular targets representing stemness, differentiation and epithelial-mesenchymal transition. This was achieved by determining genome-wide alterations induced at the transcriptome level in all of these cells which were applied to Affymetrix GeneChip® microarrays. This allowed for the analysis of gene expression alterations present in holoclones when compared to that of the relevant founder cell line.

## 4.3 Methods

Full details of methods employed are described in detail in chapter 2.

## 4.4 Results

### 4.4.1 Microarray quality control

#### 4.4.1.1 Total RNA for microarray analysis

High quality and quantity of purified total RNA is essential for successful gene expression analysis. Poor or low quality and quantity RNA can cause highly varied and un-interpretable microarray data. The quantity and purity of total RNA samples were assessed by the NanoDrop® ND-1000 spectrophotometer as per manufacteur's recommendations.

The purity of every sample was assessed on the 260/280 ratio and a value between 1.8 and 2.1 was considered pure and of high quality. For example; the sample was free of contaminants such as; proteins, alcohol, phenol chloroform and sodium chloride specifically for the holoclone samples. All 16 samples fell within this ratio range, and thus were deemed of high purity and quality for microarray experiments, see Table 4.1.

**Table 4.1: Total RNA samples for 260/280 ratios for microarray analysis**

| Cell lines & Holoclones | Quantity (ng/µl) | 260-280nm Ratio |
|---|---|---|
| PC-3 Rep1 | 744.0 | 2.1 |
| PC-3 Rep2 | 913.9 | 2.1 |
| PC-3 Rep3 | 781.9 | 2.1 |
| PC-3 Rep4 | 794.2 | 2.1 |
| LNCaP Rep1 | 1791.1 | 2.0 |
| LNCaP Rep2 | 1692.1 | 2.0 |
| LNCaP Rep3 | 2175.7 | 2.0 |
| LNCaP Rep4 | 1875.3 | 2.0 |
| PC-3 Holoclone Rep1 | 28.8 | 1.9 |
| PC-3 Holoclone Rep2 | 29.3 | 1.8 |
| PC-3 Holoclone Rep3 | 27.8 | 1.9 |
| PC-3 Holoclone Rep4 | 27.5 | 1.8 |
| LNCaP Holoclone Rep1 | 21.6 | 1.8 |
| LNCaP Holoclone Rep2 | 24.8 | 1.8 |
| LNCaP Holoclone Rep3 | 33.6 | 1.8 |
| LNCaP Holoclone Rep4 | 30.1 | 1.8 |

#### 4.4.1.2 Quality control visual assessment of microarrays

Initial direct visual inspection of each microarray was performed in order to detect the presence of image artifacts on the arrays. These include high or low intensity spots, high regional or overall background and/or scratches. The performance of the B2 oligo positive hybridization control was visually assessed. The B2 intensity at the checkerboard corners and alternating borders were inspected. If it was too high, low or skewed due to artifacts the grid over the microarray image would not align correctly. All 16 microarrays passed the initial visual quality controls as all checkerboard corners, alternating border, array names and B2 oligo controls aligned correctly on all microarray images.


**Figure 4.1: Diagrammatic representation of the B2 illuminated regions of a GeneChip® Gene Array.** Each hybridization cocktail are spiked with B2 oligos and act as a positive hybridization control. The GCOS software uses this control to place a grid over the actual microarray image. Microarray images are visualized as an alternating pattern of intensities on the border. **(A)** Checkerboard corner region and alternating region **(B)** Array name lower left region of the array **(C)** Checkerboard region (adapted from www.bcm.edu microarray core facility).

#### 4.4.1.3 Quality control metric assessment of microarrays

Quality control metrics of the microarrays were assessed in order to determine that there were no outliers present within the data set. Quality assessment metric graphs and boxplots were generated by the Affymetrix Expression Console software. The metric employed in these experiments were described in detail in section 4.3.2.2.

In all cases there was a low level of variation in the quality control metrics that were evaluated. The pm_mean assesses if any of the chips intensities were extra dim or bright, the pm_mean appears to vary between the microarrays however between the PC-3 cell lines and PC-3 holoclones there is little variation as with the LNCaP cell line and LNCaP holoclones. The signal intensity remains low thus indicating there was low levels of array variation, see Figure 4. A.

Boxplots of the relative log expression for the entire probe sets analysed indicated that there was no outlier chip and all probe sets were consistent. The mean absolute RLE is proportional to the width of the boxplots or the inter-quartile range of the RLE values. The middle bar in each box is the median RLE and these should be zero.

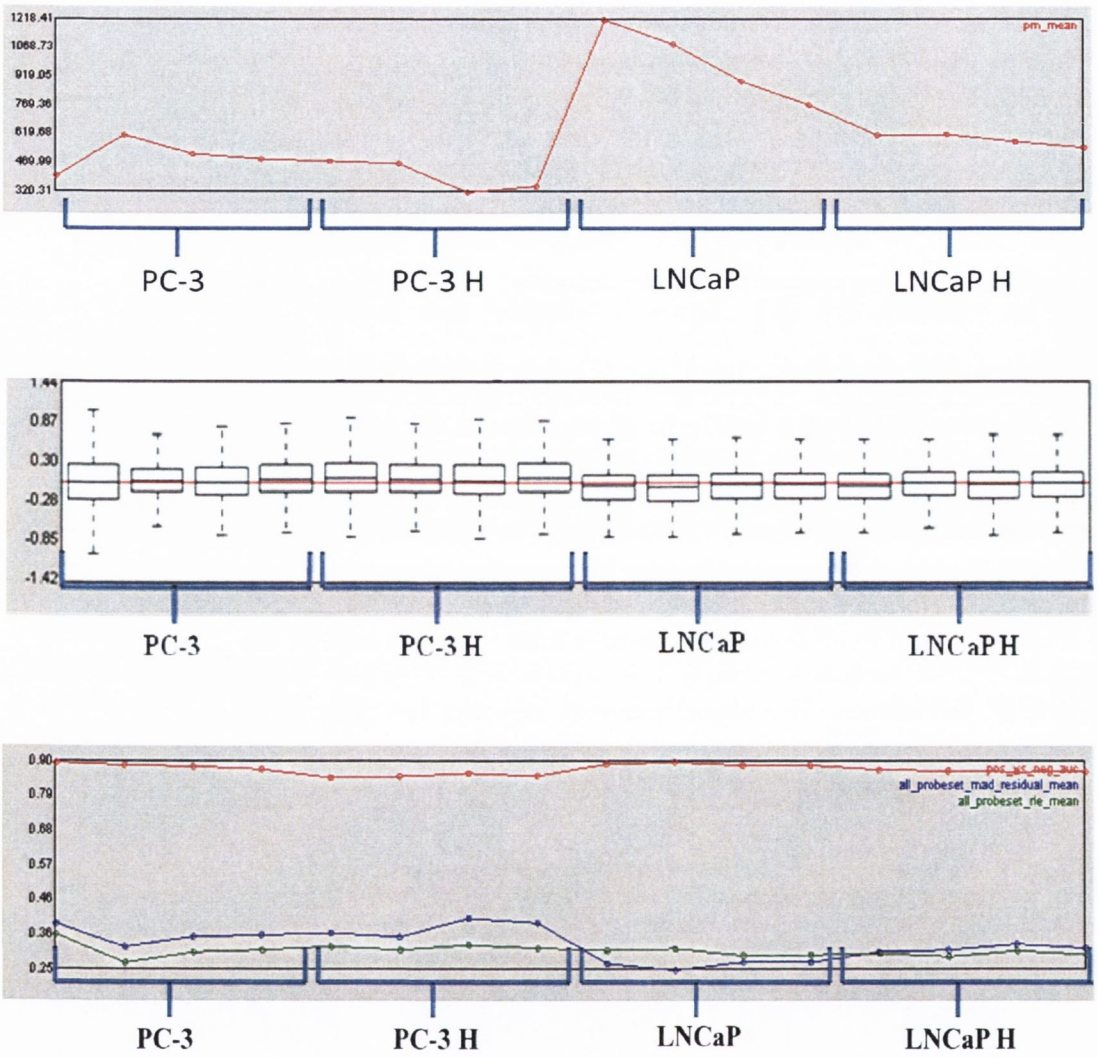



**Figure 4.2: Quality control metrics for microarrays of PC-3, PC-3 holoclones, LNCaP and LNCaP holoclones. A)** pm-mean: mean probe level intensity before normalisation and background correction. **B)** Relative log expression signal: box plots of the RLE for all probesets, the mean absolute RLE is proportional to the width of the box plots and the inter-quartile range of RLE values. The middle line/bar in each of the boxes represents the median RLE. **C)** Mean absolute: mean absolute deviation of residuals (blue), mean absolute relative log expression (green) and signal discrimination of positive and negative controls (red) in the area under the curve.

### 4.4.2 Analysis of all differentially expressed genes

In order to evaluate if the gene expression profiles show similar results, analysis was carried out using Pearson's correlation. Pearson's correlation coefficient (r) reflects the degree of linear relationships between variables. The correlation coefficient ranges from +1 to -1 and a correlation of 0 indicates that there is no relationship.

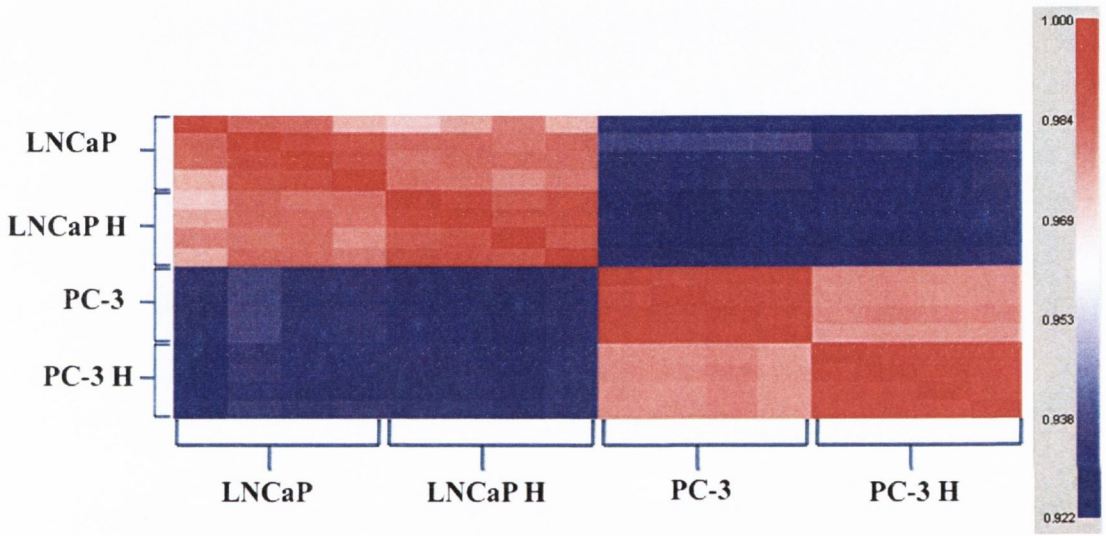



**Figure 4.3: Pearson's correlation.** Pearson's correlation between gene expression profiles of LNCaP, LNCaP holoclones, PC-3 and PC-3 holoclones. Correlation plot of both normalised and non-background corrected data (n=4). This heat map represents all possible combinations of samples used in the microarray analysis. The correlation coefficient ranges from +1 to -1, a correlation of 0 confirms that there is no relationship and a correlation of $\pm 1$ confirms that there is a perfect linear relationship.

**Table 4.2:** The top 10 genes down-regulated in LNCaP Holoclone when compared to LNCaP cell line

| Gene Symbol | Gene Annotation | NCBI Link | FC | P-value | FDR |
|---|---|---|---|---|---|
| ndufa4l2 | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 4-like 2 | NM_020142 | -4.09 | 1.09E-03 | 5.09E-02 |
| pla2g2a | phospholipase A2 group IIA (platelets synovial fluid) | NM_000300 | -3.55 | 7.04E-08 | 1.39E-04 |
| vegfa | vascular endothelial growth factor A | NM_001025366 | -3.36 | 1.40E-06 | 6.71E-04 |
| bhlhe40 | basic helix-loop-helix family member e40 | NM_003670 | -3.26 | 2.59E-06 | 9.77E-04 |
| ndrg1 | N-myc downstream regulated 1 | NM_001135242 | -2.85 | 7.47E-04 | 3.89E-02 |
| ttc6 | tetratricopeptide repeat domain 6 | NM_001007795 | -2.78 | 6.33E-06 | 1.67E-03 |
| fkbp5 | FK506 binding protein 5 | NM_004117 | -2.77 | 2.46E-05 | 4.28E-03 |
| hspa1b | heat shock 70kDa protein 1B | NM_005346 | -2.68 | 2.41E-05 | 4.27E-03 |
| dusp1 | dual specificity phosphatase 1 | NM_004417 | -2.64 | 1.68E-05 | 3.31E-03 |
| ankh | ankylosis progressive homolog (mouse) | NM_054027 | -2.42 | 5.03E-08 | 1.49E-04 |

**Table 4.3:** The top 10 genes up-regulated in LNCaP Holoclone when compared to LNCaP cell line

| Gene Symbol | Gene Annotation | NCBI Link | FC | P-value | FDR |
|---|---|---|---|---|---|
| peg3 | paternally expressed 3 | NM_006210 | 4.56 | 2.49E-05 | 4.29E-03 |
| etv1 | ets variant 1 | NM_004956 | 4.33 | 2.51E-08 | 1.48E-04 |
| fn1 | fibronectin 1 | NM_212482 | 3.04 | 1.21E-07 | 1.96E-04 |
| myo6 | myosin VI | NM_004999 | 2.86 | 4.93E-05 | 6.24E-03 |
| grb10 | growth factor receptor-bound protein 10 | NM_001001555 | 2.80 | 2.50E-05 | 4.23E-03 |
| kiaa0101 | KIAA0101 | NM_014736 | 2.62 | 1.23E-05 | 2.67E-03 |
| nup93 | nucleoporin 93kDa | NM_014669 | 2.57 | 1.11E-06 | 6.58E-04 |
| manea | mannosidase endo-alpha | NM_024641 | 2.57 | 5.06E-07 | 4.48E-04 |
| c13orf3 | chromosome 13 open reading frame 3 | NM_145061 | 2.44 | 1.40E-06 | 6.90E-04 |
| mlf1ip | MLF1 interacting protein | NM_024629 | 2.36 | 7.54E-05 | 8.10E-03 |

*LNCaP Holoclones generated a smaller gene list than the PC-3 Holoclones.

**Table 4.4:** The top 20 genes down-regulated in PC-3 Holoclone when compared to PC-3 cell line

| Gene Symbol | Gene Annotation | NCBI Link | FC | P-value | FDR |
|---|---|---|---|---|---|
| ceacam5 | carcinoembryonic antigen-related cell adhesion molecule 5 | NM_004363 | -13.33 | 1.34E-06 | 1.36E-04 |
| hpgd | hydroxyprostaglandin dehydrogenase 15-(NAD) | NM_000860 | -10.84 | 1.49E-06 | 1.42E-04 |
| mmp1 | matrix metallopeptidase 1 (interstitial collagenase) | NM_002421 | -7.99 | 5.92E-06 | 2.95E-04 |
| znf365 | zinc finger protein 365 | NM_014951 | -7.59 | 2.38E-06 | 1.77E-04 |
| ifitm1 | interferon induced transmembrane protein 1 (9-27) | NM_003641 | -7.40 | 2.07E-06 | 1.73E-04 |
| tcn1 | transcobalamin I (vitamin B12 binding protein R binder family) | NM_001062 | -7.20 | 3.66E-05 | 9.36E-04 |
| ifi44l | interferon-induced protein 44-like | NM_006820 | -6.71 | 7.50E-07 | 1.02E-04 |
| slc44a4 | solute carrier family 44 member 4 | NM_025257 | -5.95 | 4.31E-07 | 8.30E-05 |
| hla-f | major histocompatibility complex class I F | NM_001098479 | -4.52 | 3.03E-06 | 2.04E-04 |
| tff1 | trefoil factor 1 | NM_003225 | -4.38 | 1.31E-05 | 4.81E-04 |
| selenbp1 | selenium binding protein 1 | NM_003944 | -4.31 | 2.69E-06 | 1.89E-04 |
| oas1 | 2' 5'-oligoadenylate synthetase 1 40/46kDa | NM_016816 | -4.15 | 6.16E-08 | 4.20E-05 |
| gucy1a3 | guanylate cyclase 1 soluble alpha 3 | NM_000856 | -3.94 | 2.07E-08 | 3.34E-05 |
| kcnn4 | potassium intermediate/small conductance calcium-activated channel subfamily N member 4 | NM_002250 | -3.76 | 1.62E-05 | 5.46E-04 |
| ddx58 | DEAD (Asp-Glu-Ala-Asp) box polypeptide 58 | NM_014314 | -3.74 | 4.10E-06 | 2.39E-04 |
| st6galnac2 | ST6 (alpha-N-acetyl-neuraminyl-2 3-beta-galactosyl-1 3)-N-acetylgalactosaminide alpha-2 6-sialyltransferase 2 | NM_006456 | -3.63 | 8.34E-06 | 3.62E-04 |
| krt15 | keratin 15 | NM_002275 | -3.58 | 3.99E-04 | 5.61E-03 |
| hla-f | major histocompatibility complex class I F | NM_018950 | -3.42 | 9.54E-07 | 1.14E-04 |
| fam131b | family with sequence similarity 131 member B | NM_001031690 | -3.40 | 4.06E-05 | 1.01E-03 |
| shc4 | SHC (Src homology 2 domain containing) family member 4 | NM_203349 | -3.38 | 2.64E-05 | 7.56E-04 |

**Table 4.5:** The top 20 genes up-regulated in PC-3 Holoclone when compared to PC-3 cell line

| Gene Symbol | Gene Annotation | NCBI Link | FC | P-value | FDR |
|---|---|---|---|---|---|
| tgm2 | transglutaminase 2 (C polypeptide protein-glutamine-gamma-glutamyltransferase) | NM_004613 | 7.98 | 1.45E-07 | 5.72E-05 |
| flnc | filamin C gamma (actin binding protein 280) | NM_001458 | 4.53 | 2.87E-05 | 7.91E-04 |
| foxm1 | forkhead box M1 | NM_202002 | 4.52 | 2.07E-06 | 1.74E-04 |
| jub | jub ajuba homolog (Xenopus laevis) | NM_032876 | 4.50 | 2.54E-04 | 3.93E-03 |
| thbs1 | thrombospondin 1 | NM_003246 | 4.26 | 7.79E-07 | 1.04E-04 |
| tgfb2 | transforming growth factor beta 2 | NM_001135599 | 4.10 | 3.04E-05 | 8.18E-04 |
| cdh11 | cadherin 11 type 2 OB-cadherin (osteoblast) | NM_001797 | 3.79 | 7.12E-06 | 3.24E-04 |
| dlgap5 | discs large (Drosophila) homolog-associated protein 5 | NM_014750 | 3.64 | 1.75E-04 | 2.94E-03 |
| kirrel | kin of IRRE like (Drosophila) | NM_018240 | 3.64 | 1.34E-08 | 3.95E-05 |
| fam72a | family with sequence similarity 72 member A | BC035696 | 3.64 | 2.37E-06 | 1.77E-04 |
| igfbp4 | insulin-like growth factor binding protein 4 | NM_001552 | 3.62 | 3.46E-08 | 3.83E-05 |
| flna | filamin A alpha (actin binding protein 280) | NM_001456 | 3.59 | 5.58E-08 | 3.95E-05 |
| cdh2 | cadherin 2 type 1 N-cadherin (neuronal) | NM_001792 | 3.57 | 5.43E-08 | 4.18E-05 |
| ncapd2 | non-SMC condensin I complex subunit D2 | NM_014865 | 3.51 | 6.61E-06 | 3.12E-04 |
| fam72a | family with sequence similarity 72 member A | BC035696 | 3.50 | 1.95E-05 | 6.16E-04 |
| zwint | ZW10 interactor | NM_032997 | 3.33 | 1.31E-04 | 2.39E-03 |
| cav1 | caveolin 1 caveolae protein 22kDa | NM_001753 | 3.33 | 1.66E-05 | 5.53E-04 |
| ttc7b | tetratricopeptide repeat domain 7B | NM_001010854 | 3.30 | 2.41E-06 | 1.77E-04 |
| bub1 | budding uninhibited by benzimidazoles 1 homolog (yeast) | NM_004336 | 3.26 | 1.31E-06 | 1.36E-04 |
| ncaph | non-SMC condensin I complex subunit H | NM_015341 | 3.18 | 9.56E-05 | 1.87E-03 |

**Table 4.6:** The top 20 genes down-regulated in PC-3 Holoclone when compared to LNCaP Holoclone

| Gene Symbol | Gene Annotation | NCBI Link | FC | P-value | FDR |
|---|---|---|---|---|---|
| tmsb4x | thymosin beta 4 X-linked | NM_021109 | -14.80 | 2.71E-09 | 3.66E-07 |
| ctsa | cathepsin A | NM_001127695 | -8.56 | 1.88E-08 | 1.06E-06 |
| rab27b | RAB27B member RAS oncogene family | NM_004163 | -8.55 | 1.20E-09 | 2.48E-07 |
| fut8 | fucosyltransferase 8 (alpha (1 6) fucosyltransferase) | NM_178155 | -8.47 | 4.12E-08 | 1.61E-06 |
| tnfsf10 | tumor necrosis factor (ligand) superfamily member 10 | NM_003810 | -8.35 | 2.67E-08 | 1.23E-06 |
| pygl | phosphorylase glycogen liver | NM_002863 | -8.18 | 1.89E-08 | 1.05E-06 |
| fstl1 | follistatin-like 1 | NM_007085 | -8.15 | 1.18E-07 | 3.21E-06 |
| bace2 | beta-site APP-cleaving enzyme 2 | NM_012105 | -7.69 | 1.75E-07 | 4.17E-06 |
| stat1 | signal transducer and activator of transcription 1 91kDa | NM_007315 | -7.42 | 1.73E-08 | 9.97E-07 |
| cd59 | CD59 molecule complement regulatory protein | NM_203330 | -7.14 | 1.05E-10 | 8.83E-08 |
| grn | granulin | NM_002087 | -7.07 | 1.80E-08 | 1.02E-06 |
| capn2 | calpain 2 (m/II) large subunit | NM_001748 | -6.89 | 3.17E-07 | 6.21E-06 |
| tmem45b | transmembrane protein 45B | NM_138788 | -6.70 | 3.87E-09 | 4.48E-07 |
| tmsb10 | thymosin beta 10 | NM_021103 | -6.23 | 1.65E-09 | 2.87E-07 |
| gramd3 | GRAM domain containing 3 | NM_023927 | -5.89 | 7.44E-08 | 2.37E-06 |
| slc12a2 | solute carrier family 12 (sodium/potassium/chloride transporters) member 2 | NM_001046 | -5.76 | 1.99E-07 | 4.52E-06 |
| gstk1 | glutathione S-transferase kappa 1 | NM_015917 | -5.75 | 5.01E-07 | 8.53E-06 |
| arpc1b | actin related protein 2/3 complex subunit 1B 41kDa | NM_005720 | -5.51 | 5.81E-06 | 5.27E-05 |
| spred2 | sprouty-related EVH1 domain containing 2 | NM_181784 | -5.49 | 2.62E-08 | 1.22E-06 |
| tes | testis derived transcript (3 LIM domains) | NM_015641 | -5.41 | 6.77E-08 | 2.22E-06 |

**Table 4.7:** The top 20 genes up-regulated in PC-3 Holoclone when compared to LNCaP Holoclone

| Gene Symbol | Gene Annotation | NCBI Link | FC | P-value | FDR |
|---|---|---|---|---|---|
| slc30a4 | solute carrier family 30 (zinc transporter) member 4 | NM_013309 | 20.66 | 4.35E-09 | 4.67E-07 |
| sord | sorbitol dehydrogenase | NM_003104 | 14.08 | 1.28E-08 | 8.16E-07 |
| prkacb | protein kinase cAMP-dependent catalytic beta | NM_182948 | 11.86 | 7.88E-09 | 6.26E-07 |
| pik3r1 | phosphoinositide-3-kinase regulatory subunit 1 (alpha) | NM_181523 | 11.69 | 4.11E-10 | 1.46E-07 |
| efna5 | ephrin-A5 | NM_001962 | 10.42 | 9.33E-09 | 6.95E-07 |
| ppp3ca | protein phosphatase 3 (formerly 2B) catalytic subunit alpha isoform | NM_000944 | 9.63 | 4.21E-11 | 5.73E-08 |
| abhd2 | abhydrolase domain containing 2 | NM_007011 | 9.12 | 4.10E-08 | 1.62E-06 |
| rab6c | RAB6C member RAS oncogene family | NM_032144 | 8.88 | 1.40E-06 | 1.78E-05 |
| arg2 | arginase type II | NM_001172 | 8.65 | 1.03E-07 | 2.90E-06 |
| slc43a1 | solute carrier family 43 member 1 | NM_003627 | 8.52 | 3.69E-06 | 3.70E-05 |
| bdp1 | B double prime 1 subunit of RNA polymerase III transcription initiation factor IIIB | NM_018429 | 7.48 | 1.95E-07 | 4.47E-06 |
| dsel | dermatan sulfate epimerase-like | NM_032160 | 7.00 | 6.01E-07 | 9.74E-06 |
| slc4a4 | solute carrier family 4 sodium bicarbonate cotransporter member 4 | NM_001098484 | 6.98 | 5.21E-09 | 5.13E-07 |
| litaf | lipopolysaccharide-induced TNF factor | NM_004862 | 6.98 | 3.42E-06 | 3.49E-05 |
| adamts1 | ADAM metallopeptidase with thrombospondin type 1 motif 1 | NM_006988 | 6.73 | 5.55E-08 | 1.97E-06 |
| slc39a8 | solute carrier family 39 (zinc transporter) member 8 | NM_022154 | 6.72 | 2.23E-08 | 1.11E-06 |
| acpp | acid phosphatase prostate | NM_001099 | 6.71 | 8.05E-07 | 1.20E-05 |
| acsl1 | acyl-CoA synthetase long-chain family member 1 | NM_001995 | 6.46 | 1.81E-09 | 3.00E-07 |
| fasn | fatty acid synthase | NM_004104 | 6.35 | 6.01E-09 | 5.49E-07 |

**Table 4.8:** The top 20 genes down-regulated in PC-3 when compared to LNCaP cell line

| Gene Symbol | Gene Annotation | NCBI Link | FC | P-value | FDR |
|---|---|---|---|---|---|
| dkk1 | dickkopf homolog 1 (Xenopus laevis) | NM_012242 | -130.55 | 1.19E-08 | 1.94E-06 |
| cav1 | caveolin 1 caveolae protein 22kDa | NM_001753 | -125.33 | 5.6E-07 | 1.75E-05 |
| s100a16 | S100 calcium binding protein A16 | NM_080388 | -102.42 | 2.15E-09 | 8.13E-07 |
| plat | plasminogen activator tissue | NM_000930 | -99.10 | 6.31E-09 | 1.43E-06 |
| plau | plasminogen activator urokinase | NM_002658 | -94.62 | 7.91E-08 | 5.45E-06 |
| anxa2 | annexin A2 | NM_001002857 | -94.24 | 2.76E-07 | 1.13E-05 |
| met | met proto-oncogene (hepatocyte growth factor receptor) | NM_001127500 | -90.48 | 7.64E-09 | 1.46E-06 |
| fst | follistatin | NM_006350 | -66.33 | 1.17E-07 | 6.82E-06 |
| itgb4 | integrin beta 4 | NM_000213 | -65.10 | 5.75E-09 | 1.38E-06 |
| krt7 | keratin 7 | NM_005556 | -65.08 | 1.43E-09 | 6.87E-07 |
| krt81 | keratin 81 | NM_002281 | -61.58 | 6.69E-06 | 9.33E-05 |
| sulf2 | sulfatase 2 | NM_018837 | -60.76 | 1.8E-07 | 8.79E-06 |
| igfbp4 | insulin-like growth factor binding protein 4 | NM_001552 | -57.07 | 8.89E-11 | 5.25E-07 |
| ier3 | immediate early response 3 | NM_003897 | -52.83 | 1.09E-07 | 6.59E-06 |
| cd44 | CD44 molecule (Indian blood group) | NM_000610 | -52.63 | 3.7E-10 | 3.86E-07 |
| calb1 | calbindin 1 28kDa | NM_004929 | -51.15 | 3.34E-08 | 3.43E-06 |
| gstp1 | glutathione S-transferase pi 1 | NM_000852 | -50.44 | 3.76E-06 | 6.32E-05 |
| epha2 | EPH receptor A2 | NM_004431 | -49.31 | 2.14E-10 | 3.45E-07 |
| krt19 | keratin 19 | NM_002276 | -49.06 | 3.07E-08 | 3.26E-06 |
| tgm2 | transglutaminase 2 (C polypeptide protein-glutamine-gamma-glutamyltransferase) | NM_004613 | -48.63 | 8.16E-08 | 5.46E-06 |

**Table 4.9:** The top 20 genes up-regulated in PC-3 when compared to LNCaP cell line

| Gene Symbol | Gene Annotation | NCBI Link | FC | P-value | FDR |
|---|---|---|---|---|---|
| klk3 | kallikrein-related peptidase 3 | NM_001030047 | 128.46 | 1.02E-10 | 3.6E-07 |
| rab5c | RAB5C member RAS oncogene family | NM_201434 | 75.37 | 2.09E-07 | 9.73E-06 |
| folh1 | folate hydrolase (prostate-specific membrane antigen) 1 | NM_004476 | 72.49 | 2.75E-10 | 3.48E-07 |
| psmal | growth-inhibiting protein 26 | AF261715 | 69.17 | 1.06E-09 | 5.68E-07 |
| mme | membrane metallo-endopeptidase | NM_007288 | 67.21 | 1.32E-11 | 2.35E-07 |
| ncam2 | neural cell adhesion molecule 2 | NM_004540 | 63.62 | 8.7E-10 | 4.97E-07 |
| spon2 | spondin 2 extracellular matrix protein | NM_012445 | 61.94 | 1.91E-09 | 7.52E-07 |
| slc4a4 | solute carrier family 4 sodium bicarbonate cotransporter member 4 | NM_001098484 | 44.16 | 5.49E-10 | 4.23E-07 |
| tmeff2 | transmembrane protein with EGF-like and two follistatin-like domains 2 | NM_016192 | 41.92 | 9.17E-11 | 4.07E-07 |
| prune2 | prune homolog 2 (Drosophila) | NM_015225 | 39.88 | 1.19E-08 | 1.95E-06 |
| neto1 | neuropilin (NRP) and tolloid (TLL)-like 1 | NM_138966 | 39.47 | 1.38E-06 | 3.26E-05 |
| prune2 | prune homolog 2 (Drosophila) | NM_015225 | 39.46 | 1.8E-08 | 2.5E-06 |
| prkd1 | protein kinase D1 | NM_002742 | 38.09 | 4.07E-09 | 1.16E-06 |
| ar | androgen receptor | NM_000044 | 37.92 | 4.16E-11 | 3.68E-07 |
| stat3 | signal transducer and activator of transcription 3 (acute-phase response factor) | NM_139276 | 33.83 | 4.93E-10 | 4.37E-07 |
| maoa | monoamine oxidase A | NM_000240 | 32.41 | 3.25E-08 | 3.38E-06 |
| gpr158 | G protein-coupled receptor 158 | NM_020752 | 30.92 | 7.13E-09 | 1.45E-06 |
| syt4 | synaptotagmin IV | NM_020783 | 29.77 | 5.04E-07 | 1.63E-05 |
| acpp | acid phosphatase prostate | NM_001099 | 28.69 | 1.44E-07 | 7.69E-06 |
| nkx3-1 | NK3 homeobox 1 | NM_006167 | 28.51 | 8.84E-10 | 4.9E-07 |

### 4.4.3 Differential gene expression analysis

The Affymetrix GeneChip® Gene ST 1.0 microarray contains 818,005 probes which correspond to 28,869 gene transcripts. Data obtained from the microarrays was analyzed using XRAY version 3.99 software. The intensities of the probes were transformed and summarized by the application of the RMA normalization and summarization methods. XRAY software used a one sided t-test in order to evaluate the presence or absence of gene transcripts. In order to adjust for multiple comparisons, a false discovery rate (FDR) was calculated by the Benjamini and Hochberg method (1995). Genes were determined to be differentially expressed if they possessed a fold change $\geq 2$ and an FDR $\leq 0.05$.

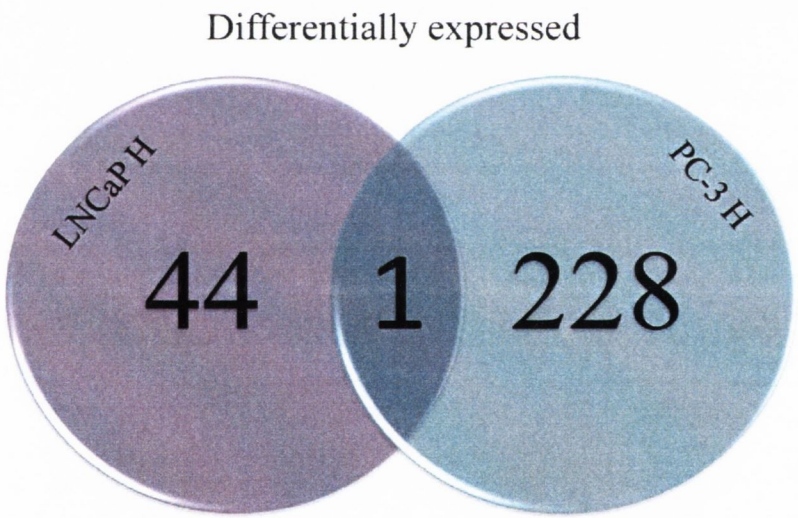



**Figure 4.4: Venn diagram illustrating differentially expressed genes in LNCap and PC-3 holoclones.** 44 genes were differentially expression in LNCaP holoclones and 228 genes were differentially expressed in PC-3 holoclones of these; 1 gene Etv1 was commonly expressed in both holoclone sets using the values of $\geq 2$ fold and a FDR $\leq$ 0.05.

### 4.4.4 Validation of selected genes by Real Time TaqMan® PCR

In order to confirm the expression generated from the microarrays, validation was carried out using a TaqMan® RT-PCR method. In total 30 genes were selected from the PC-3 and LNCap holoclone expression list for validation see Table 4.10 & 4.11. In most cases the genes that were significantly regulated in the microarray data were also significantly regulated in the TaqMan® RT-PCR data; however the fold changes values were greater for the TaqMan® RT-PCR data. This was an expected result as the normalization method used can compress values of relative expression in comparison to the PCR data. When compared to microarray data TaqMan® gene expression assays allow for a higher specificity, sensitivity and have a large dynamic range.

Affymetrix microarray data was analysed using XRAY software to determine differential gene expression and the holoclones were calibrated to the relevant resting/founder cell lines. TaqMan® RT-PCR mRNA gene expression values for each gene was normalised to the endogenous control GAPDH and calibrated to the resting cell lines PC-3 and LNCaP in order to establish differential gene expression, Figure 4.5 A & B. The majority of genes selected for validation are markers known to represent stemness, differentiation and epithelial to mesenchymal transition (EMT) and various other genes that represent apoptosis, proliferation and many other processses involved in tumour progression, Table 4.10 & 4.11. The correlation coefficient between the microarrays and the TaqMan® RT-PCR for LNCaP holoclones is 0.7935 and the correlation coefficient for the PC-3 holoclones is 0.8717.

**Table 4.10:** Genes validated in PC-3 holoclones

| Gene | Biological relevance | Microarray FC | TaqMan® FC |
|---|---|---|---|
| **Ceacam5** | Carcinoembryonic antigen-related cell adhesion molecule 5 | -13.33 | -13.98 |
| **mmp1** | Embryonic development, reproduction, and tissue remodeling & various disease processes, including metastasis | -7.99 | -11.36 |
| **Krt15** | Stemness marker | -3.58 | -8.92 |
| **Ccng2** | Involved in cell cycle | -3.01 | -2.54 |
| **tspan3** | Role in the regulation of cell development, activation, growth and motility | -2.72 | -1.68 |
| **Clu** | Involved in clearance of cellular debris and apoptosis | -2.53 | -3.34 |
| **nup37** | Causes widespread apoptosis | 2.13 | 1.73 |
| **evt1** | Androgen regulated gene | 2.34 | 5.43 |
| **Snail2** | Stemness and EMT | 2.45 | 5.79 |
| **akt3** | Involved in cell proliferation, differentiation, apoptosis, tumorigenesis | 3.09 | 3.138 |
| **Cdh2** | Key role in cancer metastasis | 3.57 | 4.174 |
| **igfbp4** | Insulin-like growth factor binding protein secreted by colon cancer cells | 3.62 | 1.75 |
| **cdh11** | Differentiation & development | 3.79 | 1.777 |
| **tgfb2** | Stemness and EMT | 4.1 | 13.5 |
| **foxm1** | key role in cell cycle progression & development, involved in many carcinomas | 4.52 | 2.131 |
| **tgm2** | Involoved in apoptosis, cellular differentiation and matrix stabilisation | 7.98 | 23.509 |

**Table 4.11:** Genes validated in LNCaP holoclones

| Gene | Biological relevance | Microarray FC | TaqMan® FC |
|---|---|---|---|
| **nudfa4l2** | Triggers apoptosis in particular, during somatic embryo development | -4.09 | -21.05 |
| **Vegfa** | Involved in inducing angiogenesis and endothelial cell growth, promoting cell migration and inhibiting apoptosis | -3.36 | -4.177 |
| **ndrg1** | Involved in stress responses, hormone responses, cell growth, and differentiation & is a member of the N-myc downregulated gene family | -2.85 | -3.14 |
| **dusp1** | Interacts with MAPkinases & negative regulation of cellular proliferation | -2.64 | -2.04 |
| **igf1r** | strong growth-promoting effect | -2.37 | -2.22 |
| **Cldn12** | Controls flow of molecules in the intercellular space between cells of epithelium | -2.25 | -3.61 |
| **SOCS2** | Mediates cell signalling & regulates growth control | -2.2 | -3.47 |
| **lifr** | Affects the differentiation, survival, and proliferation of adult & embryo cells, involved in many carcinomas | -2.13 | -2.439 |
| **gins2** | Interacts with DNA damage and is involved in cell cycle arrest | 2.01 | 1.54 |
| **nup93** | Causes widespread apoptosis | 2.57 | 1.993 |
| **kiaa0101** | Interacts with proliferating cell nuclear antigen | 2.62 | 3.779 |
| **grb10** | Encodes a growth factor receptor-binding protein that interacts with insulin receptors and insulin-like growth-factor receptors | 2.8 | 3.59 |
| **fn1** | Stemness and EMT | 3.04 | 4.457 |
| **etv1** | Androgen regulated gene | 3.09 | 9.15 |
| **peg3** | Involved in p53/c-myc-mediated apoptosis & potential cell death mediator | 4.56 | 4.45 |

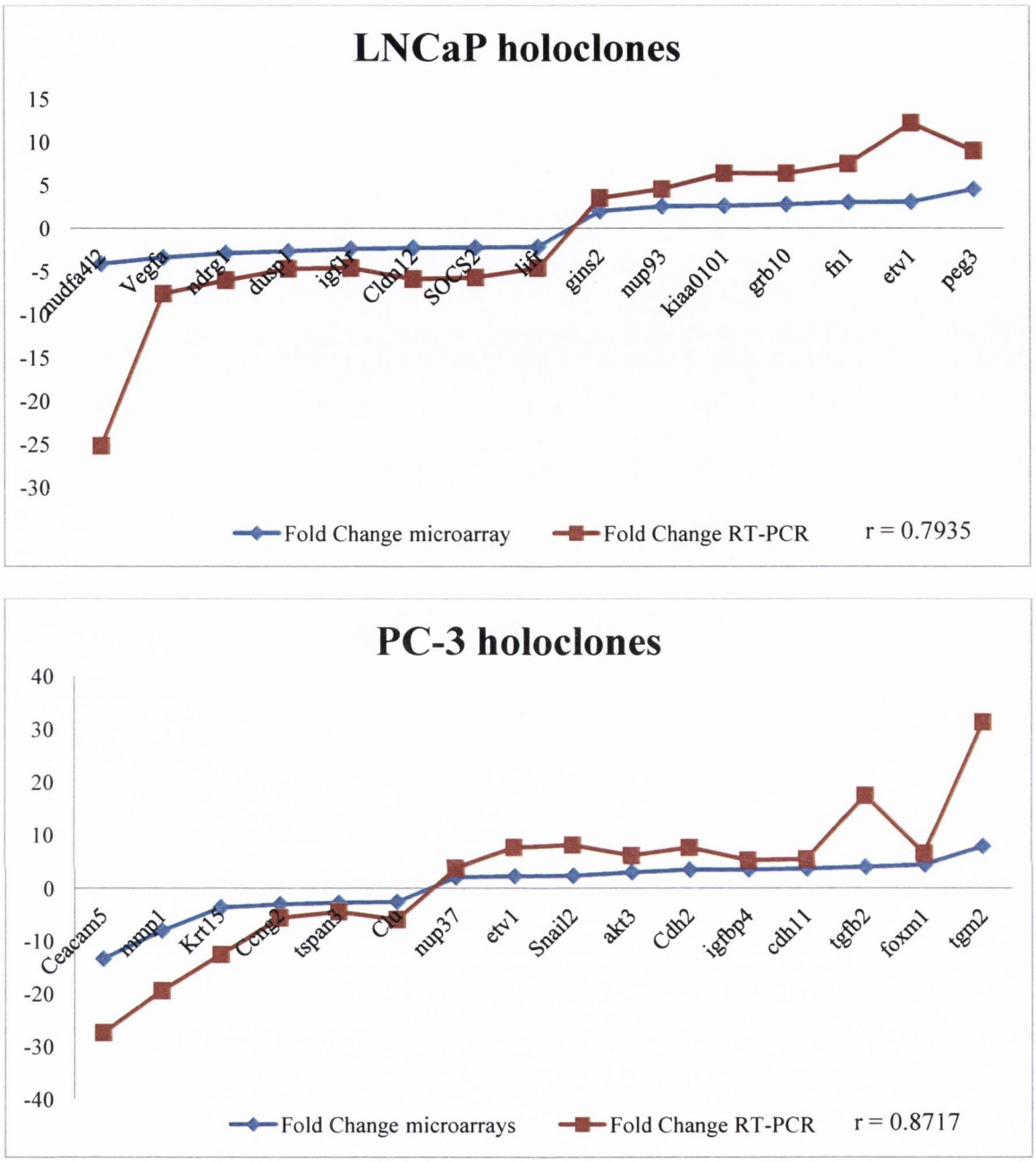



**Figure 4.5: Comparsion of fold change values between Affymetrix microarrays and TaqMan® RT-PCR gene expression. A)** Gene expression values for LNCaP holoclones **B)** Gene expression values for PC-3 holoclones.

### 4.4.5 Gene ontology analysis- DAVID database

In order to assess and identify the key processes that displayed alterations in the PC-3 and LNCaP holoclones in comparison to the resting cell lines, the gene ontology database DAVID (the database for annotation, visualization and integrated discovery) version 6.7 was used. The over-representation of key processes compared to the Homo sapiens proteome was measured using an FDR value $\leq 0.05$ in the DAVID database. Significant biological processes and pathways were identified by DAVID in both PC-3 and LNCaP holoclones, see Table 4.12 & 4.14.

DAVID determined cellular pathways that were over represented from the gene list identified from the PC-3 holoclones in comparison to their relevant resting cell line and the pathways identified were based on data from the Kegg database, see Table 4.13.

**Table 4.12: Significant biological processes generated by DAVID (Geoterm) database for the PC-3 holoclones**

| Biological processes-all (Geoterm) | Count | % | P value | FDR |
|---|---|---|---|---|
| cell division | 21 | 9.8 | 3.00E-10 | 4.90E-07 |
| mitosis | 17 | 7.9 | 7.30E-09 | 6.00E-06 |
| nuclear division | 17 | 7.9 | 7.30E-09 | 6.00E-06 |
| M phase of mitotic cell cycle | 17 | 7.9 | 9.50E-09 | 5.20E-06 |
| M phase | 20 | 9.3 | 1.20E-08 | 4.90E-06 |
| organelle fission | 17 | 7.9 | 1.30E-08 | 4.20E-06 |
| antigen processing and presentation of peptide antigen via MHC class I | 7 | 3.3 | 2.80E-08 | 7.50E-06 |
| mitotic cell cycle | 20 | 9.3 | 7.90E-08 | 1.80E-05 |
| cell cycle phase | 21 | 9.8 | 9.60E-08 | 1.90E-05 |
| cell cycle process | 28 | 13.1 | 4.10E-07 | 6.60E-05 |
| cell cycle | 28 | 13.1 | 4.10E-07 | 6.60E-05 |
| chromosome segregation | 10 | 4.7 | 4.60E-07 | 6.80E-05 |
| antigen processing and presentation of peptide antigen | 7 | 3.3 | 7.60E-07 | 1.00E-04 |
| regulation of cell cycle | 17 | 7.9 | 2.00E-06 | 2.50E-04 |
| regulation of mitotic cell cycle | 11 | 5.1 | 1.30E-05 | 1.50E-03 |
| mitotic sister chromatid segregation | 6 | 2.8 | 6.10E-05 | 6.60E-03 |
| sister chromatid segregation | 6 | 2.8 | 7.00E-05 | 7.10E-03 |
| cell cycle checkpoint | 8 | 3.7 | 1.00E-04 | 9.60E-03 |
| cytokinesis | 6 | 2.8 | 1.20E-04 | 1.00E-02 |
| chromosome condensation | 5 | 2.3 | 2.00E-04 | 1.70E-02 |
| regulation of cell cycle process | 8 | 3.7 | 4.10E-04 | 3.30E-02 |
| organelle organization | 31 | 14.5 | 4.20E-04 | 3.20E-02 |
| mitotic chromosome condensation | 4 | 1.9 | 4.30E-04 | 3.10E-02 |
| antigen processing and presentation | 7 | 3.3 | 4.50E-04 | 3.10E-02 |
| regulation of nuclear division | 6 | 2.8 | 5.10E-04 | 3.40E-02 |
| regulation of mitosis | 6 | 2.8 | 5.10E-04 | 3.40E-02 |
| interspecies interaction between organisms | 12 | 5.6 | 5.50E-04 | 3.50E-02 |
| immune system process | 25 | 11.7 | 6.80E-04 | 4.20E-02 |
| response to hypoxia | 8 | 3.7 | 1.10E-03 | 6.20E-02 |
| response to oxygen levels | 8 | 3.7 | 1.40E-03 | 8.00E-02 |

**Table 4.13: 15 significant pathways generated by DAVID (Kegg) database for the PC-3 holoclones**

| Pathways (Kegg) | Count | % | P value | FDR |
|---|---|---|---|---|
| Viral myocarditis | 8 | 3.8 | 1.0E-2 | 1.2E-2 |
| Antigen processing and presentation | 7 | 3.3 | 1.7E-2 | 9.8E-2 |
| Allograft rejection | 5 | 2.3 | 2.2E-2 | 8.5E-2 |
| Graft-versus-host disease | 5 | 2.3 | 2.9E-2 | 8.6E-2 |
| Type 1 diabetes mellitus | 5 | 2.3 | 3.9E-2 | 9.0E-2 |
| Autoimmune thyroid disease | 5 | 2.3 | 7.8E-2 | 1.5E-1 |
| Focal adhesion | 9 | 4.2 | 1.2E-2 | 1.9E-1 |
| Cysteine and methioine metabolism | 4 | 1.9 | 1.5E-2 | 2.1E-1 |
| Progesterone-mediated oocyte maturation | 5 | 2.3 | 4.4E-2 | 4.6E-1 |
| Cell cycle | 6 | 2.8 | 4.4E-2 | 4.2E-1 |
| Argine and proline metabolism | 4 | 1.9 | 4.9E-2 | 4.2E-1 |
| Cell adhesion molecules (CAMs) | 6 | 2.8 | 5.3E-2 | 4.3E-1 |
| Pathways in cancer | 10 | 4.7 | 6.6E-2 | 4.7E-1 |
| Purine metabolism | 6 | 2.8 | 8.8E-2 | 5.5E-1 |
| Renal cell carcinoma | 4 | 1.9 | 9.4E-2 | 5.5E-1 |

**Table 4.14: Significant biological processes generated by DAVID (Geoterm) database for the LNCaP holoclones**

| Biological processes-all (Geoterm) | Count | % | P value | FDR |
|---|---|---|---|---|
| Response to chemical stimulus | 12 | 29.3 | 7.1E-5 | 4.6E-2 |
| Response to organic substance | 9 | 22.0 | 1.6E-4 | 5.1E-2 |
| Response to stimulus | 18 | 43.9 | 5.4E-4 | 1.1E-2 |
| Response to endogenous | 6 | 14.6 | 2.0E-3 | 2.8E-2 |
| Response to stress | 11 | 26.8 | 3.1E-3 | 3.4E-2 |
| Cellular response to hormone stimulus | 4 | 9.8 | 3.3E-3 | 3.1E-1 |
| Protein tetramerization | 3 | 7.3 | 3.5E-3 | 2.8E-1 |
| Enzyme linked receptor protein signaling pathway | 5 | 12.2 | 7.1E-3 | 4.5E-1 |
| Negative regulation of apoptosis | 5 | 12.2 | 8.0E-3 | 4.5E-1 |
| Negative regulation of programmed cell death | 5 | 12.2 | 8.4E-3 | 4.3E-1 |
| Negative regulation of cell death | 5 | 12.2 | 8.5E-3 | 4.0E-1 |
| Anti-apoptosis | 4 | 9.8 | 1.1E-2 | 4.6E-1 |
| Cellular component assembly | 7 | 17.1 | 1.3E-2 | 5.0E-1 |
| Transmembrane receptor protein tyrosine kinase signaling pathway | 4 | 9.8 | 1.4E-2 | 4.9E-1 |
| Cell development | 6 | 14.6 | 1.4E-2 | 4.7E-1 |
| Insulin-like growth factor receptor signaling pathway | 2 | 4.9 | 2.0E-2 | 5.7E-1 |
| Macromolecular complex subunit organization | 6 | 14.6 | 2.1E-2 | 5.6E-1 |
| Cellular processes | 20 | 73.2 | 2.3E-2 | 5.7E-1 |
| Cellular component biogenesis | 7 | 17.1 | 2.3E-2 | 5.6E-1 |

* LNCaP holoclones did not generate a list of significant pathways

## 4.5 Discussion

As previously discussed in chapter 3, cancer stem cells undeniably are present in various tumour types including the prostate. Identifying and characterizing these specific tumour stem cell holoclones is necessary, thereby identifying unique cancer stem cell specific patterns of gene expression. Therefore in order to gain insight and a further understanding into the biological processes of the cancer stem cell holoclones array analysis was necessary. The microarrays analysis allowed for an unbiased approach to assess the gene expression alterations that occurred in the holoclones when compared to resting/founder cell lines.

TaqMan® RT-PCR was performed on 30 significant genes generated from a list of differentially expressed genes from the PC-3 and LNCaP holoclones in comparison to their relevant founder cell lines in order to validate the microarray results. This analysis confirms that the gene signature generated by the Affymetrix microarray analysis was truly representative of the PC-3 and LNCaP holoclones in comparison to the founder cell lines. The genes chosen to validate the microarrays were of significant relevance and represented markers of stemness, differentiation and epithelial to mesenchymal transition. These genes were selected to characterise and further confirm that the cancer stem cell like holoclones have stemess potential.

Clusterin was found to be significantly downregulated in the PC-3 holoclones in comparison to the founder PC-3 cell line and was also confirmed to be significantly downregulated by TaqMan® RT-PCR also. Clusterin is a 75 - 80 KDa disulfide linked heterodimeric protein which is associated with the clearance of cellular debris and apoptosis. Clusterin may be considered as a significant factor in predicting prostate cancer recurrence. A study by Pin *et al,* suggests that clusterin plays an important role in the stromal component of prostate cancer progression and may be useful to aid in the prediction of chance of disease recurrence in patients with a Gleason score of 6 or 7 following radical prostatectomy (Pin *et al.,* 2004). As it is an antiapoptotic mediator prostate cancer cells expressing higher levels of Clusterin and have the ability for greater survival (Sensibal *et al.,* 1995). Thus the downregulation of clusterin in the PC-3 holoclones in comparison to the founder cell lines indicates that the PC-3 holoclones are possibly involved in recurrence of prostate cancer, when compared to the resting cell line as they do not contain such a potent property.

Snail2 and TGF-β were both found to be significantly upregulated in the PC-3 holoclones in comparsion to the founder PC-3 cell line and were also confirmed to be significantly upregulated by TaqMan® RT-PCR also. Briefly, the Snail gene super-family of zinc-finger transcription factors is involved in development of vertebrate and invertebrate embryos and also in the progression of tumours and metastasis (Sefton *et al.,* 1998). TGF-β family members regulate various cellular functions in both the adult and developing embryo such as differentiation, proliferation, apoptosis and migration. In normal epithelial cells, it is an anti-proliferative factor and also at the early stages of oncogenesis. Studies have shown that Snail2 and TGF-β are both drivers and mediators of epithelial to mesenchymal transition, see Figure 4.6 (Zvaifler, 2006). Snail2 has been

shown to be expressed in the progenitor cells of developing endocrine pancreas (Rukstalis and Habener. 2006). This study demonstrated that EMT-like events seem to be implicated in the development of the mammalian pancreas *in vivo*. This analysis indicates that EMT signatures are expressed in the PC-3 holoclones further supporting that these holoclones display cancer stem cell characteristics.

EMT is known to play a role in both metastasis and proliferation. The initation of metastasis involves invasion that has phenotypic similarities to EMT such as loss of cell-cell adhesion which is mediated by E-cadherin repression and the increase in cell mobility. The loss of genes such as the members of the Hedgehog family has been shown to activate intergrin and Wnt that lead to cell-cell adhesion.

Epithelial to mesenchymal transition is a physiological process during embryonic development and then functions in adults to promote organ morphogenesis and tissue regeneration including wound healing. More recently there is emerging evidence to suggest that the acquisition of EMT is robustly associated with both cancer cell invasion and tumor metastasis. Recent studies by various groups have shown that cells with an EMT phenotype share characteristics that are consistent with the signatures of cancer stem cells, which are associated with tumor recurrence and drug resistant phenotype (Hollier *et al.*, 2009, Mani *et al.,* 2008).

Currently the literature regarding the characteristation of the PC-3 and LNCaP holoclones is limited. However in the published literature currently available, it has been shown that over-expression of Platelet-derived growth factor-D (PDGF-D) in PC3 cells induced cellular morphological changes that were coherent with the acquirement

of an EMT phenotype that was characterised by the loss of expression of epithelial markers and the gain or increased expression of mesenchymal markers (Kong *et al.*, 2010). Interestingly, the PC3 PDGF-D cells used by this group displayed an increased clonogenic and prostasphere forming capacity and these are the characteristics best known to be associated with cancer stem cell characteristics. The results reported by this group correlate with the data found in this chapter in regard to the PC-3 holoclones.

The progression of most cancers toward malignancy is usually linked with the loss of epithelial differentiation and by the switching towards a mesenchymal phenotype, which is accompanied by increased cell motility and also invasion. Recent studies have established that EMT plays an essential role in tumor metastasis and recurrence which is thought to be closly linked with cancer stem cells (Kong *et al.*, 2010). The data generated in this chapter indicates that the holoclones could potentially select for the EMT phenotype.

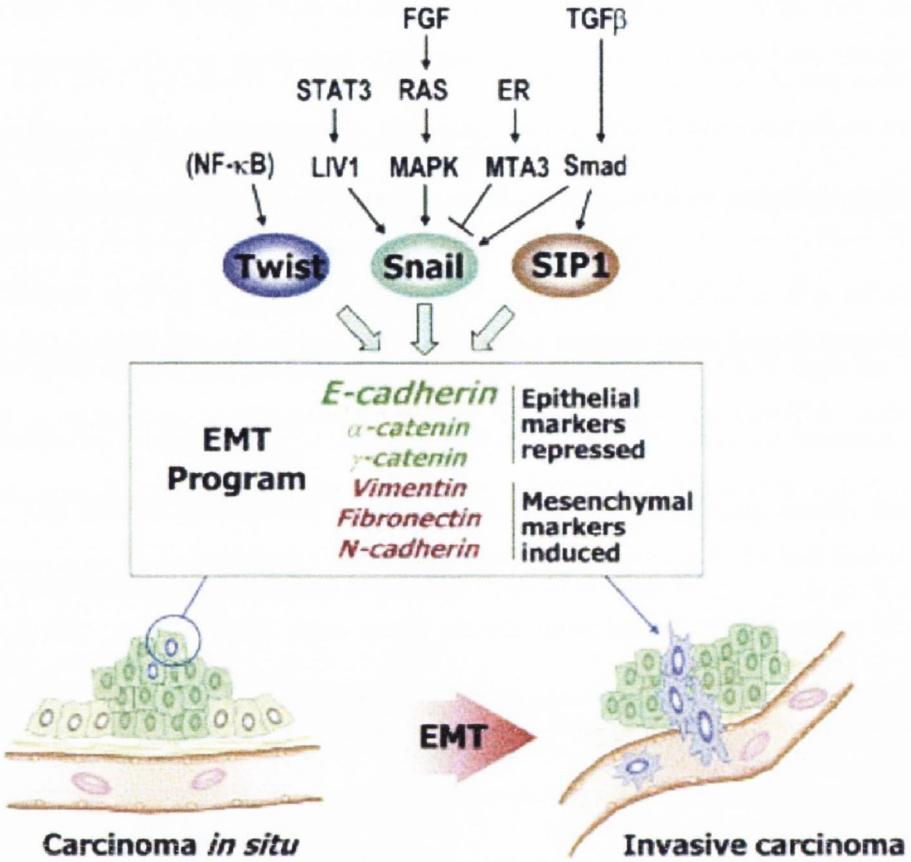



**Figure 4.6: Drivers and mediators of EMT.** Early tumour cells retain epithelial properties however the over expression of master regulators of EMT which include the transcription factors Twist, Snail and SIP1 in cancer cells causes dramatic changes in gene expression profiles and cellular behavior to occur. EMT is a characteristic feature of cells undergoing proliferation (Zvaifler, 2006).

Akt3 was found to be significantly upregulated in the PC-3 holoclones in comparison to the founder PC-3 cell line and was also confirmed to be significantly upregulated by TaqMan® RT-PCR also. RAC-gamma serine/threonine-protein kinase is an enzyme that in humans is encoded by the *AKT3* gene (Brodbeck *et al.,* 1999). The protein is encoded by the gene and is a member of the AKT serine/threonine protein kinase family. The AKT kinases are known to be regulators of cell signaling in response to both insulin and growth factors. Akt3 is known to be involved in numerous biological processes including cell proliferation, apoptosis, differentiation and tumourigenesis. Akt3 has been found to be over expressed in many cancers such as, breast, ovarian, melanoma and hepatocellular canrcinoma (Zinda *et al.,* 2001). It also has been shown to play a key role in the aggressive phenotypes of androgen-insensitive prostate cancers. Therefore the data in this chapter and the literature supports that the PC-3 holoclone is displaying potent cancer stem cell properties moreso than the resting PC-3 cell line.

Fibronectin (Fn1) was found to be significantly upregulated in the LNCaP holoclones in comparison to the founder LNCaP cell line and was also confirmed to be significantly upregulated by TaqMan® RT-PCR also. Fibronectin has been shown to play a major role in cell adhesion, growth, migration and differentiation of cells and is also very important in embryonic development (Pankov *et al.,* 2002). Altered expression of Fibronectin, its degradation and organisation have all been associated with various diseases including the development of cancer (Han *et al.*, 2006). In particular, fibronectin expression is increased in lung cancer, principally in non-small cell lung carcinoma. It is the adhesion of lung carcinoma cells to fibronectin that increases tumorigenicity and confers resistance to chemotherapeutics (Han *et al.*, 2006). Fibronectin is also involved in the EMT process.

NDRG1 was found to be significantly upregulated in the LNCaP holoclones in comparison to the founder LNCaP cell line and was also confirmed to be significantly upregulated by TaqMan® RT-PCR also. The NDRG1 gene is a member of the N-myc downregulated gene family that belongs to the alpha/beta hydrolase superfamily. It has been shown to be involved in stress and hormone responses, cell growth, and differentiation. This gene has also been shown to be upregulated during colon epithelial cell differentiation (van Belzen et al., 1997). Since it has been identified as a growth and cancer related gene, is a marker of tumour progression and cellular differentiation. Upregulation of this gene in cells induces differentiation (Ellen *et al.*, 2008). Therefore these cells are displaying differentiation characteristics which are present in cancer stem cells suggesting the holoclones are indeed cancer stem like cells. This gene is over expressed in a variety of cancer and its differential expression in cancer cells compared with normal cells makes the *NDRG1* gene an important cancer marker particularly in identifying the LNCaP holoclones from the founder cell.

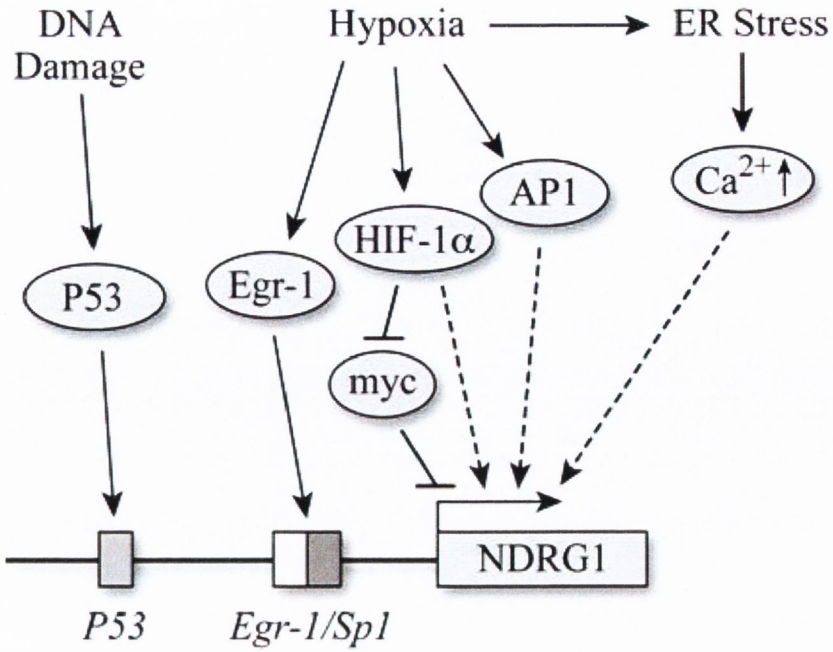



**Figure 4.7: Transcriptional regulation of NDRG1.** Summary of the numerous transcription factors directly or indirectly regulated by the transcription of the NDRG1 gene (Ellen *et al.*, 2008).

Etv1 was the only gene found to be commonly expressed in both the PC-3 and LNCaP holoclones and was significantly upregulated in both holoclones in comparison to the relevant founder cell lines. Many studies have shown that this gene is significantly over expressed in prostate cancer and have been identified as an androgen receptor regulated gene that mediates prostate cancer cell invasion. Studies have shown that ETV1 expression transitions from androgen-induced to androgen-independent as that prostate cancer cells switch from hormone-dependent to hormone-refractory. This indicates that the transition could possibly be somewhat responsible for the elevated levels of ETV1 observed in many prostate tumours and cell lines (Cai *et al.*, 2007). The analysis generated from the microarrays and TaqMan® RT-PCRs suggests that both PC-3 and

LNCaP holoclones coulds potentially play a role in driving invasion in prostate cancer further supporting the cancer stem cell and holoclones theory.

ETV1 plays an important role in prostate cancer genomic rearrangements involving the ETS family of transcription factors which occur in approximately 40-70% of prostate cancer cases (Gasi *et al.*, 2011). Both ETV1 and ERG are the most common genetic alterations. Therefore the high prevalence of these arrangements and the biological significance could potentially represent a novel therapeutic target to treat prostate cancer. Previous cell line experiments have suggested that the androgen responsive promoter elements of TMPRSS2 mediate the overexpression of the ETS family members in prostate cancer. The findings from previously published literature and from the results found in this chapter suggest that, ETV1 could potentially have major implications in the development of carcinomas and future molecular diagnosis and treatment of prostate cancer.

Gene ontology analysis was carried out using the database DAVID and demonstrated that the PC-3 and LNCaP holoclones had effects on numerous biological processes and pathways that were over represented. However it is not possible to discuss each individual biological process in detail.

A significant over representation of cell cycle pathways was predicted in the PC-3 holoclones. Mitotic cell cycle progression is achieved through a reproducible sequence of events; DNA replication known as the S phase and mitosis defined as the M phase, separated temporally by gaps known as G1 and G2 phases. Cyclin-dependent kinases (CDKs) are essential regulatory enzymes, and precise activation and inactivation of

CDKs at specific points in the cell cycle are required for orderly cell division. However as this pathway is significantly overexpressed in the PC-3 holoclones it can be deduced that cell division does not occur in an orderly manner. Clearly, the products of cell cycle regulatory genes are key determinants of cancer progression in particular in the PC-3 holoclones.

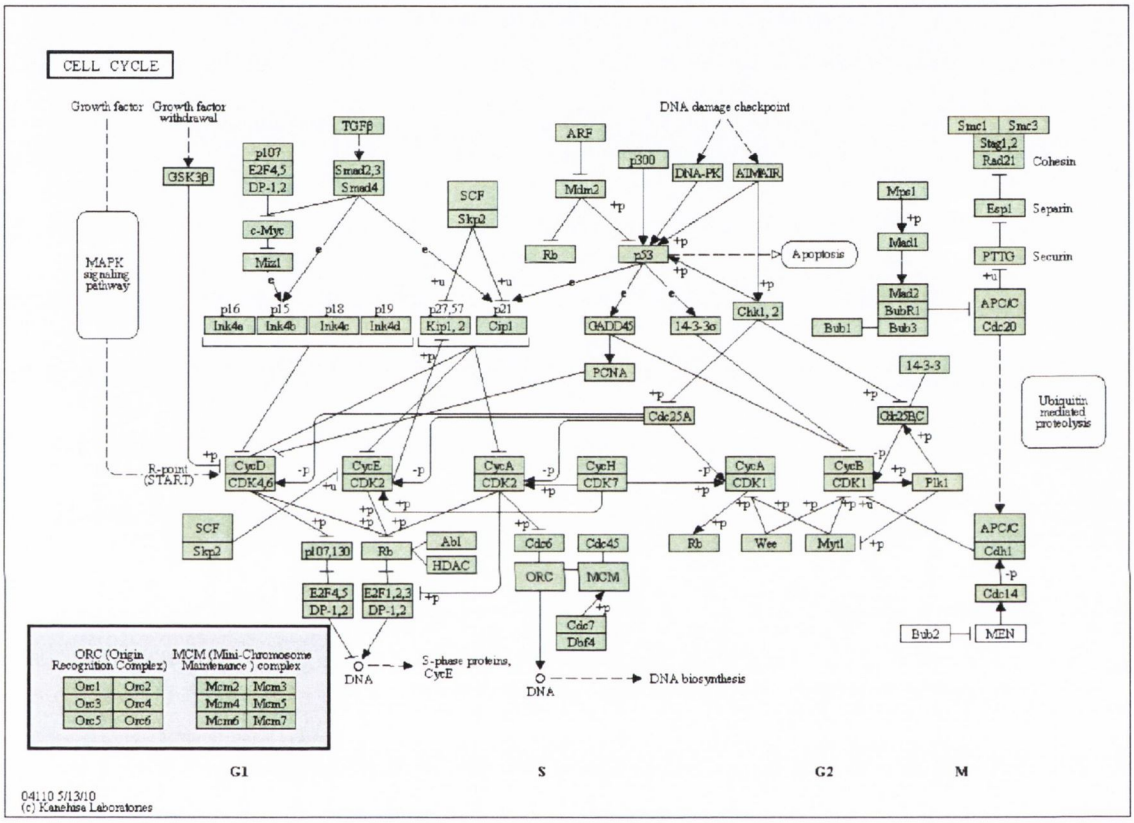



**Figure 4.8: Cell cycle Pathway**. Cell cycle pathway, over represented in PC-3 holoclones (www.genome.jp).

Another interesting pathway that was significantly over expressed in the LNCaP holoclones is the Negative regulation of apoptosis pathway. Apoptosis is a controlled mechanism of cell death which is involved in the regulation of tissue homeostasis. There are two major pathways of apoptosis; the extrinsic and the intrinsic pathways, both of which are found in the cytoplasm (Danial and Korsmeyer., 2004). More recently, studies indicate that the ER is implicated in apoptotic implementation.

Alterations and accumulation of misfolded proteins in the ER cause ER stress. Extended ER stress can result in the activation of BAD and/or caspase-12, and execute apoptosis (Heath-Engel *et al.,* 2008). This is relevant significance in the LNCaP holoclones as apoptosis could potentially be negatively regulated in the holoclones confirming that they are a more potent cancer cell than the founder cells. This analysis further supports the cancer stem cell theory.

This chapter describes for the first time a gene expression profile of the PC-3 and LNCaP cancer stem cell like holoclones when compared to founder cell lines. The analysis characterised the holoclones and also identified specific cellular targets representing stemness, differentiation and epithelial to mesenchymal transition in both the PC-3 and LNCaP holoclones in comparison to the relevant founder cell lines. This was achieved by determining genome-wide alterations induced at the transcriptome level in all of these cells from microarray analysis. In summary, this allowed for the analysis of gene expression alterations present in holoclones when compared to that of the relevant founder cell lines.

Various other genes that were validated in this chapter which represent stemness, differentiation and EMT as well as numerous other biological processes involved in cancer progression establishment, progression and maintenance. These gene and their relevant functions have been found to be either significantly downregulated or upregulated specifically in the PC-3 and LNCaP holoclones indicating that these cells have specific expression profiles for each holoclone/founder set cancer stem cell holoclone potential

# Systematic review of miRNA genes profiled in prostate cancer

Chapter 5

## 5.0 Introduction

### 5.1 Systematic review

A systematic review assembles all evidences that fit all pre-specified eligible criteria in order to address a specific question. They aim to minimise bias by using clear, systemic methods. Systematic reviews allow for the reduction of the impact on biases, promotes transparency of methods, processes and platforms (Dubben and Beck-Bornholdt, 2005). They should provide a summary of literature relevant to a research question in this case miRNA genes profiled in prostate cancer. A systematic review consists of a thorough search of the literature for published peer reviewed journals of relevance.

A traditional narrative review is different from a systematic review. Traditional, narrative reviews contain qualitative summaries of evidence on a specific area of research (Teagarden, 1989). Usually they involve subjective methods to collect and interpret studies, and tend to selectively cite literature that reinforces preconceived ideas. Narrative reviews often do not explicitly describe how the reviewers searched, selected and appraised the quality of studies In contrast, a systematic review includes a comprehensive search of the relevant literature on a specific focused question, selecting and including studies using clear and reproducible eligibility inclusion criteria, assessment of the quality of each study and then synthesis of the compiled results (Pai *et al.*, 2004). A systematic review uses an objective and transparent methodology for research combination, with the overall aim of minimizing bias (Savoie *et al.*, 2003). Many systematic reviews are based on available data sets from various published groups, there are also qualitative reviews that gather, analyse and report the findings of these various published groups (Moher *et al.*, 2007).

Systematic reviews can scrutinise either quantitative or qualitative evidence. When the two or more types of evidence are inspected within one review it is referred to as a mixed-method systematic review. The strength of a mixed-method systematic review includes the preservation of the integrity of the findings of different types of studies i.e.; groups that use different technical platforms and by using the appropriate type of analysis that is specific to each type of reported finding from the various groups. All systematic reviews should contain both narrative and quantative elements, the following questions should be considered and addressed;

- Which comparisons should be included?
- Which study results should be included in each comparison?
- What is the best summary of effect for each comparison?
- Are the results of studies similar within each comparison?
- How reliable are the generated summaries?

A systematic review aims to convey the same level of rigor to reviewing research evidence. A high quality systematic review aims to; identify all relevant published studies, select appropriate studies for inclusion, assess the quality of each study and finally synthesise the finding from each study in an unbiased way.

Unlike conventional narrative reviews, in which the author writes about their particular area of research interest, systematic reviews use explicit and reproducible criteria in order to reduce bias within the systematic review. This process includes a comprehensive search approach, which usually involves both an electronic search methods of all potentially relevant peer reviewed publications and the application of

predetermined inclusion and exclusion criteria in the selection of publications for review. The relevant and eligible studies are critically evaluated in terms of methodological rigour, the data is synthesised and the results are summarised and interpreted using a standardised format i.e.; normalised in order to reduce bias. The usefulness of the end outcome of results depends greatly on whether a clear and relevant question was proposed and the extent to which methodological rigour has been used to minimise both error and bias (Kaczorowski., 2009).

Systematic reviews can assist in examining and better understanding inconsistencies and evolution of various scientific and clinical studies over time. By pooling the results of several studies and normalising and formatting the findings of all revelant published studies, systematic reviews can summarise findings between the results generated by the individual studies and identify the common findings presented by each study.

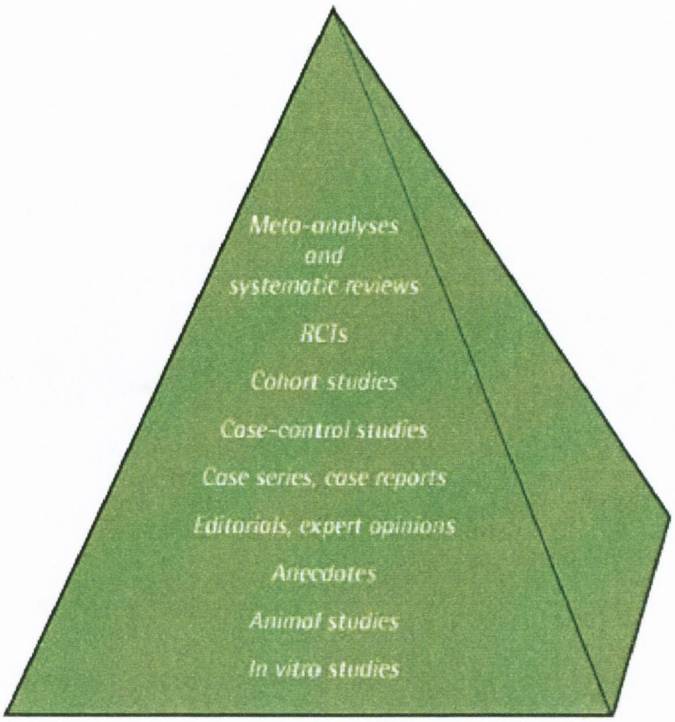



**Figure 5.1: Pyramid of evidence.** Pyramid depicting the hierarch of the numerous types of scientific and medical research reviews and methodology in order of significance (Kaczorowski., 2009).

Variation across studies is known as heterogeneity should be considered and random effects meta-analyses allow for heterogeneity by assuming that underlying effects follow a normal distribution (Engels *et al.,* 2000). Sensitivity analyses are also needed in order to examine if the overall findings are robust and could potentially influence decisions.

In this chapter data was merged from several different studies that performed miRNA profiling on different technical platforms, tumour sample types and cell line models (versus normal samples) and the systematic review allows for the investigation into specific or common miRNA profile patterns. This analysis distinguishes common miRNAs that may play a crucial role in prostate cancer.

## 5.2 Chapter aims

The aim of this chapter was to compile a qualitative systematic review of various different independent studies on miRNA expression profiling in prostate cancer, in cell line models and prostatectomy specimens. This was achieved by merging data from several different technical platforms, tumour sample types and cell line models and compiling all the data findings from all relevant eligible published studies. This analysis distinguished specific miRNA profile patterns and any common miRNAs that could potentially play a crucial role in prostate cancer. The benefit of the systematic review evaluates the knowledge base in relation to miRNA profiles in prostate cancer. The final aim of this chapter was to carry out bioinformatic analysis on the most common miRNA genes identified by the systematic review.

## 5.3 Methods

Full details of methods employed are described in detail in chapter 2.

## 5.4 Results

### 5.4.1 Study inclusion for the generation of the systematic review

A total of 6 eligible studies were identified from the PubMed database that miRNA profiled prostate cancer specimens and cell line models that had not been identified in previous systematic reviews, see Table 5.1. Various other studies were identified however some studies were not included as they were not eligible and did not meet inclusion criteria. Such studies did not have sufficient reported data to be included in the systematic review and this could possibly have introduced heterogeneity.

**Table 5.1**: Summary of studies identified for miRNA profiling in prostate cancer.

| Reference | Tumour tissue | Cell lines | Profiling method |
|---|---|---|---|
| Ambs *et al* 2008 | Tumour vs. Non-tumour | LNCaP & PC-3 | Microarray validated by quantitative real-time PCR |
| Jiang *et al* 2005 | — | LNCaP, PC-3, PPC-1, DU145 & TSU-PR1 | Real-time PCR & Northern blotting validation SYBR green |
| Mattie *et al* 2006 | Tumour vs. Non-tumour | PC-3, & LNCaP | Microarray validated by quantitative real-time PCR |
| Porkka *et al* 2007 | Tumour vs. Non-tumour | — | Oligonucleotide array hybridization method |
| Szczyrba *et al* 2010 | Tumour vs. Non-tumour | — | Microarray, Quantitative real-time PCR determined by miRNA *in situ* hybridization. |
| Tong *et al* 2008 | Tumour vs. Non-tumour | — | Microarray validated by quantitative real-time PCR |

### 5.4.2 Generation of miRNA gene lists and bioinformatic analysis

A list of miRNA genes profiled in prostate cancer specimens and cell lines was compiled from 6 original published studies, see Table 5.2. MiRNA genes were reported as upregulated, downregulated, undetected and no change. MiRNA genes that were not profiled by groups were not reported and were displayed as blank fields in Table 5.2. The top 10 predicted gene targets for 20 of the most common miRNA genes were generated using the online database miRGen, see Table 5.3. The top 5 predicted metabolic pathways were generated for the most common 15 miRNA genes using the online database miRNApath, see Table 5.4.

**Table 5.2: MicroRNA genes profiled in prostate cancer cell lines and tumour tissue compare to normal tissue from 6 eligible studies.**

| miRNA | Szczyrba Tumours | Porkka Tumours | Mattie Cell line | Mattie Tumours | Ambs Both | Tong Tumours | Jiang Cell lines |
|---|---|---|---|---|---|---|---|
| let-7-a | up | down | up | down | | up | |
| let-7-a* | | | up | down | | | |
| let-7a-1 | | | | | | | 5down |
| let-7a-2 | | | | | | | 4up&1down |
| let-7a-3 | | | | | | | 1NC&4down |
| let-7b | | down | NC&up | down | down | | 3NC&2down |
| let-7b* | | | NC&up | down | | | |
| let-7c | up | down | NC&up | down | | | 2und&2down&1up |
| let-7c* | | | NC&up | down | | | |
| let-7d | | down | up | down | | | 2NC&3down |
| let-7d* | | | up | down | | | |
| let-7e | | | up | down | | | 4down&1und |
| let-7e* | | | up | down | | | |
| let-7-f | up | down | up | down | | | |
| let-7-f* | | | up | down | | | |
| let-7f-1 | | | | | | | 5down |
| let-7f-2 | | | | | | | 5down |
| let-7g | | down | down&NC | down | | | 1NC&4down |
| let-7g* | | | down&NC | down | | | |
| let-7i | | | down&NC | down | up | | 5down |
| let-7i* | | | NC&down | down | | | |
| MIR-001* | | | down&up | down | | | |
| MIR-001 | | | down&up | down | | | |
| MIR-001-1 | | | | | | | 3down&2NC |
| MIR-001-2 | | | | | down | | 3Nc&1up&1down |
| MIR-007-1 | | | | | | | 4down&1NC |
| MIR-007-2 | | | | | | | 5down |
| MIR-007-3 | | | | | | | 5down |
| MIR-007-1,2 | | | | | down | | |
| MIR-009-1-P | | | | | | | 5down |
| MIR-009-2-P | | | | | | | 5down |
| MIR-009-3-P | | | | | | | |
| MIR-010a* | | | up&down | down | | | |

| miRNA | Szczyrba Tumours | Porkka Tumours | Mattie Cell line | Mattie Tumours | Ambs Both | Tong Tumours | Jiang Cell lines |
|---|---|---|---|---|---|---|---|
| MIR-010a | | | up&down | down | | | |
| MIR-010b* | | | down | down | | | |
| MIR-010b | | | down | down | | | |
| MIR-010a,b | | | | | | | 1NC&1und&3down |
| MIR-015a* | | | | down | | | |
| MIR-015a | | | down&NC | down | | | 4down&1NC |
| MIR-015b* | | | down | up | | | |
| MIR-015b | up | | down&NC | down | | | 2up&3NC |
| MIR-16b* | | | | down | | | |
| MIR-16* | | | down | down | | | |
| MIR-16 | | down | down | down | | | |
| MIR-016-1 | | | | | | | 3up&2NC |
| MIR-016-2 | | | | | | | 3NC1up&1down |
| MIR-017-P | | | | | | | 4NC&1up |
| MIR-018 | | | | | | | 5up |
| MIR-19a* | | | down&NC | down | | | |
| MIR-19a | | | down&NC | down | | | |
| MIR-019a-P | | | | | | | 1NC&3down&1up |
| MIR-19b* | | | down&NC | down | | | |
| MIR-19b | | down | down&NC | down | | | |
| MIR-019b-1-P | | | | | | | 3NC&2up |
| MIR-019b-2-P | | | | | | | 2down&3und |
| MIR-020* | | | NC&up | down | | | |
| MIR-020 | | | NC&up | down | | | 3down&2und |
| MIR-020a | up | | | | | up | |
| MIR-21* | | | up&down | down | | | |
| MIR-021 | up | | up&NC | down | | | 5up |
| MIR-22* | | | down | down | | | |

| miRNA | Szczyrba Tumours | Porkka Tumours | Mattie Cell line | Mattie Tumours | Ambs Both | Tong Tumours | Jiang Cell lines |
|---|---|---|---|---|---|---|---|
| MIR-22 | down | down | down | down | | | |
| MIR-022-P | | | | | | | 2up&3NC |
| MIR-023a | | down | up&down | down | | | 3NC&3down |
| MIR-23a* | | | up&down | | | | |
| MIR-23b* | | | NC&down | down | | | |
| MIR-23b | | down | NC&down | down | | down | |
| MIR-023b-P | | | | | | | 3up&21down&1up |
| MIR-024-1,2 | | | | | | | 5up |
| MIR-024 | down | | NC&down | down | | down | |
| MIR-024* | | | NC&down | down | | | |
| MIR-025 | up | | NC&up | down | up | up | 1down&4NC |
| MIR-025* | | | NC&up | down | | | |
| MIR-026a | | down | down | down | | | |
| MIR-026a* | | | down | down | | | |
| MIR-026a-1,2 | | | | | up | | 4down&1NC |
| MIR-026b* | | | down | down | | | |
| MIR-026b | up | down | down | down | | | 3up&2down |
| MIR-027a* | | | NC&down | down | | | |
| MIR-027a | down | down | NC&down | down | | | |
| MIR-027b* | | | down | down | | | |
| MIR-027b | down | down | down | down | | | |
| MIR-027a,b | | | | | | | 3up&2NC |
| MIR-028 | | | down&NC | down | | | 5down |
| MIR-028* | | | down&NC | down | | | |
| MIR-029a* | | | NC&down | down | | | |
| MIR-029a | down | down | NC&down | down | | | |
| MIR-029c* | | | down | down | | | |
| MIR-029a,c | | | | | | | 1NC&2down&2up |
| MIR-29b* | | | down | down | | | |

| miRNA | Szczyrba Tumours | Porkka Tumours | Mattie Cell line | Mattie Tumours | Ambs Both | Tong Tumours | Jiang Cell lines |
|---|---|---|---|---|---|---|---|
| MIR-29b | | down | down | down | | | |
| MIR-029b-1,2 | | | | | | | 4down&1NC |
| MIR-029c | | | down | down | | | |
| MIR-30a_5p * | | | | down | | | |
| MIR-30a_5p | | down | down | down | | | |
| MIR-30a_3p * | | | NC&down | down | | | |
| MIR-30a_3p | | | down | down | | | |
| MIR-030a,e | | | | down | | | 3down&2NC |
| MIR-030b* | | | down | down | | | |
| MIR-030b | up | down | down | down | | | 3up&2Nc |
| MIR-30c* | | | down | down | | | |
| MIR-30c | | down | down | down | | | |
| MIR-030c-1,2 | | | | | | | 3Nc&1up&1down |
| MIR-030d | | | down&NC | down | | | |
| MIR-030d* | | | down | down | | | 5down |
| MIR-030e | down | | down&und | up | | | |
| MIR-031-P | | | | | | | 5up |
| MIR-031 | | | up&down | down | up | | |
| MIR-031* | | | up&down | down | | | |
| MIR-032 | | | | | up | | 3down&2NC |
| MIR-033 | | | | | | | 5down |
| MIR-034a | | | und&NC | down | down | | 2NC&2doown&1up |
| MIR-034a* | | | down&NC | down | | | |
| MIR-034b | | | | | up | | 5down |
| MIR-034c | | | | | | | 5undetected |
| MIR-92 | | down | NC&up | down | | | |
| MIR-92* | | | NC&up | down | | | |
| MIR-092-1,2 | | | | | up | | |

| miRNA | Szczyrba Tumours | Porkka Tumours | Mattie Cell line | Mattie Tumours | Ambs Both | Tong Tumours | Jiang Cell lines |
|---|---|---|---|---|---|---|---|
| MIR-092-1 | | | | | | | 5down |
| MIR-092-2-P | | | | | | | 4down&1NC |
| MIR-093* | | | NC&up | down | | | |
| MIR-093 | | | NC&up | down | up | | 3Nc&2down |
| MIR-095 | | | | | | | 3down&2NC |
| MIR-096 | | | | | | | |
| MIR-096-P | | | | | | | 5up |
| MIR-098* | | | up | down | | | |
| MIR-098 | | | up | down | | | 5up |
| MIR-099a | | down | down&NC | down | | | 5down |
| MIR-099a* | | | down&NC | down | | | |
| MIR-099b* | | | NC | down | | | |
| MIR-099b | | | NC | down | up | | 1up&1down&3NC |
| MIR-100 | | down | NC&down | down | | down | 4up&1down |
| MIR-101* | | | down | down | | | |
| MIR-101 | down | | down | down | | | |
| MIR-101-1 | | | | | | | 5down |
| MIR-101-2 | | | | | | | 3NC&2down |
| MIR-103 | | down | NC | down | | | |
| MIR-103* | | | NC&up | down | | | |
| MIR-103-1,2 | | | | | | | 5up |
| MIR-105-1,2 | | | | | | | 2NC&3down |
| MIR-106a | up | | NC | down | | | |
| MIR-106a* | | | NC&up | down | | | |
| MIR-106a-P | | | | | | | 5down |
| MIR-106b* | | | down&NC | down | | | |
| MIR-106b | up | | down&NC | down | up | | 5up |
| MIR-107 | | | down | | | | 5up |
| MIR-107* | | | | undetected | | | |

| miRNA | Szczyrba Tumours | Porkka Tumours | Mattie Cell line | Mattie Tumours | Ambs Both | Tong Tumours | Jiang Cell lines |
|---|---|---|---|---|---|---|---|
| MIR-108 | | | | | | | 3NC&2down |
| MIR-122a | | | und&NC | down | | up | 5down |
| MIR-122a* | | | und&up | down | | | |
| MIR-124a-1,2,3 | | | | | | | 5down |
| MIR-125a | | down | NC&down | down | up | | 2NC&2up&1down |
| MIR-125a-5p | down | | | | | | |
| MIR-125a* | | | NC&down | down | | | |
| MIR-125b | down | down | down&NC | down | | down | |
| MIR-125b* | | | down&NC | down | | | |
| MIR-125b-1 | | | | | | | 2up&2NC&1down |
| MIR-125b-2 | | | | | | | 4down&1NC |
| MIR-126 | | | down | down | down | | 5down |
| MIR-126* | up | | down | down | | | |
| MIR-127* | | | down | down | | | |
| MIR-127 | | | down | down | | | 3up&2NC |
| MIR-128a* | | | down&NC | down | | | |
| MIR-128a | | | NC | down | down | | 4NC&1up |
| MIR-128b* | | | NC&up | down | | | |
| MIR-128b | | | NC | down | | | 5down |
| MIR-129 | | | | | | up | |
| MIR-129-2 | | | | | | | 5up |
| MIR-130a-P | | | | | | | 5up |
| MIR-130a | | | down | down | | | |
| MIR-130a* | | | down | down | | | |
| MIR-130b* | | | down | down | | | |
| MIR-130b | | | down | down | | | 5down |
| MIR-132* | | | down | down | | | |
| MIR-132 | | | down | down | | | 5down |

| miRNA | Szczyrba Tumours | Porkka Tumours | Mattie Cell line | Mattie Tumours | Ambs Both | Tong Tumours | Jiang Cell lines |
|---|---|---|---|---|---|---|---|
| MI R - 133a* | | | down | up | | | |
| MI R - 133a | | | | up | | | |
| MI R - 133a-1 | | | | | down | | |
| MIR-133a-1,2 | | | | down | | | 3NC&2down |
| MIR-133b* | | | down | down | | | |
| MIR-133b | | | down | down | | | 2NC&2up&1down |
| MIR-134 | | | | | | | 3up2NC |
| MIR-135a | | | down | up | | | |
| MIR-135a* | | | down | undetected | | | |
| MIR-135a-1,2 | | | | | | | 5down |
| MIR-135b-P | | | | | | | 4down&1NC |
| MIR-136 | | | | | | | 5down |
| MIR-137 | | | | | | | 4down&1NC |
| MIR-138-1,2 | | | | | | | 5down |
| MIR-139 | | | | | | | 2up&3NC |
| MIR-140 | | | | | | | 4und&1down |
| MIR-141 | | down | und&down | up | | up | 5down |
| MIR-142-P | | | | | | | 3down&2NC |
| MIR-143* | | | down | down | | | |
| MIR-143 | down | down | down | down | | down | 4down&1NC |
| MIR-144 | | | | | | | 3und&2down |
| MIR-145* | | | down | down | | | |
| MIR-145 | down | down | down | down | down | down | |
| MIR-145-P | | | | | | | 3down&2NC |
| MIR-146* | | | | down | | | |
| MIR-146 | | | up&und | | | | 2und&3down |
| MIR-147 | | | | | | | 4down&1Nc |
| MIR-148a | up | down | down&NC | down | | | |

| miRNA | Szczyrba Tumours | Porkka Tumours | Mattie Cell line | Mattie Tumours | Ambs Both | Tong Tumours | Jiang Cell lines |
|---|---|---|---|---|---|---|---|
| MIR-148a* | | | down&NC | down | | | |
| MIR-148a-P | | | | | | | 5down |
| MIR-148b-P | | | | | | | 2NC&2down&1up |
| MIR-148b | | | down&NC | down | | | |
| MIR-148b* | | | down | down | | | |
| MIR-149* | | | down | down | | | |
| MIR-149 | | | down | down | | | 1up&4NC |
| MIR-150* | | | | down | | | |
| MIR-150 | | | down | down | | | 5NC |
| MIR-151* | | | NC&down | NC | | | |
| MIR-151 | | | up&NC | down | | | 4down&1Nc |
| MIR-152* | | | down | down | | | |
| MIR-152 | down | | down | down | | | 5down |
| MIR-153-1,2 | | | | | | | 5down |
| MIR-154 | | | | | | | 5down |
| MIR-155 | | | | | | | 5down&1und |
| MIR-175p | | | NC&up | down | | | |
| MIR-175p* | | | NC&up | down | | | |
| MIR-173p* | | | und&down | up | | | |
| MIR-181a-1,2 | | | | | up | | |
| MIR-181a* | | | NC&down | down | | | |
| MIR-181a | | | NC&down | down | | | |
| MIR-181a,c | | | | | | | 3und&2up |
| MIR-181b | | | up&down | down | | | |
| MIR-181b* | | | up&down | down | | | |
| MIR-181b-1,2 | | | | down | | | 5down |
| MIR-181b-1,2 | | | | | | | |

| miRNA | Szczyrba Tumours | Porkka Tumours | Mattie Cell line | Mattie Tumours | Ambs Both | Tong Tumours | Jiang Cell lines |
|---|---|---|---|---|---|---|---|
| MIR-182* | | | NC | down | | | |
| MIR-182 | | | NC | down | up | | 5up |
| MIR-183 | | | up | down | | | 1up&4down |
| MIR-183* | | | up&NC | down | | | |
| MIR-184 | | | | | | | 3up&2NC |
| MIR-185 | | | down | down | | | 3up&2NC |
| MIR-185* | | | NC | down | | | |
| MIR-186 | | | down&NC | down | | | up(LNCap)/ down(PC3) |
| MIR-186* | | | down | down | | | |
| MIR-186-P | | | | | | | 5up |
| MIR-187* | | | down&NC | down | | | |
| MIR-187 | | | down | down | | | 5down |
| MIR-188 | | | | | up | | |
| MIR-188b-P | | | | | | | 5down |
| MIR-189* | | | NC | down | | | |
| MIR-189 | | | down | down | | | |
| MIR-190 | | | | | | | 3und&2down |
| MIR-191 | | | NC&up | down | | | 5down |
| MIR-191* | | | NC&up | down | | | |
| MIR-192 | | | | | | | 5undetected |
| MIR-193* | | | down&NC | down | | | |
| MIR-193 | | | down | down | | | 5down |
| MIR-194-1,2 | | | | | up | | 5down |
| MIR-195 | | down | down | down | | | |
| MIR-195* | | | down&NC | down | | | |
| MIR-195-P | | | | | | | 5up |
| MIR-196a-1 | | | | | | | 4down&1Nc |
| MIR-196a-1,2 | | | | | up | | 3NC&2down |

| miRNA | Szczyrba Tumours | Porkka Tumours | Mattie Cell line | Mattie Tumours | Ambs Both | Tong Tumours | Jiang Cell lines |
|---|---|---|---|---|---|---|---|
| MIR-196a-2 | | | | | | | |
| MIR-196b | | | | | | | 5up |
| MIR-197 | | | down&NC | down | | | 5up |
| MIR-197* | | | down&NC | down | | | |
| MIR-198 | | up | | | | | 1NC&1up&3down |
| MIR-199a | | down | down | down | | | |
| MIR-199a** | | | down | down | | | |
| MIR-199a* | | down | down | down | | | |
| MIR-199a-5p | down | | | | | | |
| MIR-199a-1-P | | | | | | | 3up&2NC |
| MIR-199a-2-P | | | | down | | | 3down&2NC |
| MIR-199b* | | | down | down | | | |
| MIR-199b | | | down | | | | |
| MIR-199b-P | | | | down | | | 4up&1NC |
| MIR-200a | | | down | down | | | |
| MIR-200a* | | | down | | | | |
| MIR-200a-P | | | | down | | | 5up |
| MIR-200b* | | | NC&up | down | | | |
| MIR-200b | | | NC&up | | | | 5down |
| MIR-202 | | up | | down | | | |
| MIR-200c* | up | | | down | | | |
| MIR-200c | | | down&up | down | up | | 5down |
| MIR-203 | | | down&NC | down | | | 5down |
| MIR-203* | | | down&und | down | | | |
| MIR-204* | | | down | down | | | |
| MIR-204 | | | down&und | down | | | 3up&1NC&1down |
| MIR-205 | | down | NC&down | down | down | down | 5down |
| MIR-205* | | | down&NC | down | | | |

| miRNA | Szczyrba Tumours | Porkka Tumours | Mattie Cell line | Mattie Tumours | Ambs Both | Tong Tumours | Jiang Cell lines |
|---|---|---|---|---|---|---|---|
| MIR-206 | | | NC&up | down | | | 5down |
| MIR-206* | | | up&und | down | | | |
| MIR-208 | | | | down | | | 5down |
| MIR-210 | | up | up&NC | down | | | 5up |
| MIR-211 | | | | | | | 5up |
| MIR-212 | | | | | | | 5NC |
| MIR-213 | | | | down | | | 2NC&2down |
| MIR-214 | | | down&NC | down | | | 4up&1NC |
| MIR-214* | | | down&NC | | | | |
| MIR-215 | | | | | | | 5down |
| MIR-216 | | | | | | | 5down |
| MIR-217 | | | | down | | | 2und&3down |
| MIR-218* | up | | NC&down | down | | | |
| MIR-218 | | | down | down | | | |
| MIR-218-2 | | | | | down | | |
| MIR-218-1,2 | | | | | | | 1NC&4down |
| MIR-219-1,2 | | | | | | | 1NC&4down |
| MIR-220 | | | | down | down | | 3down&2NC |
| MIR-221 | down | down | NC&down | down | down | down | 1up&2down&2NC |
| MIR-221* | | | NC&down | down | | | |
| MIR-222 | | down | up&down | down | | down | |
| MIR-222* | | | up&down | | | | |
| MIR-222-P | | | | | | | 3down&2NC |
| MIR-223 | down | | NC&down | down | | | 5down |
| MIR-223* | | | down | down | | | |
| MIR-224 | | | down | down | | | 4down&1NC |
| MIR-224* | | | down&NC | | | | |
| MIR-296 | | up | | | | | 3down&2NC |
| MIR-299-P | | | | | | | 2up&1NC |

| miRNA | Szczyrba Tumours | Porkka Tumours | Mattie Cell line | Mattie Tumours | Ambs Both | Tong Tumours | Jiang Cell lines |
|---|---|---|---|---|---|---|---|
| MIR-301 | | | | | | | 3NC&1down&1up |
| MIR-302 | | | | | | up | 5down |
| MIR-302b | | | | | | | 5down |
| MIR-302c | | | | | | | 5down |
| MIR-302c* | | up | | | | | |
| MIR-302d | | | | down | | | 5down |
| MIR-320 | down | up | up&NC | down | | | 3down&2NC |
| MIR-320* | | | up&NC | | | | |
| MIR-321 | | | | | | | 5up |
| MIR-323 | | | | | | | 5down |
| MIR-324-P | | | | | | | 4NC&1down |
| MIR-325 | | | | | | | 5down |
| MIR-326-P | | | | down | | | 5down |
| MIR-328 | | | down&NC | down | | | 3up&2NC |
| MIR-328* | | | down&NC | down | | | |
| MIR-329 | | | | | down | | |
| MIR-330 | | | | down | | | 4NC&1down |
| MIR-331 | | | NC | down | | | |
| MIR-331* | | | | | | | |
| MIR-331-P | | | | down | | | 3NC&2down |
| MIR-335 | | | NC | down | | | 5down |
| MIR-335* | | | down | down | | | |
| MIR-337 | | | | down | | | 3down&2NC |
| MIR-338 | | | down | down | | | 3NC&2down |
| MIR-339 | | | down&NC | down | | | 3up&1NC&1down |
| MIR-339* | | | down&NC | | | | |
| MIR-340 | | | | down | down | | 4NC&1up |
| MIR-342 | | | down | down | | | 3down&2NC |
| MIR-342* | | | down | down | | | |

| miRNA | Szczyrba Tumours | Porkka Tumours | Mattie Cell line | Mattie Tumours | Ambs Both | Tong Tumours | Jiang Cell lines |
|---|---|---|---|---|---|---|---|
| MIR-345 | | up | down&NC | down | down | | 3up&2NC |
| MIR-345* | | | | | | | |
| MIR-346 | | | | | | | 3up&2NC |
| MIR-361 | | | | | | | 4down1NC |
| MIR-367 | | | | | | | 4down&1und |
| MIR-368 | | | | | | | 3und&2down |
| MIR-369 | | | | | | | 4und&1down |
| MIR-370 | | up | | | up | | 5down |
| MIR-371 | | | | | | | 3NC&2up |
| MIR-372 | | | | | | | 5down |
| MIR-373 | | | | | | | 5down |
| MIR-373* | | up | | | | | |
| MIR-374-P | up | | | | | | 3down&2und |
| MIR-375 | | | | | up | | 5down |
| MIR-376 | | | | | | | 5down |
| MIR-377 | | | | | | | 4down&1NC |
| MIR-378 | | | | | | | 5down |
| MIR-379 | | | | | | | 4down&1und |
| MIR-380 | | | | | | | 3und&2down |
| MIR-381 | | | | | | | 5down |
| MIR-382 | | | | | | | 4down&1NC |
| MIR-383 | | | | | | | 5down |
| MIR-384 | | | | | | | 5undetected |
| MIR-410 | | | | | down | | |
| MIR-422b | | | | | | | 3NC&1down&1up |
| MIR-423 | | | | | | | 5down |
| MIR-424 | down | | | | | | 5down |
| MIR-425 | | down | | | up | | 2up&3down |
| MIR-449 | | | | | up | | |

| miRNA | Szczyrba Tumours | Porkka Tumours | Mattie Cell line | Mattie Tumours | Ambs Both | Tong Tumours | Jiang Cell lines |
|---|---|---|---|---|---|---|---|
| MIR-487 | | | | | down | | |
| MIR-497 | | | | | | | |
| MIR-490 | | | | | down | | |
| MIR-491 | | up | | | | | |
| MIR-494 | | | | | down | | |
| MIR-497 | | down | | | | | |
| MIR-498 | | up | | | | | |
| MIR-499 | | | | | down | | |
| MIR-503 | | up | | | | | |
| MIR-513 | | up | | down | | | |
| MIR-520h | | | | | down | | |
| MIR-1423-p | | | down | down | | | |
| MIR-1423-p* | | | down | down | | | |
| MIR-3243p* | | | down | down | | | |
| MIR-3243p | | | down&NC | down | | | |
| MIR-3245p | | | NC | down | | | |
| MIR-3245p* | | | NC | down | | | |

Both; Ambs data was reported as Tumours and cell lines combined, Up; upregulated, Down; downregulated, NC; no change, und; undetected, MIR; microRNA, p; prime.

**Table 5.3: Top 10 predicted gene targets for the 20 most common miRNA genes identified by the systematic review**

| miRNA | Predicted Targets (miRGen) |
|---|---|
| let-7-a | TUSC2, BZW2, XAGE1, CHD9, XAGE1, UHRF2, COL1A2, GDF6, COL4A2, E2F5 |
| let-7b | XAGE1, COL3A1, GHR, GDF6, GATM, CCND2, COL4A2, COL15A1, E2F5, IL13 |
| let-7c | COL1A2, GHR, GDF6, GATM, COL4A2, E2F5, IL13, MYC, VSNL1, MAP4K3 |
| let-7d | E2F5, BZW2, XAGE1, COL1A2, COL3A1, GDF6, GATM, IL13, AKAP6, GNG5 |
| let-7e | XAGE1, CDC34, GNG5, SLC20A1, BZW2, LIN28, KIAA1539, ZCCHC5, COL1A2, ITGB3 |
| let-7-f | COL3A1, E2F5, BZW2, XAGE1, ADRB2, COL1A2, IL13, KPNA4, MAP4K3 |
| let-7g | BZW2, CHD7, XAGE1, UHRF2, COL1A2, CACNA1D, CCND2, COL4A2, COL15A1, E2F5 |
| MIR-021 | JAG1, NTF3, CHD7, RECK, PITX2, TGFBI, SKI, NFIB, SPRY2, SMAD7 |
| MIR-099a | HS3ST2, SMARCA5, FRAP1, SMAD7, TRIB2, FZD8, HOXA1, INSM1, HS3ST3B1, EIF2C2 |
| MIR-099b | SMARCA5, HS3ST2, INSM1, FRAP1, UST, HS3ST3B1, EIF2C2, EPDR1, C4orf16, MTMR3 |
| MIR-125b | MYT1, PAFAH1B1, GPC4, KCNS3, KCNS3, MAP3K11, MSI1, OAZ2, SMARCD2, NEF3 |
| MIR-143 | MLLT3, GLI3, IGFBP5, VAPB, SFRS11, SMNDC1, TARDBP, KIAA1012, ITM2B, ATP10A |
| MIR-145 | ACTG1, TBPL1, CCNL1, SOX9, EIF4A2, FOXO1A, INHBB, FZD7, ACTR3, APPBP2 |
| MIR-152 | KLF4, NOG, EDG1, CUL5, PDIA3, MEOX2, TOMM70A, NLK, CHD7, XPO4 |
| MIR-205 | C21orf63, E2F5, ACSL1, LRP1, UBE2N, HS3ST1, CLDN11, ZFHX1B, PAFAH1B1, NR3C2 |
| MIR-221 | FOS, NLK, MESDC1, KHDRBS2, BMF, ARF4, IRF2, MEIS1, MAP3K10, NTF3 |
| MIR-222 | FOS, DMRT3, MAP3K10, NTF3, CDKN1B, RAB1A, IRX5, PBX3, ZFPM2, YWHAG |
| MIR-223 | ACVR2A, HLF, STIM1, HSP90B1, LMO2, NFIA, NFIB, PDE4D, TCERG1, FBXO8 |
| MIR-320 | PHOX2B, GSPT1, PLK3, COPS2, MLLT3, TOMM70A, C15orf24, RAI2, GPBP1, CDH20 |
| MIR-345 | IRF1, SSR2, MAGEA2B, MAGEA6, MAGEA2, PHTF1, ADA, CHRNE, COL7A1, EPOR |

**Table 5.4: Top 5 predicted metabolic pathways for the 15 most common miRNA genes identified by the systematic review.**

| miRNA | Predicted Pathways (miRNApath) |
|---|---|
| let-7c | Cell cycle, Melanogenesis, Glycerophospholipid metabolism |
| Let-7g | Cell cycle, Aminophosphonate metabolism, Glycerophospholipid metabolism |
| MIR-021 | Calcium signaling, Neuroactive ligand-receptor interaction, Glycolysis/Gluconeogenesis, Alanine & aspartate metabolism, Valine leucine & isoleucine biosynthesis |
| MIR-099a | Calcium signaling, Neuroactive ligand-receptor interaction, Sphingolipid metabolism, Glycan structures-biosyntheis 2, Apoptosis |
| MIR-099b | Calcium signaling, Neuroactive ligand-receptor interaction, Sphingolipid metabolism, Glycan structures-biosyntheis 2, Apoptosis |
| MIR-125b | Natural killer cell mediated cytotoxicity, T cell receptor signaling pathway , Focal adhesion, ECM-receptor interaction, Hematopoietic cell lineage |
| MIR-143 | Neuroactive ligand-receptor interaction, Melanogenesis, Complement and coagulation cascades, Purine metabolism, Pyrimidine metabolism |
| MIR-145 | Glycosphingolipid biosynthesis – ganglioseries, Glycan structures - biosynthesis 2, Inositol phosphate metabolism, Calcium signaling pathway, Phosphatidylinositol signaling system |
| MIR-152 | Focal adhesion, ECM-receptor interaction, Hematopoietic cell lineage, Regulation of actin cytoskeleton, ABC transporters - General |
| MIR-205 | Inositol phosphate metabolism, Calcium signaling pathway, Phosphatidylinositol signaling system, ErbB signaling pathway, Dorso-ventral axis formation |
| MIR-221 | Purine metabolism, Pyrimidine metabolism, RNA polymerase, SNARE interactions in vesicular transport, Glutamate metabolism |
| MIR-222 | Glycan structures - biosynthesis 1, SNARE interactions in vesicular transport, Glutamate metabolism, Aminoacyl-tRNA biosynthesis, Insulin signaling pathway |
| MIR-223 | nositol phosphate metabolism, Calcium signaling pathway, Phosphatidylinositol signaling system, Ribosome, Androgen and estrogen metabolism |
| MIR-320 | SNARE interactions in vesicular transport, Natural killer cell mediated cytotoxicity , T cell receptor signaling pathway, Adherens junction, Regulation of actin cytoskeleton |
| MIR-345 | Glycosphingolipid biosynthesis – ganglioseries, Glycan structures - biosynthesis 2, Inositol phosphate metabolism, Calcium signaling pathway, Phosphatidylinositol signaling system |

## 5.5 Discussion

Recently systematic reviews have shown that there is much knowledge to be gained from collecting and compiling existing published research studies however approaches used by traditional reviews have largely failed to extract and present this knowledge in a detailed, accurate and precise way that is useful in the area that particular review area. What is now needed is the same rigour in generating secondary research as is expected from the original primary research.

A high quality systematic review strives to identify all relevant published data in the literature, select appropriate and relevant studies or reports that meet the inclusion criteria for the systematic review that seeks to answer a defined set of questions. It should assess the quality of each study or report before the data is included in the report and then synthesise the findings from individual studies or reports in an unbiased way; for example all data is collected and reported in a standardised format. All collected findings should be interperted and presented in a balanced and impartial summary and then consider any flaws in the evidence reported.

A systematic review was generated using 6 original peer reviewed publications, which profiled miRNA genes in prostate cancer specimens and cell line models (Ambs *et al.*, 2008, Jiang *et al.*, 2005, Mattie *et al.*, 2006, Porkka *et al.*, 2007, Szczyrba *et al*, 2010, Tong *et al.*, 2008). All groups used normal tissue as their calibrator. The 6 studies included in the review used various technical platforms in order to generate a miRNA profile and for validation, the technologies include microarrays, quantitative real-time PCR and northern blotting methods.

The need for rigour in the generation of this systematic review was a crucial step in the process. In order to minimise bias as much as possible understanding the approach taken by each group was essential and all data was then standardised and reported in this format. All reported data included in the review was standardised and formatted using the following criteria; only data that displayed a fold change value between 0.5 and 2 was considered significant despite study groups reporting data of various different fold change values of significance. MiRNA genes displaying a fold change of 2 or above were reported as upregulated and any miRNA gene displaying a fold chine of 0.5 or below was considered as significantly downregulated. Any miRNA gene within the fold change value of 0.5 and 2 was reported as displaying no significant change. Any miRNA gene that was unamplified was reported in the review as undetected and many miRNA genes were also unprofiled by the groups and these genes were not reported in the review.

In this chapter the systematic review identified common miRNA genes expressed in both prostate cancer specimens and cell line models. The systematic review generated a list of common miRNAs that are grouped into upregulated, downregulated and undetected in prostate cancer when compared to normal/non cancerous tissues. The systematic review has identified 20 miRNAs that are all commonly upregulated, downregulated or unchanged within all cell lines and cancerous tissues, these include; let-7a, let-7b, let-7c, let-7d, let-7e, let-7g, miR-21, miR-99a, miR-99b, miR-125b, miR-143, miR-145, miR-152, miR-205, miR-221, miR-222, miR-223, miR-320 and miR-345.

MiR-21 was profiled by many groups and was upregulated in all 5 cell lines profiled by Jiang *et al.*, 2005 and was also found to be upregulated in tumour tissue profiled by Szczyrba *et al.*, 2010 Mattie *et al.*, 2006 showed inconsistencies as in the cell lines miR-21 was found to be upregulated and also displayed no change, however in tumour tissue it was found to be downregulated. Bioinformatic analsysis predicted gene targets such as TGFβ1 and SMAD7. SMAD7 belong to the SMAD protein family and also belong to the TGFβ super-family of ligands. SMAD7 has also been shown to be involved in cell signaling and differentiation. Overall the systematic review identified miR-21 to be a common gene profiled in prostate cancer and was found to be the mostly upregulated.

MiR-99a was profiled by many groups and was downregulated in all 5 cell lines profiled by Jiang *et al.*, 2005 and was also found to be downregulated in tumour tissue profiled by Porkka *et al.*, 2007, Mattie *et al.*, 2006 showed small inconsistencies as in the cell lines miR-99a was found to be downregulated and also displayed no change, however in tumour tissue it was found to be downregulated also. Bioinformatic analsysis predicted gene targets involved in cancer such as SMAD7 and HOXA1. The HOX genes are involved in and regulate gene expression, morphogenesis and cellular differentiation. Metabolic pathway predictions showed pathways such as Apoptosis which has been shown to be involved in the progression of cancer.

MiR-143 was profiled by many groups and was found to be downregulated in 4 out of 5 cell lines profiled by Jiang *et al.*, 2005 and was also found to be downregulated profiled by Szczyrba *et al.*, 2010, Porkka *et al.*, 2007, Mattie *et al.*, 2006 and Tong *et al.*, 2008 Bioinformatic analysis predicted gene targets involved in cancer such as SOX9. Many of the SOX genes are known to be involved in different types of development and many

of the SOX genes have been identified and found useful in the early diagnosis of childhood brain tumours (Rex *et al.,* 1998). Overall the systematic review identified miR-143 was a common gene profiled in prostate cancer and was found to be mostly downregulated with only one exception.

MiR-221 was profiled by many groups and was found to be downregulated by many groups such as; Szczyrba *et al.,* 2010, Porkka *et al.*, 2007, Ambs *et al.,* 2008 and Tong *et al.,* 2008. MiR-221 as found to be downregulated in tumour tissue and downregulated in LNCaP cell lines and displayed no change in the PC-3 cell line by Mattie *et al.,* 2006 and was also downregulated in 2 out of 5 cell lines profiled by Jiang *et al.,* 2005. Bioinformatic analysis predicted gene targets displayed genes involved in cancer such as; FOS, IRF2 and MAP3K10. Members of the FOS family are known to upregulate transcription in a diverse range of genes and are involved in both proliferation and differentiation in order to defend against invasion and cell damage (Garofalo *et al.,* 2009). Both proliferation and differentiation have been shown to drive the progression of cancer. The IRF gene family have play major roles in the immune response, regulating apoptosis, tumour suppression and also DNA damage. MAP3K10 functions through the JNK signaling pathway, and is reported to be involved in nerve growth factor and also induces apoptosis. Overall the systematic review identified miR-221 was a common gene profiled in prostate cancer and was found to be mostly downregulated in most cell lines and tumour tissue.

Systematic reviews generated from high quality studies are considered to represent the height of published evidence. However, in order to have confidence in the findings presented in any systematic review, it is crucial that the review contains a comprehensive assessment of the data in the current literature and that the final interpretation incorporates information regarding features of each individual study such as the quality of every study included. It is also of importance to ensure that the review process does not introduce any publication bias. Due to the increasing number of studies published and the various different technical platforms generating vast amounts of data it is important for reviews to compile and identify the relevant information and more importantly assess the quality of the reported evidence is critical. However, the systematic review generated a panel of 20 miRNAs that are all commonly upregulated, downregulated or unchanged within all cell lines and cancerous tissues, these include; let-7a, let-7b, let-7c, let-7d, let-7e, let-7g, miR-21, miR-99a, miR-99b, miR-125b, miR-143, miR-145, miR-152, miR-205, miR-221, miR-222, miR-223, miR-320 and miR-345.

# Differential expression of miRNAs in prostate cancer cell lines and Holoclones

Chapter 6

## 6.0 Introduction

## 6.1 MicroRNAs

MicroRNAs (miRNAs) are short single stranded RNAs that are 21-25nt in length and they are a group of regulatory molecules of non-coding RNAs that control protein synthesis. There are approximately 800 known miRNAs, however their exact function remains unknown but they are involved in the regulation of cellular differentiation, proliferation and apoptosis. miRNAs are thought to function in the sequestration and degradation of messenger RNAs by gene silencing, this is a RNA mediated mechanism. They are also thought to be involved or controlled by epigenetic alterations (Lujambio A *et al*, 2007; Lujambio A *et al* 2008).

MicroRNAs are currently thought to be mechanistically involved in the development of various malignancies and could potentially be a new class of biomarkers. Currently there is evidence for a link between miRNAs and cancer such as; they are involved in cell proliferation and apoptosis. Several different groups have demonstrated that there are many significant different populations of miRNAs found to be deregulated in various malignancies when compared to the relevant normal tissues (Calin and Croce., 2006, Gaur *et al.*, 2007, Lu *et al.,* 2005). This clearly indicates that miRNAs are involved in malignancy and stemness and suggests that they play a key role in cancer and cancer stemness.

Expression of abnormal miRNAs in cancer has been well described in the literature. The discovery and examination of miRNAs expression profiles in cancer has further progressed the classification of cancer. MiRNAs in cancer can act as both oncogenes and tumour suppressors. This depends on the cellular environment and the target genes

that they regulate and are involved in the tumours progression and metastasis. The various mechanisms that miRNAs are involved in the aggressiveness of the tumour include; migration, invasion, cell proliferation, EMT, angiogenesis and apoptosis (White *et al.,* 2011). There are many different miRNAs that are specifically involved with each of these mechanisms in cancer.

Prior to this study the involvement of miRNAs in prostate cancer stemness in particular the holoclone model has not been previously established or described. MiRNAs are likely to represent a key target group involved in prostate cancer stemness. Currently there are relatively few studies analysing the role of miRNAs in prostate cancer and there are no studies so far solely addressing the role or even characterising the miRNAs in prostate cancer stem cell or the holoclone cell model.

## 6.2 Aims

The initial aim of this chapter was to investigate the miRNA expression of prostate cancer cell lines LNCaP and PC-3 compared with the normal cell line PWR-1E and to generate a miRNA profile for the PC-3 and LNCaP holoclones, in order to assess the validity of the miRNA screening technology usd in this study. The second aim of this chapter was to identify prostate cancer holoclone stem cell specific miRNA populations. This was achieved by TaqMan Low Density Array Microfludic cards which are a technology used to evaluate and quantitate the expression profile of 384 miRNAs. Finally the last aim of this chapter was to carry out bioinformatic analysis on significant profiled miRNA genes.

## 6.3 Methods

Full details of methods employed are described in detail in chapter 2.

## 6.4 Results

### 6.4.1 Total RNA purified for miRNA expression profiling

In order to obtain meaningful results from the miRNA expression profiling analysis, the concentration and quality of miRNA extracted from cell lines, in particular the holoclones was determined using the *mir*Vana miRNA isolation kit (Ambion at Applied Biosystems, Foster City, CA, USA), TRIzol (Invitrogen, Life Technologies Incorporation, California, 92008) and the RNeasy Midi Kit from Qiagen (Qiagen, Fleming Way, Crawley, West Sussex, RH 10 9NQ, UK). Agarose gel electrophoresis and the nanodrop 1000 determined that the *mir*Vana kit demonstrated the best concentration and the only useable quality of small RNAs.

#### 6.4.1.1 Asessment of RNA quantity and purity

Quantity and purity of total RNA isolated and purified from both cell lines in the presence and absence of RA was examined prior to quantative TaqMan® PCR analysis. In all cases, total RNA was determined by micro-volume quantitation using a Nanodrop 1000 spectrophometry. As recommended by manufactures instructions (Qiagen, Fleming Way, Crawley, West Sussex, UK) only highly pure RNA with ration 1.8 to 2.1 measured at the absorbance 260nm to 280nm was included in the analysis, see Table 6.1.

**Table 6.1: Total RNA quantity and purity of PWR-1E, PC-3, LNCaP and derived Holoclones.**

| Cell lines & Holoclones | Quantity (ng/µl) | 260-280nm Ratio |
|---|---|---|
| PWR-1E Rep1 | 406.4 | 2.0 |
| PWR-1E Rep2 | 486.8 | 2.0 |
| PWR-1E Rep3 | 439.2 | 2.0 |
| PC-3 Rep1 | 744.0 | 2.1 |
| PC-3 Rep2 | 913.9 | 2.1 |
| PC-3 Rep3 | 781.9 | 2.1 |
| LNCaP Rep1 | 1791.1 | 2.0 |
| LNCaP Rep2 | 1692.1 | 2.0 |
| LNCaP Rep3 | 2175.7 | 2.0 |
| PC-3 Holoclone Rep1 | 28.8 | 1.9 |
| PC-3 Holoclone Rep2 | 29.3 | 1.8 |
| PC-3 Holoclone Rep3* | 278.4 | 1.9 |
| LNCaP Holoclone Rep1 | 21.6 | 1.8 |
| LNCaP Holoclone Rep2 | 24.8 | 1.8 |
| LNCaP Holoclone Rep3 | 33.6 | 1.8 |

*PC-3 Holoclone Rep3 generated higher yields due to improved isolation and washing leading to better removal of agarose to yielgs 278.4 ng/µl.

### 6.4.2 Evaluation of MammU6 gene as an endogenous control

MammU6 was set as the endogenous control (pre-designed TLDAs by Applied Biosystems, Foster City, CA) as it was the most consistent control and was therefore used to normalise TaqMan® Low Density Array data, see Figure 6.1. MammU6 was also recommended by Applied Biosystems as the standard endogenous control and ath-miR159a as the negative control (GAPDH was not contained within the pre-designed arrays).

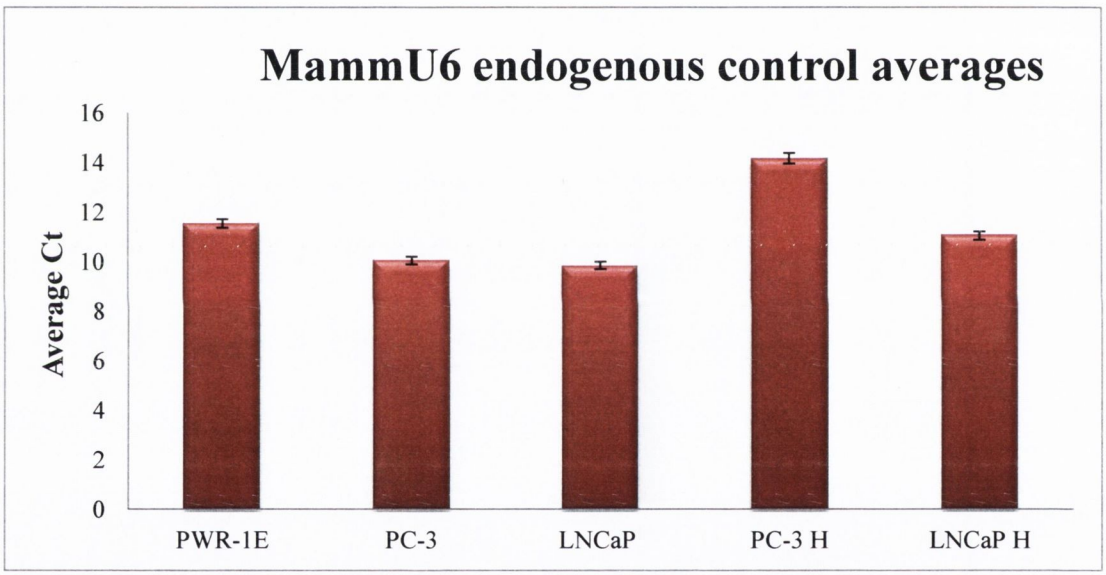



**Figure 6.1: Engogenous control averages.** The Ct average values of each of the 5 prostate cancer cell lines miRNA profiled, PC-3, LNCaP and their derived holoclones and normal prostate cell line PWR-1E, for the endogenous control MammU6 generated by the TaqMan® Low Density miRNA Arrays, displaying the standard deviation between all biological replicates, n=3.

### 6.4.3 TaqMan® Low Density Array quality control

Three biological replicates of each of the cell lines and the holoclones were performed and the biological replicates showed good concordance. Figure 6.2 provides a visual display of a scatter plot demonstrating the distribution of the raw Ct values generated across two biological replicates of the LNCaP holoclones generated from two different LNCaP cell line passage numbers.

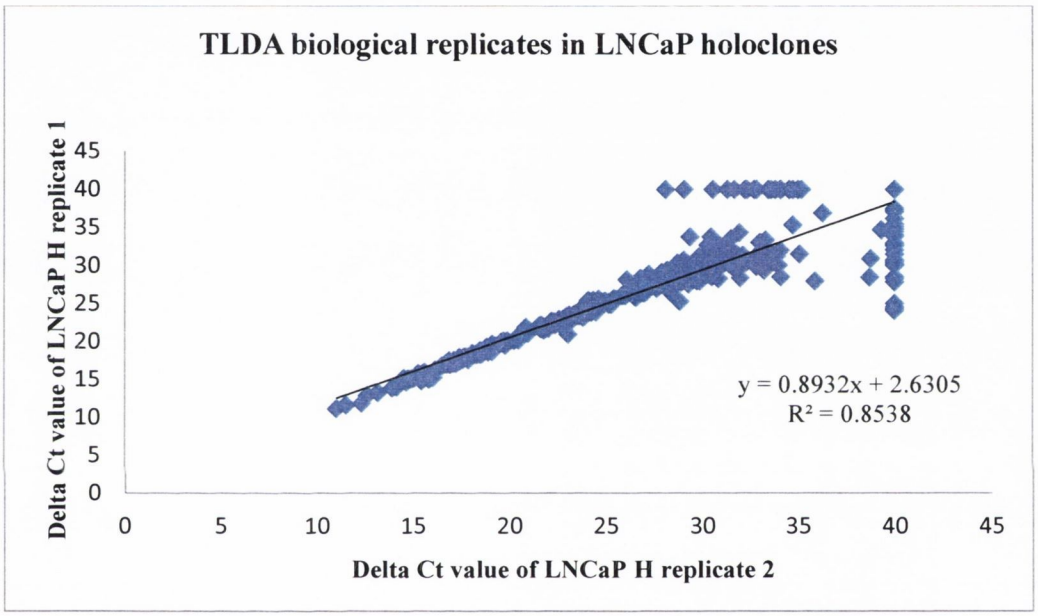



**Figure 6.2: LNCaP holoclone scatter plot representing a biological linear relationship.** Scatter plot comparing two biological replicates of the LNCaP holoclones of the initial raw Ct values generated by the TaqMan® Low Density miRNA Arrays demonstrating a linear relationship. Ct values not contained within the linear relationship that have a Ct value of 40 are undetected miRNAs.

### 6.4.4 TaqMan® Low Density Array-Megaplex Pools for miRNA expression

The expression of 380 miRNAs and controls were profiled using TLDAs in the cell lines PC-3 which represents a grade 4 adenocarcinoma and LNCaP which represents a metastatic carcinoma and the normal cell line PWR-1E. Both PC-3 and LNCaP top 10 up and down regulated miRNAs are graphically displayed in comparsion to the normal cell line, see Figure 6.3A&B. P-values were calculated using a t-test and significant miRNAs had a P value of $P \leq 0.05$.

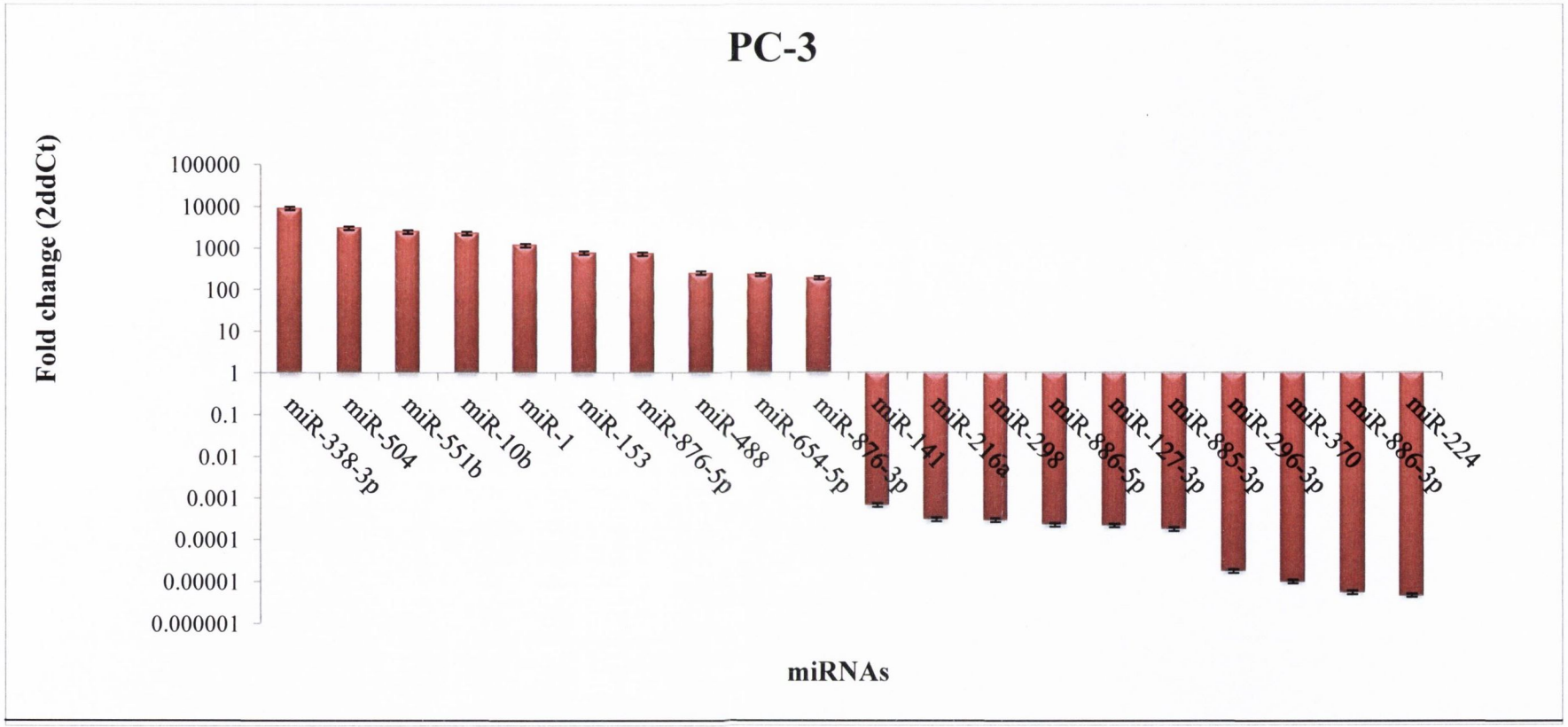



**Figure 6.3A: MicroRNA gene expression profiling of cell lines PC-3 compared to PWR-1E.** Top 10 upregulated and downregulated miRNAs for PC-3 versus PWR-1E.

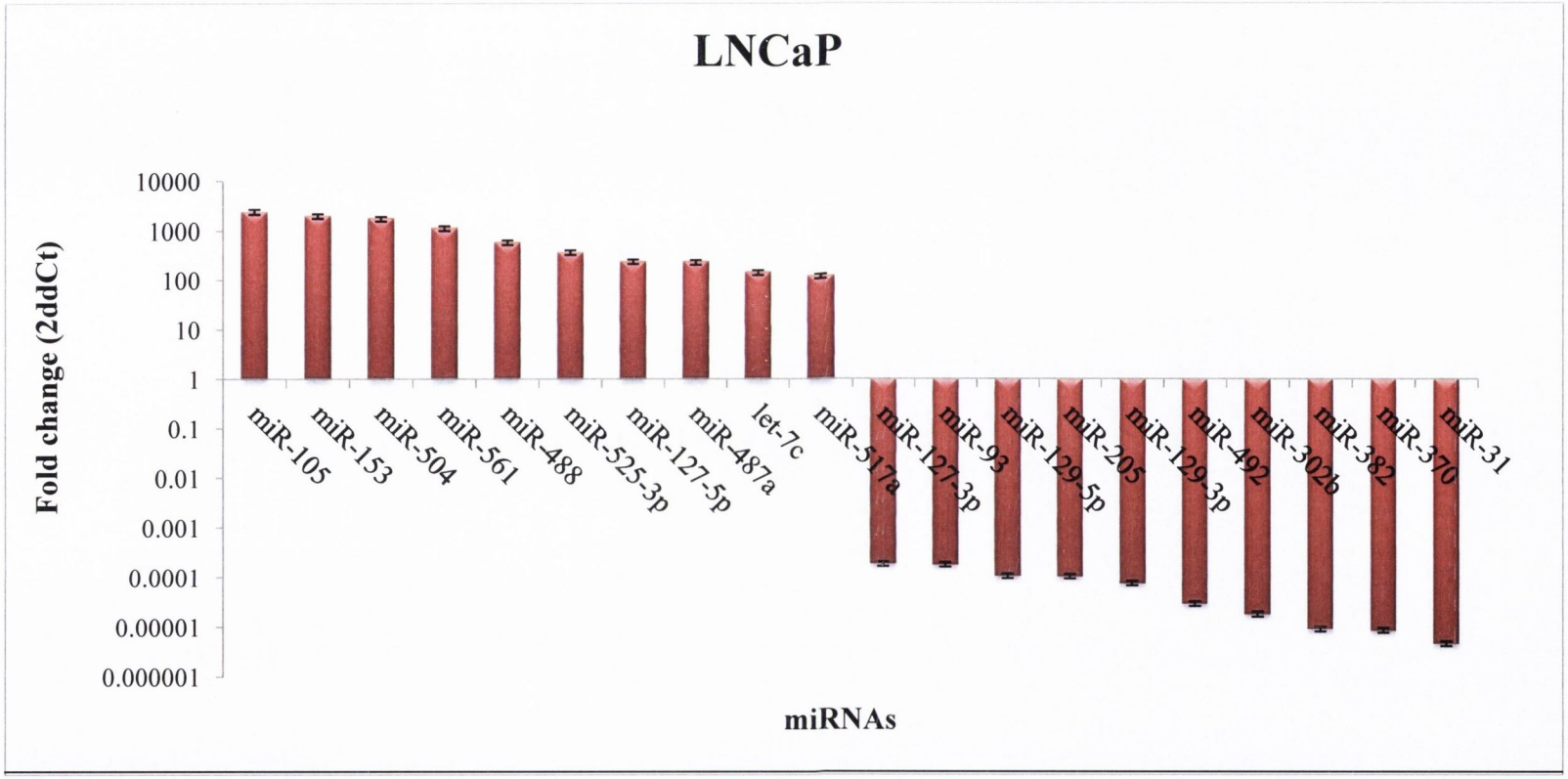



**Figure 6.3B: MicroRNA gene expression profiling of cell lines LNCaP compared to PWR-1E.** Top 10 upregulated and downregulated miRNAs for LNCaP.versus PWR-1E.

**Table 6.2:** Summary of miRNA expression detected in PC-3, LNCaP in comparsion to the normal cell line PWR-1E.

| Description of 380 miRNAs profiled | Number of miRNAs |
|---|---|
| miRNAs detected in PC-3 but not LNCaP | 30 |
| miRNAs detected in LNCaP but not PC-3 | 16 |
| miRNAs undetected in normal | 88 |
| miRNAs undetected in PC-3 versus normal | 84 |
| miRNAs undetected in LNCaP versus normal | 98 |

**Table 6.3:** Summary of miRNA expression detected in PC-3 and LNCaP Holoclones compared to the normal cell line PWR-1E.

| miRNA status | PC-3 H | LNCaP H |
|---|---|---|
| Upregulated | 81 | 71 |
| Downregulated | 147 | 132 |
| No change | 99 | 87 |
| Undetected | 54 | 88 |

### 6.4.5 MicroRNA expression profiling of PC-3 and LNCaP holoclones

The expression of 380 miRNAs and controls were profiled using TLDAs in the PC-3 and LNCaP holoclones in comparsion to the normal cell line PWR-1E. Both PC-3 and LNCaP holoclones top 10 up and down regulated miRNAs are graphically displayed in comparsion to the normal cell line, see Figure 6.4A&B. P-values were calculated using a t-test and significant miRNAs had a P value of $P \leq 0.05$.

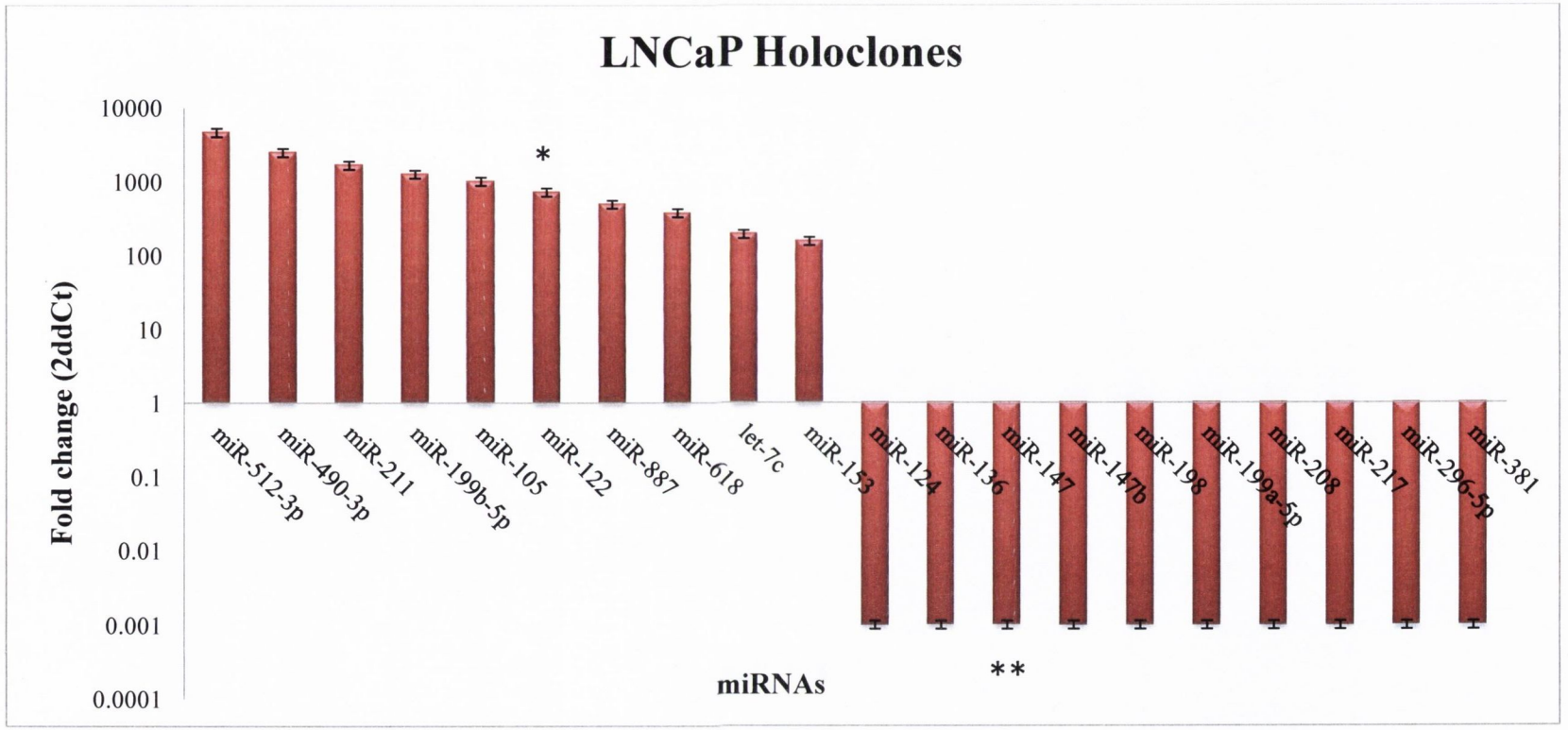



**Figure 6.4A: MicroRNA gene expression profiling of LNCaP holoclones compared to the normal cell line PWR-1E.** Top 10 upregulated and downregulated miRNAs LNCaP holoclone. n=3, * $P < 0.0021$, ** $P < 0.00057$.

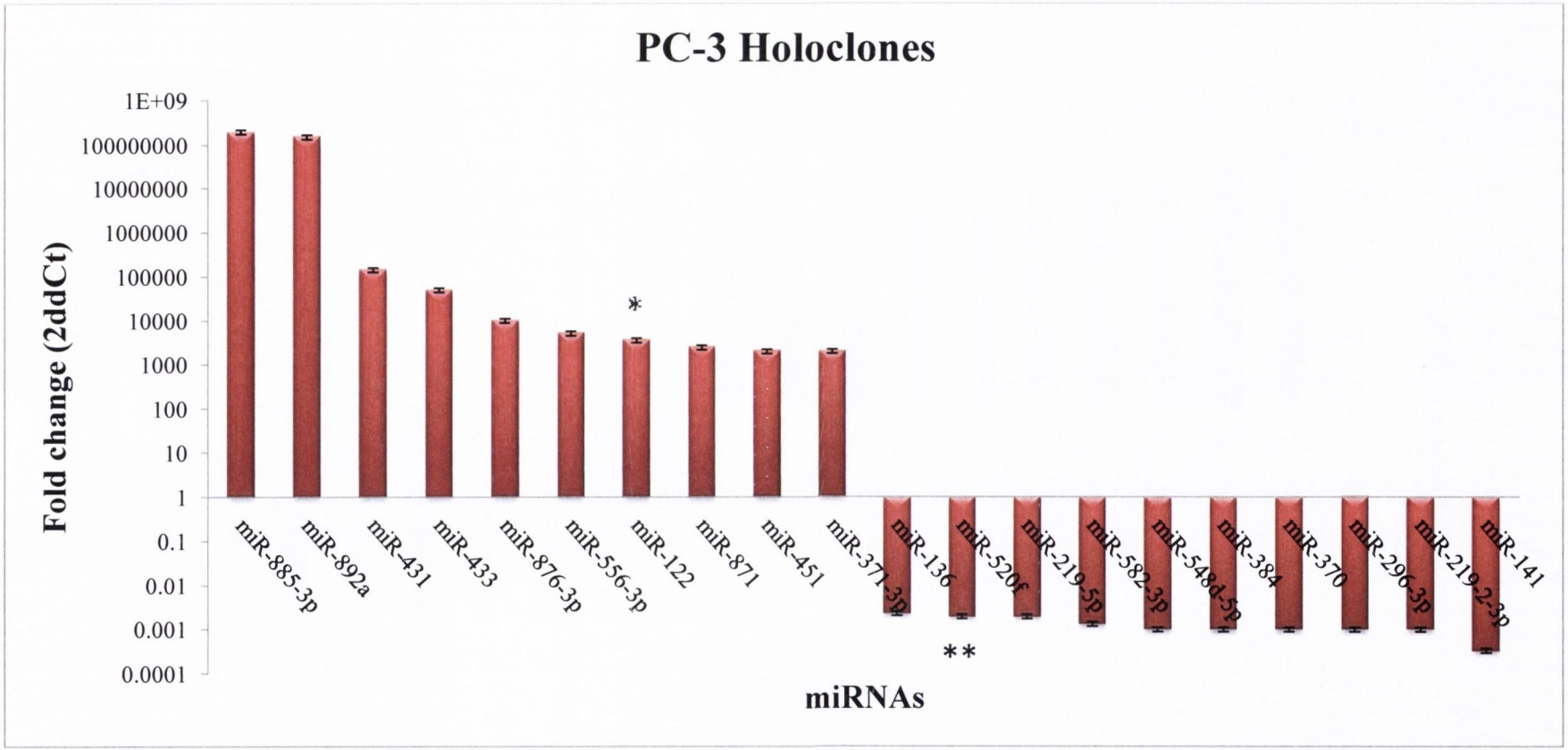



**Figure 6.4B: MicroRNA gene expression profiling of PC-3 holoclones compared to the normal cell line PWR-1E.** Top 10 upregulated and downregulated miRNAs for PC-3 holoclone. n=3, * $P < 0.0043$, ** $P < 0.0061$.

### 6.4.6 Identification of PC-3 and LNCaP holoclone specific miRNA populations

The top 10 most significantly upregulated and downregulated miRNAs were identified in PC-3 and LNCaP holoclones in comparsion to the relevant cell from which they were derived from, see Table 6.4 and 6.5.

**Table 6.4: Top 10 upregulated and downregulated prostate cancer stem cell specific miRNAs identified in PC-3 holoclones compared to PC-3 cell lines.**

| miRNA | T-test P-value | PC-3 (FC) | PC-3 H |
|---|---|---|---|
| **1.) miR-892a** | (P<0.0042) | undetected | upregulated |
| **2.) miR-885-3p** | (P<0.0001) | undetected | upregulated |
| **3.) miR-450b-3p** | (P<0.005) | undetected | upregulated |
| **4.) miR-548-3p** | (P<0.00021) | undetected | upregulated |
| **5.) miR-431** | (P<0.007) | no change (0.7069) | upregulated |
| **6.) miR-1** | (P<0.00035) | up (399.301) | upregulated |
| **7.) miR-433** | (P<0.004) | up (2291.907) | upregulated |
| **8.) miR-891b** | (P<0.00016) | undetected | upregulated |
| **9.) miR-871** | (P<0.0003) | undetected | upregulated |
| **10.) miR-490-3p** | (P<0.001) | undetected | upregulated |
| **1.) miR-141** | (P<0.0004) | undetected | downregulated |
| **2.) miR-219-2-3p** | (P<0.0005) | undetected | downregulated |
| **3.) miR-296-3p** | (P<0.0009) | undetected | downregulated |
| **4.) miR-370** | (P<0.00062) | undetected | downregulated |
| **5.) miR-384** | (P<0.0008) | undetected | downregulated |
| **6.) miR-216a** | (P<0.00031) | undetected | downregulated |
| **7.) miR-200c** | (P<0.001) | undetected | downregulated |
| **8.) miR-199a-5p** | (P<0.005) | undetected | downregulated |
| **9.) miR-412** | (P<0.0002) | up (636.125) | downregulated |
| **10.) miR-672** | (P<0.0005) | no change (1.020069) | downregulated |

**Table 6.5: Top 10 upregulated and downregulated prostate cancer stem cell specific miRNAs identified in LNCaP holoclones compared to LNCaP cell lines.**

| miRNA | T-test P-value | LNCaP (FC) | LNCaP H (FC) |
|---|---|---|---|
| **1.) miR-512-3p** | (P<0.001) | up (259.36) | upregulated |
| **2.) miR-490-3p** | (P<0.0035) | undetected | upregulated |
| **3.) miR-211** | (P<0.0005) | (1.247) | upregulated |
| **4.) miR-887** | (P<0.0025) | undetected | upregulated |
| **5.) miR-511** | (P<0.00036) | undetected | upregulated |
| **6.) miR-433** | (P<0.001) | undetected | upregulated |
| **7.) miR-556-5p** | (P<0.003) | undetected | upregulated |
| **8.) miR-499-5p** | (P<0.0015) | undetected | upregulated |
| **9.) miR-448** | (P<0.0034) | undetected | upregulated |
| **10.) miR-142-5p** | (P<0.00045) | (0.718138) | upregulated |
| **1.) miR-124** | (P<0.0003) | (0.72951) | downregulated |
| **2.) miR-147** | (P<0.00024) | undetected | downregulated |
| **3.) miR-147b** | (P<0.0008) | undetected | downregulated |
| **4.) miR-198** | (P<0.00014) | undetected | downregulated |
| **5.) miR-199a-5p** | (P<0.008) | undetected | downregulated |
| **6.) miR-208** | (P<0.0006) | (0.76172) | downregulated |
| **7.) miR-217** | (P<0.0003) | undetected | downregulated |
| **8.) miR-381** | (P<0.002) | undetected | downregulated |
| **9.) miR-526b** | (P<0.007) | undetected | downregulated |
| **10.) miR-655** | (P<0.00049) | undetected | downregulated |

### 6.4.6.1 All differentially expressed miRNAs

A heatmap with Hierarchical Clustering was generated using Spotfire software for all differentially expressed miRNAs for the cell lines PC-3 and LNCaP and their derived holoclones, see Figure 6.5.

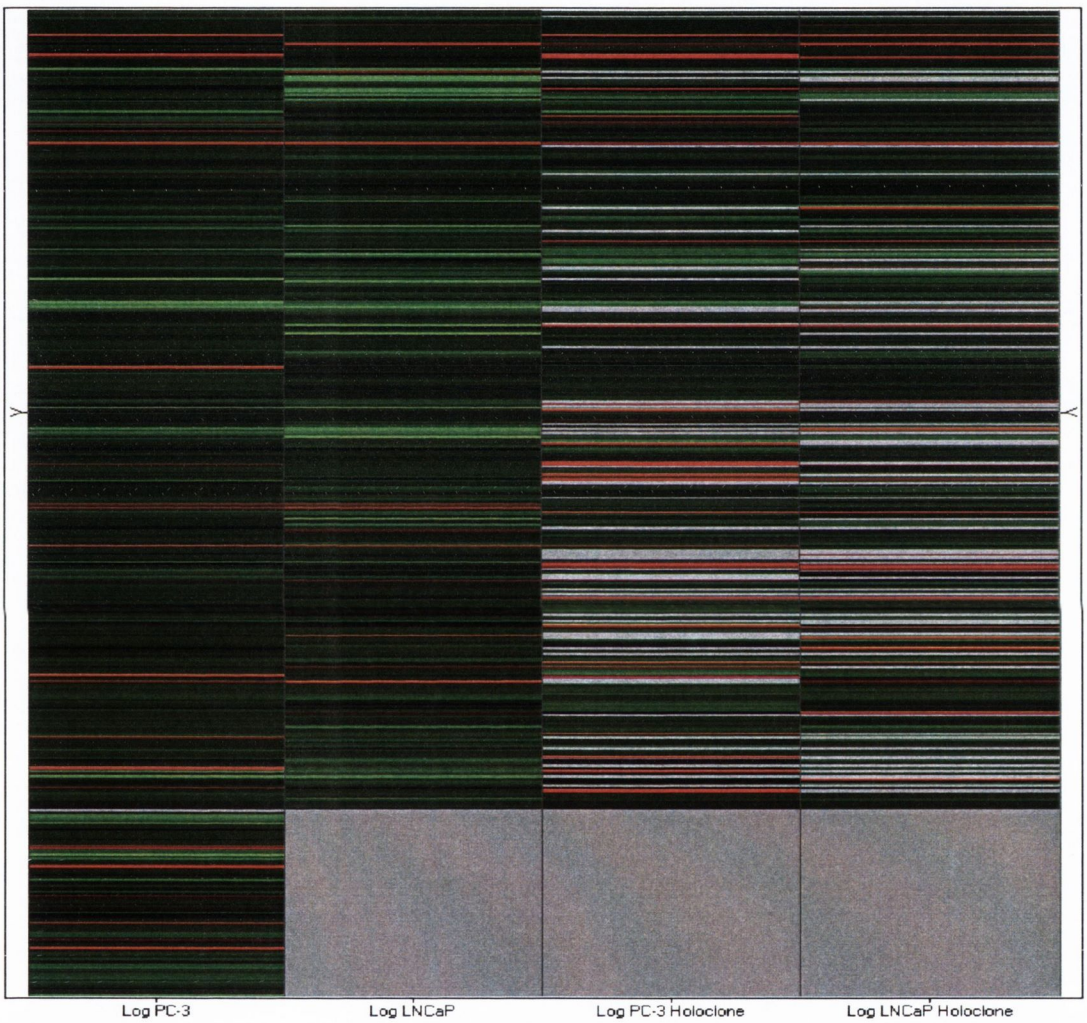



**Figure 6.5: Heatmap of differentially expressed miRNAs.** MiRNAs differentially expressed in represented in log of RQ for the following cell lines and holoclones; PC-3, LNCaP, PC-3 holclones and LNCaP holoclone. Green; downregulated, Red; upregulated, Grey; undetected.

### 6.4.7 PC-3 and LNCaP holoclones predicted gene targets

Gene targets were predicted for the top 10 upregulated and downregulated miRNAs in the PC-3 and LNCaP holoclones cell lines using the databases; miRGen version 2007(v3) and microrna.org (version September 2008). Mirgen is an intergrated database, which the target interface allows access to union and intersections of 4 widely used target prediction programmes and experimentally supports targets from Tarbase. The top 10 upregulated and downregulated miRNAs and the top 10 predicted gene targets were predicted using the databases miRGen and microrna.org, see Table 6.6 and 6.7.

**Table 6.6: Top 10 predicted gene targets in PC-3 holoclones**

| miRNA | Predicted Targets miRGen & miRNA.org |
|---|---|
| miR-892a | LOC642236, NAA16, HECTD2, XPO1, NPTX1, C11orf1, PRKAR2B, PPM1D, SRGN, KANK1 |
| miR-885-3p | PRPF3, ARHGAP18, ARMCX3, NRG1, C10orf26, PON1, CAP2, NAA11, TUBA4B, CLIP4 |
| miR-450b-3p | GLS, HMCN1, HEY2, ZNF169, TMEM139, KCNMA1, GPC4, KCNA5, LMBR1L, ACP1 |
| miR-548-3p | unavailable |
| miR-431 | LHX8, SSX4B, SSX4, CST7, CHST2, C20orf103, LAMC2, MAGED4, SPATA8, MAGED4 |
| miR-1 | E2F5, DDX5, DHX15, EIF4E, CLTC, FNDC3A, HOXB4, GJA1, TIMP3, NOTCH3 |
| miR-433 | PCCB, HIVEP1, RGS2, SFRS3, SRP54, SSR2, NIPSNAP1, B4GALT3, TRIP12, SLC17A5 |
| miR-891b | DAZAP2, MTF2, DKK3, TRAM1, MAST4, LDB2, FAM76B, C8orf34, SNAP23, UBE2D3 |
| miR-871 | unavailable |
| miR-490-3p | TNKS2, COMMD10, TMTC3, CLCC1, RBPJ, VDAC1, LOC646096, PPME1, SUV420H1, PPP6C |
| miR-141 | ATXN1, CTNND2, OAZ2, YY1, DDX5, ELAVL2, ZFHX1B, CHD9, JAG1, TTR |
| miR-219-2-3p | GABRP, DSCAM, MTHFD2L, YY1, FUBP3, SLC30A5, ZNF706, HNRNPK, PGM3, ANKRD49 |
| miR-296-3p | ZNF732, LOC643955, PFN2, ZNF138, CD200R1, RPL15, ZNF117, ZNF253, C10orf81, NLK |
| miR-370 | DMRTB1, C2, CACNG1, KCNK1, LRPAP1, CCL21, SFTPA1B, SFTPA1, SERPINA4, CIT |
| miR-384 | BTK, GPR143, LTA, PTGER2, ACVR1, BMP2, GTF2B, HLA-DOB, LAMC1, PTN |
| miR-216a | NPIPL3, DMXL2, LOC100132247, C9orf93, TMEM161B, HNRPLL, CMPK1, RANBP17, LEF1, NGFRAP1 |
| miR-200c | RHOA, PTPRZ2, PTPRZ1, FOXG1C, FOXG1B, NOG, ZFPM2, ZFHX1B, ANLN, NPC1 |
| miR-199a-5p | ZNF763, LOC643955, ZNF844, ZNF544, ZNF440, EXOSC9, ZNF439, ZNF419, NTNG1, FAM108C1 |
| miR-412 | XAGE1, GUCY2D, HLA-DOB, SFTPD, CST7, EFNB1, ROR2, FAM101A, COMP, HADHA |
| miR-672 | unavailable |

**Table 6.7: Top 10 predicted gene targets in LNCaP holoclones**

| miRNA | Predicted Targets miRGen & miRNA.org |
|---|---|
| miR-512-3p | HSD17B3, CHRNB1, SERPINF2, LRPAP1, PPAPDC3, CTGLF1, CREBL1, TNXB, ACADVL, ADSL |
| miR-490-3p | TNKS2, COMMD10, TMTC3, CLCC1, RBPJ, VDAC1, LOC646096, PPME1, SUV420H1, PPP6C |
| miR-211 | ATF2, AKAP1, CDH2, FBN2, VIL2, COX5A, EEF1E1, KHDRBS3, YWHAG, PPARGC1A |
| miR-887 | P2RY12, PLD2, NPAT, GSK3A, TMEM38B, DUS4L, CNPY2, STAG1, HEATR6, PDK1 |
| miR-511 | F8A1, F8A3, F8A2, HLA-DOB, SFTPA1B, SFTPA1, PHTF1, RAB32, SFTPA2B, SFTPA2 |
| miR-433 | PCCB, HIVEP1, RGS2, SFRS3, SRP54, SSR2, NIPSNAP1, B4GALT3, TRIP12, SLC17A5 |
| miR-556-5p | NR3C2, TAF9B, PTPRM, LSM5, CASC2, YBX1, AP4S1, MEIS2, PI4K2B, CRYZ |
| miR-499-5p | DNAJC25, ANGPT1, HNRNPC, MAMDC2, DDX1, MYLK, SULF1, C12orf35, RAP2C, ZNF85 |
| miR-448 | PCDHGA12, PCDHGA3, PCDHGC3, PCDHGC4, PCDHGC5, RAB7, PCDHA13, PCDHAC1 |
| miR-142-5p | TSC22D2, HDAC2, BAI3, DNAJC7, LRP2, JARID2, ZFPM2, APRIN, LRP1B, OSR1 |
| miR-124 | SNAI2, NID1, SIX4, TMC7, VAMP3, B4GALT1, CD164, ITGB1, PTBP2, TNFSF11 |
| miR-147 | LTA, CCK C1GALT1C1 C20orf134 PAIP2 EDN1 SSR1 AXIN2 RAB33A GPA33 |
| miR-147b | NDUFA4 TINAG RGS7 NDRG4 RORB CLSTN1 REG1A ZNF503 KCNA3 WHSC1L1 |
| miR-198 | ALPL TSHZ1 TCF7L1 LMNA GUCY2D IL10 ACHE FBXL10 ADAM2 GFRA2 |
| miR-199a-5p | ZNF763, LOC643955, ZNF844, ZNF544, ZNF440, EXOSC9, ZNF439, ZNF419, NTNG1, FAM108C1 |
| miR-208 | THRAP1, CSNK2A2, OMG, SP3, ETS1, IL1RAPL1, NLK, CXXC5, BHLHB3, AFTPH |
| miR-217 | CHN2, ANLN, BAI3, EIF4A2, NEUROD1, PPM1D, STX1A, FOXG1C, FOXG1B, UBL3 |
| miR-381 | LRRC4, RPL35A, SIAH1, IGFBP3, NDEL1, ARNTL, SEC14L1, CSTF3, ELAVL1, ACTG1 |
| miR-526b | ALPL, PSMD13, ECHS1, BMP1, ACADVL, EPOR, GCDH, GCK, SERPIND1, ICAM1 |
| miR-655 | NIPBL, LILRP2, BNIP2, PHF20L1, ZEB1, IL7, BAZ2B, AK7, SYT4, ATAD2 |

### 6.4.8 PC-3 and LNCaP holoclone- metabolic pathway predictions

Metabolic pathways were predicted for specific upregulated and downregulated miRNAs in the PC-3 and LNCaP holoclones using the database miRNApath, see table 6.8. MiRNApath offers a relationship between miRNAs, target genes and metabolic pathways. This database allows for the investigation of miRNAs and shows the pathways affected by these miRNAs. It also allows for the possible determination of the disease caused by them.

**Table 6.8: Top 5 predicted metabolic pathways for specific miRNAs up and downregulated in PC-3 and LNCaP holoclones.**

| miRNA | Predicted metabolic pathways (miRNApath) |
|---|---|
| miR-892a | unavailable |
| miR-141 | Cell cycle, Fatty acid elongation in mitochondria, Fatty acid metabolism, Valine leucine and isoleucine degradation, Lysine degradation |
| miR-220c | Cell cycle, Purine metabolism, Pyrimidine metabolism, RNA polymerase, Glycolysis/ Gluconeogenesis |
| miR-512-3p | Glycosylphosphatidylinositol(GPI)-anchor biosynthesis, Glycan structures - biosynthesis 2, Glycolysis / Gluconeogenesis, Pentose phosphate pathway, Fructose and mannose metabolism |
| miR-124 | unavailable |

## 6.5 Discussion

Recently, high-throughput studies carried out by various groups have displayed deregulation of miRNAs in most types of cancer (Volinia *et al.*, 2006). Many of these groups have shown a mixed tumour specific pattern of downregulated miRNA gene expression also with the upregulation of some miRNA genes. More importantly, specific miRNA gene expression patterns in numerous cancers can be associated with diagnosis, prognosis and therapeutics (Calin and Croce., 2006) Moreover, it has also been suggested that specific miRNAs could potentially play crucial roles in both the initation and progression of cancers through their effects on the numerous molecular pathways involved. Therefore an inproved understanding of miRNA gene expression patterns in prostate cancer and specifically prostate cancer stem cells is necessary as miRNAs patters have not been previously described in the literature for prostate cancer stemness.

The identification of specific miRNAs populations of the PC-3 and LNCaP holoclones are of great importance as this has not been previously identified in the literature available to date. This analysis allows for the identification of the actual holoclone population through miRNA profiling in order to potentially find putative biomarkers and also for future developments of therapies and treatments. The analysis also allows for further characterisation of the holoclone populations as many significant miRNAs identified in this chapter have been shown in the literature to have strong associations with stemness, differentiation and carcinogenesis.

In the PC-3 holoclones miR-892a is the most significantly upregulated miRNA in comparsion to the PC-3 cell line. It is thought that several microRNAs are associated with the normal function in regulating neural stem cell self-renewal and differentiation that occurs during development. However, dysfunction of these miRNAs in many carcinomas could possibly contribute to the maintenance of an undifferentiated proliferative phenotype by preventing the expression of differentiation targets, in turn allowing for the expression of targets that promote renewal of the stem cell (Tysnes, 2010). Abnormal function of the regulation of stem cells could potentially, contribute to malignant transformation and the formation of a cancer stem cell–like phenotype such as the holoclone population of cells. MiR-892a is an example of this, and is also described in the literature; miR-892a is a predicted miRNA that targets Oct4/Poufl, a master regulator of pluripotency and differentiation, predicted by TargetScan 5.1 (Tysnes, 2010). This suggests that MiR-892a is an important miRNA associate with the cancer stem cell phenotype however there is no published literature currently available in relation to this miRNA gene in the PC-3 cancer stem cell phenotype.

MiRNA-1, miR-133a and miR-133b are significantly upregulated in the PC-3 holoclones when compared to the founder cell lines. MiR-1 and miR-133 are clustered on the same chromosomal loci and are transcribed together in a tissue specific manner during development. Both miRNAs have been shown to play a distinct role in the modulation of skeletal muscle proliferation and differentiation in myoblasts (embryonic progenitor cell that gives rise to myocytes) *in vitro* (Chen *et al.*, 2005). More recent studies have displayed findings that both miR-1 and miR-133 are also involved in apoptosis leading to the progression of the tumour and metastasis (White *et al.*, 2011).

MiR-1 predicted gene targets shows that both NOTCH3 and TIMP3 are significantly over represented in this miRNA. The NOTCH signalling pathway has been shown to play a crucial role in proliferation in many cancers such as; breast and ovarian. In particular, NOTCH3 mediated signalling plays a significant role in proliferation of ErbB2-negative breast tumour cells (Yamaguchi *et al.,* 2008). The gene TIMP3 is also an over represented gene target of miR-1, tissue inhibitor metalloproteinases 3 is known to inhibit the effects of metalloprotease enzymes in tissue extracellular matrix. In prostate cancer and benign prostatic hyperplasia (BPH) the balance of tissue glycosaminoglycans is disturbed. Specifically, versican (a chondroitin sulfate proteoglycan) is shown to be highly expressed in BPH and prostate carcinoma where it is also associated with poor clinical outcomes (Cross *et al.,* 2005).

In the PC-3 holoclones miR-141 is the most significantly downregulated miRNA in comparsion to the PC-3 cell line as it is undetected. MiR-200c is the seventh most significantly downregulated miRNA in the PC-3 holoclone in comparison to the PC-3 dell line which was also undetected. Predicated metabolic pathways included the cell cycle pathway which is a pathway involved in the progression of cancer.

Recently there is evidence that suggests miRNAs can contribute to the generation of the EMT phenotype, this contributing to the aggressiveness of cancer cells (White *et al.,* 2011). The miR-200 family which consists of miR-200, miR200b, miR-200c, miR-141and miR-429 are known to be involved in EMT (Burk *et al.*, 2008, Gregory *et al.*, 2008, Korpal & Kang, 2008, Park et al., 2008, Paterson et al., 2008). EMT is a fundamental process in which an epithelial cell loses epithelial characteristics and

acquires mesenchymal and stemness related features. The miR-200 family have emerged as major players in the control of EMT (White *et al.*, 2011).

The miR-141/200c cluster in particular, has been shown to be involved in the epithelial to mesenchymal transition (EMT) process. The expression of these two miRNAs is inversely related with both tumorigenicity and invasiveness in many cancers. These miRNAs play a role in cancer progression largely due to the ability of their capacity to target the transcription factors ZEB1 and ZEB2 which are EMT activators that can repress expression of E-cadherin. Studies have demonstrated that the expression of the miR-141/200c cluster is regulated by DNA methylation, indicating epigenetic regulation of this miRNA locus in both aggressive breast cancer cell lines and in untransformed mammary epithelial cells (Neves *et al.*, 2010). EMT is known to be an essential early step in tumour metastasis formation and is achieved by controlling the detachment of invasive cancer cells from the actual primary tumour itself. These two miRNAs are significantly downregulated in the PC-3 holoclones and specific to the holoclone population and could possibly support the cancer stem cell/ holoclone theory as previously described in chapter 3. According to a study carried out by Kong *et al.*, 2010 MiR-200b and miR-200c expression has been shown to be linked to cancer stem cell signatures with the EMT phenotype and the group also found that the down-regulation of Notch1 by miR-200 was partially responsible for the inhibition of colonogenic and prostasphere-forming ability of the generated PC-3 PDGF-D cells. This study further supports and correlates with the data generated in regard to the miR-200 family in PC-3 holoclones.

MiR-141 has also been described in the literature as a stable blood based circulating miRNA detected in patients with advanced prostate cancer (Mitchell *et al.,* 2008). Currently there is very little known about the regulation of the mir141/200c cluster, although their targeting has been proposed as new biomarker and possible therapy for the many aggressive tumours. This analysis has identified these miRNAs as specifically associated with the PC-3 holoclone population.

In the LNCaP holoclones miR-512-3p is the most significantly upregulated miRNA in comparsion to the LNCaP cell line. Human embryonic stem cells have the ability to self-renew and differentiate into various different types of cells and the behaviors of miR-512-3p has been identified during differentiation of hESCs into a specialized cell type has been described by Kim et al. This group have identified that miR-512-3p is a significantly upregulated miRNA in human embryonic stem cells during hepatocyte differentiation (Kim *et al.,* 2011). The expression profile of miR-512-3p has been found to be of significant importance during differentiation in human embryonic stem cells. The findings of this group are of great importance as this miRNA could potentially be involved in differentiation of the holoclone stem cell/ stem-like population.

In the LNCaP holoclones miR-124 is the most significantly downregulated miRNA in comparsion to the LNCaP cell line. MiR-124 has been described in the literature as a novel tumor-suppressive miRNA for hepatocellular carcinoma (Furuta *et al.,* 2009). MiR-124 is also a miRNA that is associated with transcription factors that regulate pluripotence and self-renewal, miR-124 promoter regions bind to Oct4/Sox2?NANOG and TCF3 in both human and mouse embryonic stem cells (Marson *et al.,* 2008).

Bioinformatic analysis predicted gene targets displayed genes involved in cancer and stemness such as Snail2. Snail2 was previously described in chapter 3. The Snail gene super-family have been previously described to be involved in development of vertebrate and invertebrate embryos and also in the progression of tumours and metastasis (Sefton *et al.,* 1998). Snail is highly expressed in patients with breast carcinoma and can result in poor outcome and is also associated with process of breast cancer recurrence (Moody *et al.,* 2005). Snail has been found to cause resistance to cell death which allows embryonic cells to migrate and colonise and also allows for malignant cells to separate from the primary tumour and invade, therefore leading to metastasis (Vega *et al.*, 2004). Epithelial cells that display Snail expression ectopically adapt a fibroblastoid phenotype and develop tumorigenic and invasive properties. This analysis indicates that miR-124 is a very significant miRNA in prostate cancer LNCaP holoclones as a stem cell specific miRNA as the predicted gene target displays genes that are associated with cancer stemness.

In the LNCaP holoclones miRNA-21 is significantly downregulated in comparison to the LNCaP founder cell lines. Recently miR-21 has emerged as a key oncomir in tumorigenesis (White *et al.,* 2011). MiRNA-21 is currently defined as a metastamir in the literature. A metastamir is a miRNA that is associated with metastasis either as having prometastatic or antimetastatic effects or as previously mentioned the LNCaP cell line represents a metastatic carcinoma.

Recently, miR-21 has emerged as a key oncomir in tumorigenesis and many functional studies have been carries out into the effect of miR-21 on the progression of tumours and metastasis (White *et al.,* 2011). Thus, numerous miR-21 targets have been identified. MiR-21 has been shown to target both PTEN in human hepatocellular cancer (Meng *et al.,* 2007). PTEN is a known tumour suppressor gene which negatively regulates cell proliferation and survival (White *et al.,* 2011). MiR-21 in human hepatocellular cancer cells has been shown to increase PTEN expression and decrease cell proliferation, migration and invasion (Meng *et al.,* 2007). MiR-21 also targets PDCD4 and TMP1, as PDCD4 is a tumour suppressor gene which inhibits transformation, tumour promotion and progression and is also significantly downregulated in numerous carcinomas (Meng *et al.,* 2007).

MiR-21 is also involved in various different cellular processes and pathways which include upstream effectors and downstream targets as represented in Figure 6.9. MiR-21 has been shown to increase cell invasion through many pathways, such as; targeting inhibitors of matrix metalloproteinases (MMPs), reversion inducing cysteine rich protein with Kazal motifs (RECK) and tissue inhibitor of metalloproteinase 3 (TIMP3). Many studies have shown that miR-21 enhance tumourigenesis and has a key involvement in metastasis and the results found in this study are in keeping with published literature.

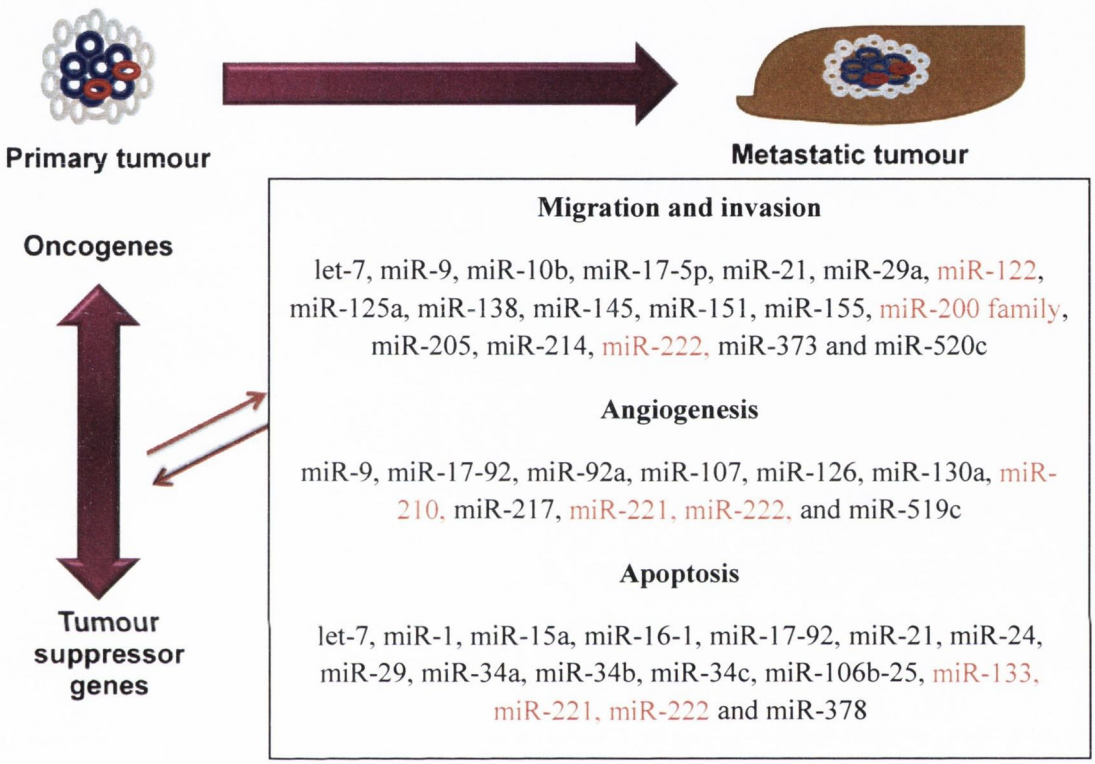



**Figure 6.6: Effects of miRNAs on metastasis.** miRNAs indicated in red are miRNAs generated from the profiled data in this chapter that correlate with previously published literature that are associated with migration and invasion, angiogenesis and apoptosis (Adapted from Winter *et al.*, 2011).

In summary, potential miRNA populations have been identified and bioinformatically analysed in both PC-3 (Grade 4 adenocarcinoma) and LNCaP (metastatic carcinoma) holoclones. These miRNA populations have not been identified or characterised previously in a holoclone population and are now available for future analysis. The results in this chapter indicate that there are different miRNAs have various different functions in the holoclone population in comparison to the actual original cell line. Many of the most significantly regulated miRNAs mentioned have been shown in the literature to be involved in processes such as; stemness, differentiation, EMT and cancer. This further indicates that the holoclone population of cells could potentially play a major role in driving tumourgenesis and recurrence and this chapter has identified popentially important cancer stem cell or stem-like, holoclone specific miRNA populations.

This analysis clearly demonstrates that the regulation of miRNAs is a key component of both holoclone cell types to their differentiation and stemness characteristics. This chapter has now characterised and identified the involvement of specific miRNAs in the PC-3 and LNCaP cells resting state and in the generated holoclone cancer stem cell phenotype. These newly identified miRNAs are now available for future functional analysis.

# General discussion

Chapter 7

## 7.1 General discussion

Prostate cancer is one of the five most prevalent malignancies in all male age groups worldwide. Prostate cancer is a heterogeneous disease and is the second most common cause of cancer resulting in male deaths caused by cancer and is the most commonly diagnosed cancer in Irish males. There are over 1500 new cases diagnosed every year in Ireland, the second leading cause of fatal cancer in Irish men (National Cancer Registry Ireland, 2010). Currently there is no curative treatment for locally advanced or metastatic disease and treatments for localised disease causes many complications such as, impotence and incontinence. Prostate cancer can be divided into two forms: sporadic and hereditary however they cannot be identified at the molecular level. (Hughes *et al.*, 2007). There are many unanswered questions regarding the molecular pathology of prostate cancer, recurrence and prostate cancer stem cells.

The overall aims of this project were to generate a holoclone model representing cancer stemness and characterise the specific genes, pathways and miRNAs involved. The holoclone model was derived using a high salt soft-agar assay in order to select for a pure population of holoclones. Microarray data was generated and numerous key stemness genes and pathways were identified and subsequently validated. A systemic review was generated by merging data from previously published studies in order to create a specific miRNA profile pattern that distinguishes common miRNAs that may play a crucial role in prostate cancer. The miRNA expression profiles of 380 known miRNAs were also analysed. Subsequently specific genes, pathways and miRNAs of relevance were subjected to further bioinformatics analysis.

Cancer stem cells have been identified in several malignancies including prostate cancer and are thought to drive primary tumorigenesis through self-renewal and differentiation (Richardson *et al.*, 2004). Additionally the persistence of stem cells post-intervention has been proposed as an explanation for metastasis and recurrence. Holoclones are a tightly packed clone of small cells generally thought to contain stem cells and progenitors. This analysis has demonstrated that key stemness genes and pathways and markers of differentiation have significant altered gene expression in both the holoclones and the retinoic acid treated cell lines also referred to as the "super-differentiated" cell lines.

When comparing self-renewal and differentiated data sets, genes involved in self-renewal should be downregulated upon retinoic acid treatment and genes facilitating differentiation should be upregulated. Stem cells can self-renew and differentiate to produce three cells indicative of one or more of the three germ layers representing; endoderm, ectoderm and mesoderm. Prostate cancer cell lines were stimulated with retinoic acid in order to further differentiate the cells and this was indicated at the morphology level and then confirmed by quantative TaqMan® RT-PCR. This was shown by the expression of biological markers of differentiation of AFP and NCAM1 were significantly upregulated in the retinoic acid treated cell lines when compared to the untreated cell lines. Changes in expression were detected at the time points of one and two weeks. This analysis indicates that these cell lines retain their ability and potential to further differentiate. This analysis also demonstrated that these cell lines have differentiation potential as they show a significant increase in expression in two out of the three differentiated markers examined; AFP and NCAM1.

In this study, holoclones were cultured using a high salt-soft agar assay for LNCaP (metastatic carcinoma) and PC-3 (non-metastatic adenocarcinoma) cell lines. It is the high sodium content that allows for the selective growth of a pure holoclone population. The expression of a panel of key stemness genes and pathways, was assessed in prostate holoclones derived from cell lines LNCaP and PC-3 using a quantitative Real Time TaqMan® PCR method. LNCaP holoclones were maintained for twenty four days and PC-3 holoclones were maintained for six days. The differential duration of the time points denotes that the LNCaP cell line represents a metastatic prostatic carcinoma and should contain a higher number of cancer stem cells. In combination stemness genes were used to exemplify the stem cell potential and characteristics of these hololcones. LNCaP and PC-3 holoclones both indicated stemness characteristics. However both LNCaP and PC-3 holoclones expressed different stemness markers and this disparity was also further represented by the duration differences in time points.

The main focus of the microarray analysis was to characterise and generate a genome wide expression profile of the PC-3 and LNCaP holoclones, as prior to this study holoclones have remained largely uncharacterised at the molecular level. The differences between the holoclones and founder cell lines were determined and genes and pathways representing stemness, differentiation, EMT and cancer associated genes were identified. PC-3 compared to the PC-3 holoclones demonstrated 228 significant differentially regulated genes and LNCap compared to the LNCaP holoclones demonstrated 44 significant differentially regulated genes. Both PC-3 and LNCaP holoclones displayed a unique stemness expression signature. The microarray analysis demonstrated that holoclone derived cells display significant stemness gene expression

when compared to the founder cell lines also indicated different stemness characteristics between PC-3 and LNCaP holoclones. This analysis revealed significant deregulated genes and pathways involved in stemness. Therefore the results obtained from the microarray study, which characterised both holoclone models confirmed the stemness potential of the holoclones derived from prostate cancer. The microarray study was validated and these finding were confirmed.

MiRNAs have recently emerged as an important group of regulatory molecules. MicroRNAs are currently thought to be mechanistically involved in the development of various malignancies and could have the potential to become a new class of biomarkers and eventually used as a therapeutic approach. Therefore the molecular events associated with the holoclones and the resting cell lines must be characterised. A miRNA profile was generated for the following reasons;

- Deregulated miRNA expression has been identified in many cell line models and malignant tumours, indicating their role in the molecular regulation mechanism.
- Different groups of miRNAs are expressed in normal and malignant tissues and cell line models.
- Various different miRNAs are expressed in many types of stem cells, such as undifferentiated and differentiated stem cells.

A systematic review was compiled and generated a specific miRNA profile pattern that distinguishes common miRNAs that are grouped into upregulated, downregulated, undetected and unchanged in prostate cancer tissues and cell line models, all groups used normal tissue as their calibrator. A systematic review was generated using 6 original peer reviewed publications, which profiled miRNA genes in prostate cancer specimens and cell line models.

The expression of approximately 380 miRNAs were assessed by TaqMan® Low Density Arrays a quantative PCR method as described in Chapter 6. MiRNA profiles were generated and identified groups of both specific and common miRNAs for each cell type, indicating specific miRNAs involved in stemness. This study demonstrated the different populations of miRNAs expressed in cell line models of stemness and ascending malignancy representing;

- Normal
- Metastatic carcinoma
- Adenocarcinoma (Gleason score: Grade 4)
- Holclones of metastatic carcinoma
- Holoclones of adenocarcinoma (Gleason score: Grade 4)

Prostate cancer stem cell specific holoclone miRNA populations have been identified. Significant miRNAs associated with the PC-3 and LNCaP holoclones have been identified when compared to the relevant founder cell line. This analysis indicates that there are different groups or populations of miRNAs associated with the holoclone model of stemness. In particular, miRNAs involved metastasis and epithelial to mesenchymal transition (EMT) have been identified in the PC-3 and LNCaP holoclone cell models. Potential miRNAs involved in tumour aggressiveness such as; migration, invasion, cell proliferation, epithelial to mesodermal transition, angiogenesis and apoptosis have been identified in the miRNA holoclone population in comparison to the founder cell lines.

Due to the high variability in the course of prostate cancer, novel biomarkers are strongly needed for early detection and at the time of diagnosis in order to facilitate correct treatment planning. Currently used prognostic tools depend solely on pathological and clinical parameters. Therefore understanding alterations at the molecular level which distinguish progressive from non-progressive disease is crucial. This study has allowed for the generation of a potential panel of biomarkers necessary for androgen-sensitive and independent adenocarcinomas and their derived cancer stem cells, see Table 7.1. This panel of novel biomarkers could potentially be useful for further alanysis in the future such as; immunohistochemistry.

**Table 7.1:** Suggested panel of biomarkers for androgen-sensitive and independent adenocarcinomas.

| Biomarker Type | Androgen-sensitive Adenocarcinoma (LNCaP) | Androgen-independent Adenocarcinoma (PC-3) |
|---|---|---|
| **Stemness & Differentiation** | Fn1, Snail2, NCAM1, AFP, Nanog, SHH, miR-122, miR-136 | Oct4, TGF-β, Snail2, miR-122, miR-136, miR-885-3p, miR-876-3p |
| **Metastasis** | let-7c, miR-153, miR-105, miR-504, miR-488, miR153 | — |
| **Androgen independence** | — | miR-504, miR-488, miR153 |

To conclude, in this study a number of important phenomena relating to prostate cancer and a prostate cancer stem cell holoclone model have been identified. Firstly; holoclones were generated and a population was isolated from the relevant cell lines, which were characterised and their stemness, differentiation and EMT potential was identified. Secondly a prostate cancer stem cell specific miRNA population was identified for these holoclones. Genetic changes identified in this project may become useful in screening patients at risk of prostate cancer earlier and possibly identify biomarkers involved in disease progression and metastasis and recurrence and an overall understanding of the holoclone model at the molecular level.

Currently there are a number of peer reviewed publications on miRNA expression profiling in prostate cancer however, there are not enough to generate a statistical meta-analysis. Future work will consist of carrying out statistical analysis on the systemic review in order to generate a meta-analysis on the compiled list of miRNAs. A meta-analysis is not feasible until such time as there are a sufficient number of these publications.

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