

The role of homeobox gene *NKX3.1* in prostate cancer

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This thesis is dedicated to my family and friends for their unconditional love and encouragement; and to Prof. Sir Walter Bodmer for giving me the opportunity and providing continual support.

ABSTRACT

NKX3.1, a prostate specific homeobox gene is a known marker of prostate epithelium during embryogenesis and is also expressed subsequently through different stages of prostate differentiation. However, all studies on *NKX3.1* are focused on its regulation by androgen receptor (AR). The aim of this project is to establish the role of *NKX3.1* in differentiation in prostate cancer, independent of AR regulation. In this thesis, I characterize the cell lines in terms of their differentiation capabilities in 3D, expression levels of *NKX3.1* and the mismatch repair status. The genes potentially involved in differentiation and regulators of *NKX3.1* are also identified using microarray data of the cell lines (**Chapter 3**). Although *NKX3.1* plays a key role in prostate development no studies have been conducted on the effect of *NKX3.1* expression on differentiation capabilities of prostate cell lines. In **Chapter 4**, this was investigated by siRNA mediated knockdown of *NKX3.1* in 22Rv1 cell line and overexpression of *NKX3.1* in PC3 (designated PC3-Nkx3.1) and PNT1a cells followed by growth in 3D. These functional studies show that the expression of *NKX3.1* is vital for lumen formation in 3D, which is used as a measure of differentiation. The microarray data and overexpression of *NKX3.1* studies suggest that this gene may also be involved in inhibiting epithelial to mesenchymal transition (EMT). Homeobox B13 (*HOXB13*) was identified as one of the downstream targets of *NKX3.1*. *NKX3.1* and *HOXB13* expression levels are positively correlated not only in the panel of prostate cell lines but also in the *NKX3.1* overexpression and knockdown studies (**Chapter 5**). The results of the work presented in this thesis demonstrate that there is a striking parallel between the function of *NKX3.1* in prostate and Caudal-type homeobox 1 (*CDX1*) in the colon and rectum. In conclusion, *NKX3.1* plays a key role as a tumour suppressor in prostate cancer by controlling differentiation of prostate cancer cells.

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DECLARATION

I, Ruchi Kamlesh Patel, hereby declare that the work on which this thesis is based is my original work and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university. The work is original, except where indicated by reference in the text.

Signed:

Date:

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LIST OF ABBREVIATIONS

AR	Androgen Receptor
α -SMA	Alpha-smooth muscle actin
bp	Base pair
CDH1	E-cadherin
cDNA	Complementary DNA
CK5	Keratin 5
CK8/18	Keratin 8/18
CRC	Colorectal cancer
CSC	Cancer stem cells
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
ECM	Extracellular matrix
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
FOXA1	Forkhead box protein A1
HIF	Hypoxia inducible factor
HOXB13	Homeobox B13
μ g	Microgram
μ L	Microlitre
μ m	Micro-meter
μ M	Micro-molar
mA	Milliamperes
mAb	Monoclonal antibody

mg	Milligram
mL	Milliliter
MMR	Mismatch repair
mRNA	Messenger ribonucleic acid
MSI	Microsatellite instability
NKX3.1	NK3 Homeobox 1
ng	Nanogram
nM	Nanomolar
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with 0.02% Tween
PCR	Polymerase chain reaction
PSA	Prostate specific antigen
qPCR	Quantitative polymerase chain reaction
RER	Replication error
rpm	Revolutions per minutes
RQ	Relative quantification
RT-PCR	Real time polymerase chain reaction
SEM	Standard error of mean
siRNA	Small interfering RNA

Chapter 1

Introduction

Overview

1.1 Anatomy and Histology

1.1.1. *Anatomy of the prostate*

Embryologically, the prostate, seminal vesicles, and ductus (vas) deferens originate from 2 separate structures. The seminal vesicles and the ductus deferens are formed from the mesonephric duct. A budding collection of tissue in the urogenital sinus gives rise to the prostate.

The adult morphology of the prostate greatly differs between mouse and human. At adulthood, the rodent prostate gland consists of four distinct lobes: anterior (also known as coagulating gland), dorsal and lateral (collectively referred to as the dorsolateral lobe), and ventral. In contrast, the human prostate lacks discernible lobular organization, but can be divided into four zones (**Figure 1.1**). Each of these zones has a variable content of exocrine acini and fibro-muscular stroma (McNeal, JE *et al* 1981, Fine and Reuter, 2012). The four zones of the human prostate are:

- 1) Peripheral zone (PZ): This zone is mesodermal in origin and comprises of about 70% of the glandular prostate. It surrounds the urethra and being the largest part it is the most common site of origin of prostate cancer.
- 2) Central zone (CZ): The central zone contains the ejaculatory ducts. It is the second largest zone and about 20-25% of prostate cancers arise from this zone.

- 3) Transitional zone (TZ): This zone surrounds the urethra and is the site of BPH origin. It comprises 5-10% of the prostate gland and about 20% of prostate cancers arise from TZ.
- 4) Anterior zone (AZ): The anterior zone is a fibro-muscular zone and lacks glands.

The most common animal model for prostate physiology and pathology is mouse. However, the human prostate is remarkably different from the rodent prostate. The rodent prostate does not have zones, but very distinct lobes (Marker et al., 2003).

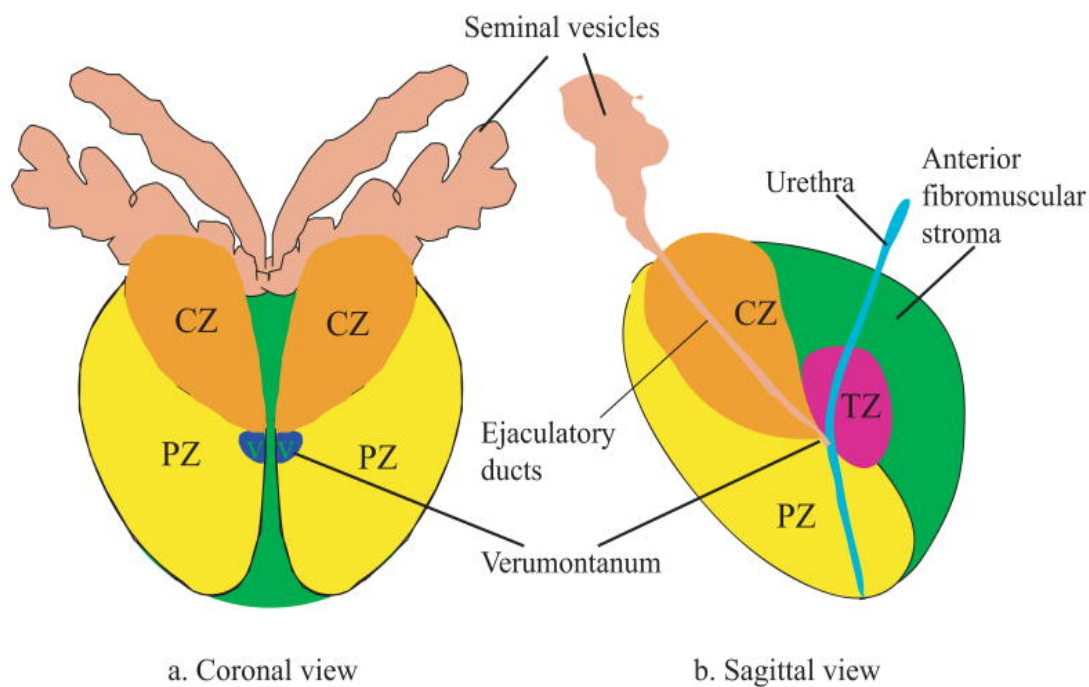


Figure 1.1

Zonal anatomy of the human prostate. It divides a human prostate into one non-glandular zone (the anterior fibro-muscular stroma), and three glandular zones: peripheral zone, central zone and transition zone denoted by PZ, CZ and TZ respectively. (a) Coronal section of the prostate. (b) Sagittal section of the prostate (Zhai, L. *et al*, 2010).

The function of the prostate is to store, secrete and control the flow of the fluid that constitutes 30% of the volume of the semen along with spermatozoa and seminal vesicle fluid. Prostate-specific antigen (PSA), also known as kallikrein3 is a protease in the form of a glycoprotein secreted in the prostate acini that helps to keep the semen in its liquid form.

1.1.2 Histology of the prostate

The prostate is a ductal gland situated at the base of the bladder and contributes secretory proteins to seminal fluid. The human prostate has a high level of organization with an epithelial acini and fibro-muscular stroma. The epithelial and stromal components are separated from each other by a basal membrane called the basal lamina (**Figure 1.2**).

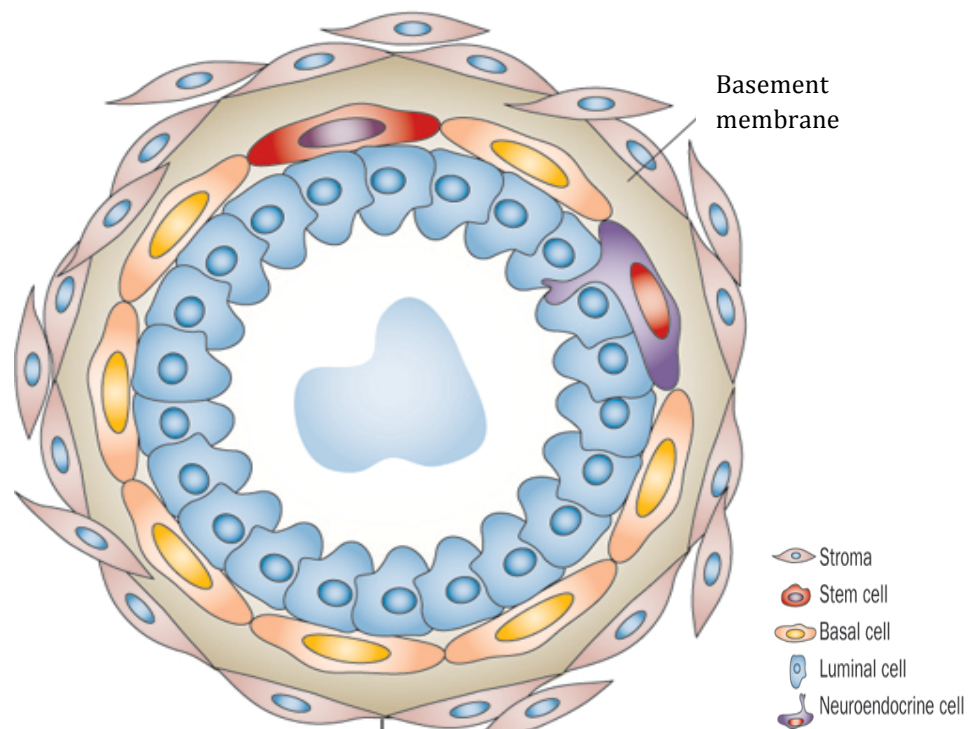


Figure 1.2

Schematic representation of the prostate acinus. The prostate is composed of stromal and epithelial compartments that communicate reciprocally. The epithelium consists of several identifiable cell types, including basal, luminal, and neuroendocrine cells as shown in the figure. (The proportion of the different cell types is not to scale) (Rane, J.K et al., 2012).

1.1.2.1 Prostate epithelium

The prostate epithelium is composed of basal, luminal and a small fraction of neuroendocrine cells.

- 1.) Luminal cells: The luminal layer is made up of tall columnar cells that are terminally differentiated and produce prostatic secretions. These secretions are composed of enzymes such as prostatic acid phosphatase (PAP) and Prostate Specific Antigen (PSA). These cells have no proliferative potential and almost all cells (85%-100%) show a negative expression of proliferation markers PCNA and Ki-67 (De

Marzo, A.M. *et al.*, 1998). Luminal cells are characterized mainly by the expression of androgen receptor (AR) (Nakada, S.Y. *et al.*, 1993, Wang, X. *et al.*, 2006a), NKX3.1 (Wang, X., *et al.* 2009), CD24 (Liu and True, 1997, Lawson, D.A. *et al.*, 2007), and cytokeratin 8/18 (Verhagen, A.P. *et al.*, 1992, Robinson E.J. *et al.*, 1998, Van Leenders, G. *et al.*, 2000).

2.) Basal cells: The basal layer that is made up of cuboidal epithelial cells lying below the luminal layer. These cells are surrounded by the basement membrane or the basal lamina, which acts as a structural barrier between the prostate epithelia and stroma. They do not express AR and their growth is independent of androgen regulation, but they are androgen responsive. These cells are non-secretory and do not produce any PSA or other secretory proteins. Most studies suggest that basal cells are pre-cursors of luminal cells in adult prostate (Issacs, W.B. *et al.*, 1989, Robinson, E. *et al.*, 1998, Richardson, G.D. *et al.*, 2004, Frame, F.M. *et al.*, 2010, Ousset, *et al.*, 2012, Signoretti, S. *et al.*, 2005). On the contrary, it is also proposed that adult prostate basal and luminal cells are derived from a common pluripotent progenitor/stem cell in the basal compartment (Wang, Y. *et al.*, 2001, Choi, N. *et al.*, 2012). Basal cells are characterized mainly by the expression of P63 (Signoretti, S. *et al.*, 2006), CK5 and CK14 (Verhagen, A.P., *et al.*, 1992) and CD44 (Liu, A.Y. *et al.*, 1997). It has been proposed that the prospective epithelial stem cells and cancer stem cells (CSCs) reside in the basal layer of the prostate (Wang, Y. *et al.*, 2001, Lawson, DA. *et al.*, 2010, Maitland, NJ. *et al.*, 2011, Taylor, RA *et al.*, 2012).

3.) Neuroendocrine cells: The neuroendocrine cells are scattered sparsely between the luminal and basal cells and are androgen insensitive (Bonkhoff, H. *et al.*, 2001). It is hypothesized that these cells regulate growth and differentiation as well as exocrine secretory activity through endocrine, paracrine, neurocrine, and lumenocrine mechanisms (di Sant'Agnese PA. *et al.*, 1998). These intraepithelial regulatory cells secrete serotonin, bombesin, calcitonin and parathyroid hormone-related peptide (Nakada, S.Y. *et al.*, 1993, Rumpold, H., *et al.*, 2002). Expression of Chromogranin A, neuron-specific enolase, blc-2 and the AR are modulated during neuroendocrine differentiation and serve as molecular markers for neuroendocrine cells (Amorino, GP *et al.*, 2004).

In addition to these 3 cell types, there is a population of heterogeneous cells that express an intermediate phenotype between early progenitor basal cells and terminally differentiate luminal cells. These cells are called intermediate or transit amplifying cells. The transit amplifying cells may or may not express AR.

1.1.2.2 Prostate stroma

The prostate epithelium is surrounded by stroma, mainly consisting of smooth muscle cells and fibroblasts, and extracellular matrix rich in collagen fibers that intervenes between the secretory acini. Interaction and AR signaling between the prostate epithelium and stroma is essential for normal function and differentiation of the prostate. Derived from a mesenchymal lineage, myofibroblasts possess features common with fibroblasts and smooth muscle cells, in that their morphology is an ovoid-stellate hybrid and they are positive

for both vimentin and α -smooth muscle actin protein expression (Andoh, A *et al.*, 2005). Wnt signaling is important in regulating epithelial cell proliferation, and myofibroblasts have been shown to secrete an array of Wnt proteins *in vitro*, implying a role in supplying proper Wnt ligand signaling to prostate acini. Myofibroblast-conditioned medium promotes migration and invasion of the T84 colorectal cancer cell line (McKaig, BC *et al.*, 1999), while such medium can promote an undifferentiated state in primary colorectal cancer stem cells (Vermeulen, L *et al.*, 2010). Breast-associated fibroblasts can induce increased clone formation within the UACC-812 breast cancer cell line (Samoszuk, M *et al.*, 2005). Clearly, a strong role exists for associated fibroblasts in regulating tumour promotion. The stromal microenvironment in prostate cancer, also known as reactive stroma, is altered compared with normal stroma. The normal prostate stroma consists mainly of fibroblasts, while the reactive stroma comprises of myofibroblasts and cancer-associated fibroblasts (CAFs), which may have evolved from existing fibroblasts (Barron, DA *et al.*, 2012, Tuxhorn, JA *et al.*, 2002). It is also possible that a small sub-population of myofibroblasts already exist in the normal stroma in the neighbourhood of the normal stem cells; and this population is increased in prostate carcinoma. While normal fibroblasts are viewed as being instrumental in maintaining tissue homeostasis, their activated counterparts (myofibroblasts/CAFs) promote tumour progression.

1.2 Disorders of the prostate

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

1.2.1 Prostatitis

This is the most common disorder of the prostate. The prevalence of prostatitis is approximately 8.2 percent (range: 2.2 to 9.7 percent) (Krieger, JN *et al.*, 2008). Prostatitis is defined as microscopic inflammation of the tissue of the prostate gland (predominantly of the central zone), which spans a broad range of clinical conditions. The disease presents as an acute or chronic (most common presentation) lower abdominal pain with increased urinary frequency, or it could remain asymptomatic (Sharp, VJ *et al.*, 2010). There are four classifications of prostatitis: acute bacterial, chronic bacterial, chronic prostatitis/chronic pelvic pain syndrome, and asymptomatic. A number of diagnostic tests are available to differentiate and categorize the four types of prostatitis. These include localization tests and expressed prostatic secretions. The retrograde transmission of infectious agents and damage caused by passing urinary toxins, autoimmunity, and physical stress in the central zone are some of the most common aetiological factors of prostatitis (Krieger, JN *et al.*, 2004, Sharp, VJ *et al.*, 2010). Chronic prostatitis is considered to be a risk factor for the development of BPH and even prostate cancer (Nickel, J.C. *et al.*, 2008, Sciarra, AM *et al.*, 2008).

1.2.2 Benign Prostatic Hyperplasia (BPH)

BPH is a non-malignant nodular hyperplasia of both the stroma and the epithelium of the prostate, almost exclusively arising from the periurethral transitional zone (Roehrborn, C. *et al.*, 2007). It results in an increase in the size of the prostate in middle-aged to elderly men. **Figure 1.3** shows the difference between a normal prostate and a prostate with BPH. The large prostate may constrict the urethra and impede the urine flow causing pain during urination. Age-related deregulation in a balance between androgenic and peptide growth is considered to be the prime aetiological factor for the development of BPH (Thorpe, A. *et al.*, 2003). It is often a slow growing lesion and usually presents after the age of 50 years, with symptoms such as dribbling at the end of urinating, urinary hesitancy, dysuria, and urinary retention (Roehrborn, C. *et al.*, 2011). The severity of symptoms and personal preference of patients determine the treatment choice (Lepor, H. *et al.*, 2011). 5- α reductase inhibitor treatment is usually recommended for mild symptoms. For patients with more severe symptoms, minimally invasive operations, such as laser prostatectomy are available. However, the most common treatment for BPH is trans-urethral resection of prostate (TURP) (Roehrborn, C. *et al.*, 2011). TURP is known to cure BPH patients in 95% of the cases (Varkarakis, J. *et al.*, 2004). The remaining patients need a second operative procedure. It is widely accepted that BPH is not a precancerous lesion, although it increases the coincidental diagnosis of incipient prostate cancer (Chang, RT. *et al.*, 2012). It is possible that a very small proportion of BPHs lead to cancer and once the cancer has developed, the BPH can no longer be seen.

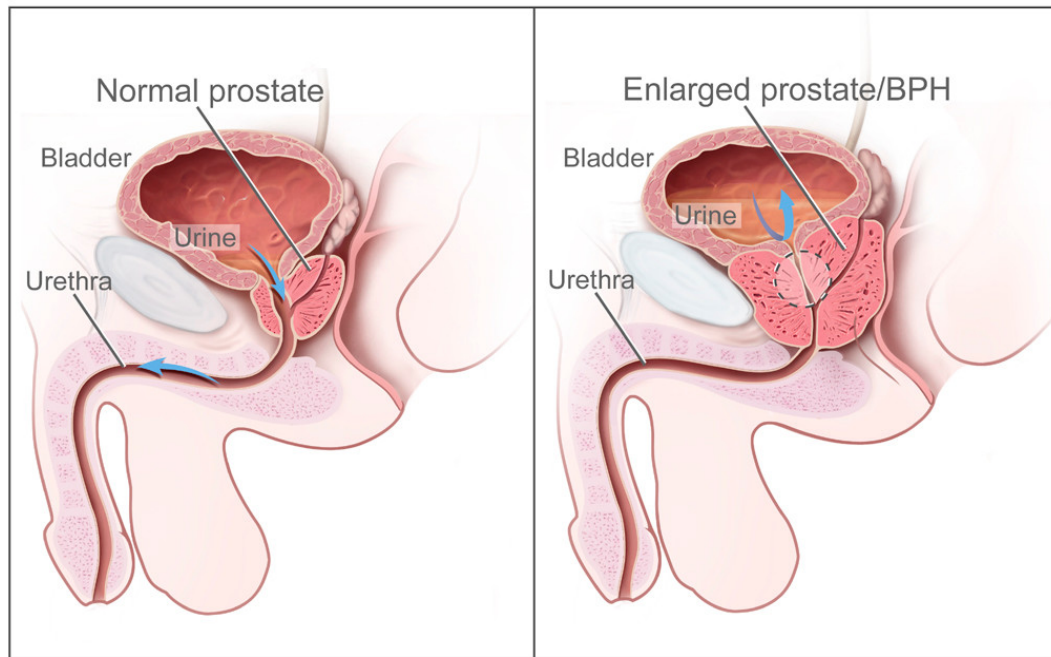


Figure 1.3

Enlargement of the prostate due to benign prostatic hyperplasia (BPH). (Leveillee, RJ *et al.*, 2009).

1.2.3 Prostatic Intraepithelial Neoplasia (PIN)

Prostatic intraepithelial neoplasia (PIN) is a condition defined by neoplastic growth of epithelial cells within preexisting benign prostatic acini or ducts (Brawer, MK *et al.*, 2005). PIN has been widely accepted as a precursor to prostate cancer and has been identified as the most likely progenitor of the majority of prostatic adenocarcinomas (Bostwick, DG *et al.*, 2004, Brawer, MK *et al.*, 2005)

1.2.4 Prostate cancer

Prostate cancer is the most frequently diagnosed non-skin cancer and third leading cause of cancer mortality in men. More than 40,000 incident cases of prostate cancer and more than 10,000 prostate cancer related deaths occur each year in the United Kingdom (Wilt, TJ *et al.*, 2013). The vast majority of these

cancers are adenocarcinomas (99%), with minimal incidence of ductal carcinoma, mucinous adenocarcinoma, small cell carcinoma, carcinosarcoma and embryonal carcinosarcoma variants (Kendal, W. *et al.*, 2010). Increasing age is the most important risk factor for developing cancer. Therefore henceforth, prostate cancer will be discussed only in relation to prostate adenocarcinoma.

1.2.4.1 Symptoms

Most cancers arise in the periphery of the prostate gland, and cause symptoms only when they have grown to compress the urethra, or invade the sphincter or neurovascular bundle (Guess, HA *et al.*, 2001, Wang, J *et al.*, 2001). The stage of the cancer is helpful in understanding what symptoms (if any) it produces. Four staging systems have been described, and the tumour, node, metastasis (TNM) system is the most widely used (Mohler, JL *et al.*, 2013). In the TNM system, T1 tumours are by definition impalpable; T2 are palpable rectally, T2a tumours are confined to one lobe and are less than 1.5cm in size, and T2b are diffuse, larger, or present in both lobes, T3 tumours have invaded local structures, such as the bladder, seminal vesicles or the prostatic capsule; and T4 tumours have invaded more widely. The symptoms are associated with signs, such as bone marrow suppression, a hard mass on digital rectal examination, and high PSA.

1.2.4.2 Diagnosis

The contemporary challenge of prostate cancer diagnosis has been changed in the past decade from the endeavour to increase detection to that of detecting only those tumours that are clinically significant. The main diagnostic tools used presently in practice to look for evidence of prostate cancer are serum

concentration of PSA, digital rectal exam, and transrectal ultrasound guided biopsies. Conclusive diagnosis of prostate cancer can only be established after histological examination of biopsies.

1.) PSA: The widespread incidence and relative ease in treatment of localized disease has prompted a call for routine serum prostate specific-antigen (PSA) screening. A blood test to measure PSA has been long considered the most effective test currently available for the early detection of prostate cancer. An increase in serum PSA is considered as an indicator of increasing prostate gland volume and prostate cancer progression (Hudson, MA *et al.*, 1989). However, other prostate diseases namely prostatitis and BPH have also been found to produce elevated levels. Although, PSA screening is routine in some countries like USA and Austria there is hesitancy in a lot of countries to routinely use PSA tests due to two principal reasons:

- High levels of false positives and false negatives with PSA testing
- Inability of PSA to distinguish between aggressive and indolent cancers (Andriole, GL *et al.*, 2012)

New markers that are more specific and sensitive biomarkers, such as PCA3 and circulating tumour cells, although not widely used, could be a more promising option for the screening and risk stratification of prostate cancer (Hessels, D *et al.*, 2003, Danila, DC *et al.*, 2007).

2.) Digital rectal exam: Digital rectal exam is a simple technique where the doctor inserts a lubricated finger (digit) through the patient's rectum to feel for suspicious areas of the prostate gland, in particular to search for hard, lumpy or abnormal areas on the prostate. The exam is limited to detecting lesions protruding from the surface of the prostate.

3.) Transrectal ultrasound-guided biopsies

Elevated PSA levels or detection of suspicious lump during digital rectal exam is followed by an ultrasound-guided biopsy. This involves a probe about the size of a finger that is inserted into the rectum. The probe produces ultrasound waves that are transformed into a video to help target a needle of the probe, through the rectum wall into specific areas of the prostate to take a sample of the tissue for histological analysis. Biopsies are usually taken at 10 points around the prostate to reduce false negatives (Hansel, DE *et al*, 2007).

Better interpretation of the role of Prostate-specific antigen (PSA) and its kinetics as a diagnostic tool, the adoption of extended prostate biopsy schemes, and perhaps implementation of new transrectal ultrasound (TRUS) technologies are key in more effective diagnosis. Conclusive diagnosis of prostate cancer can only be established after histological examination of biopsies. Gleason grade is universally used for pathologic scoring of the differentiation of prostate cancer. This grading system is based entirely on the histologic pattern of arrangement of carcinoma cells in H&E- stained sections (Gleason, DF *et al*, 1966). Prostate cancer can be graded from 1 to 5, based on the histological architecture of the prostate gland and differentiation status of the tumour (grade 1 being closer to the normal histology and 5 being the most disrupted architecture) (**Figure 1.4**). The overall Gleason score is calculated by adding the score of the most common grade (primary grade pattern) and the second most common grade (secondary grade pattern). For example, for Gleason grade 7 could be (4+3) or (3+4). 4+3 means, the most prevalent grade is 4 and the second most prevalent grade is 3

and vice versa in case of a (3+4) grading. Higher Gleason scores indicate a higher grade of prostate cancer, indicating a more advanced or more aggressive type of cancer. In Dr. Gleason's original study, it was shown that prostate cancer behaves in proportion to its average grade, rather than according to the most severe grade.

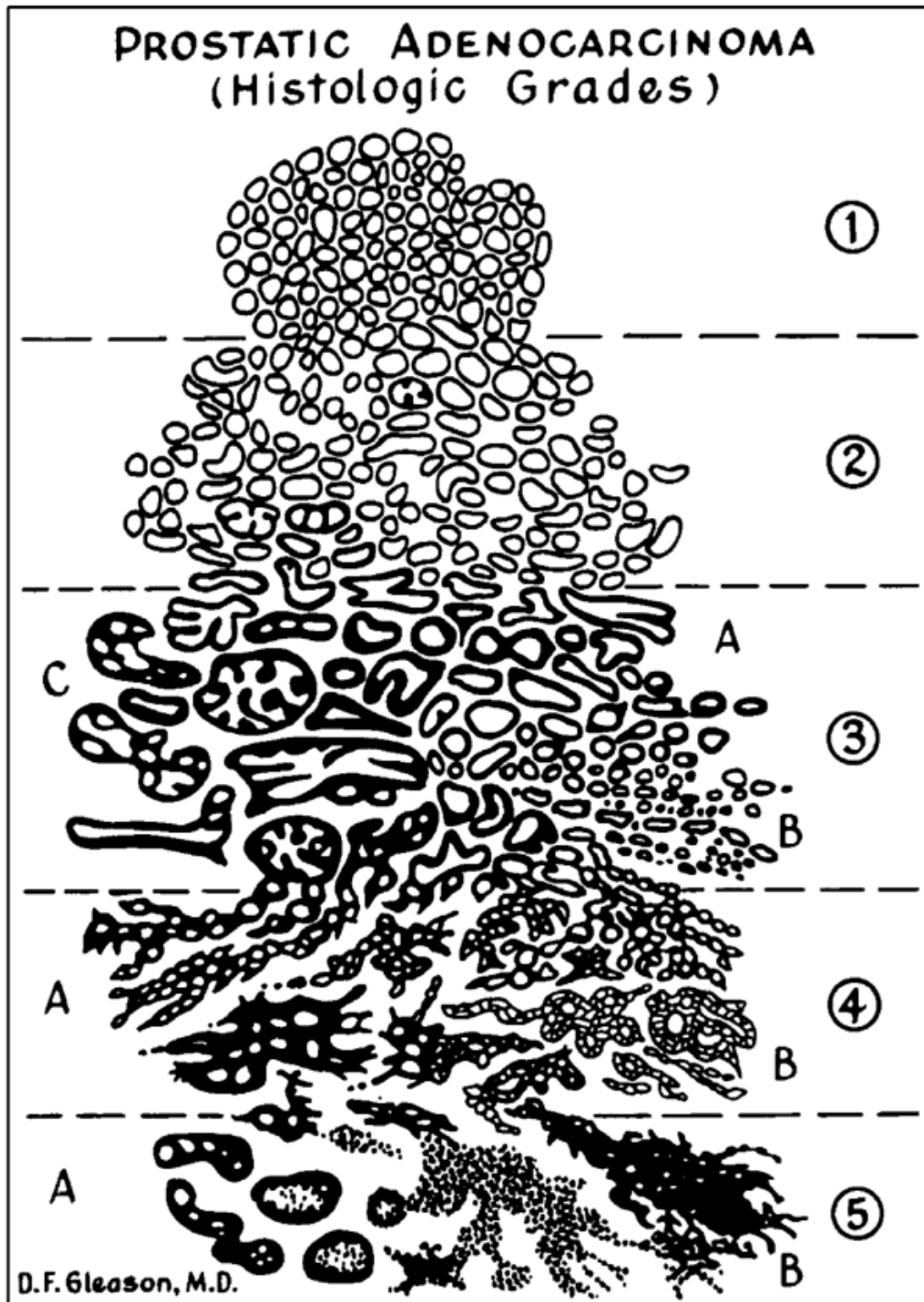


Figure 1.4

Schematic representation of the Gleason grading of prostate cancer. Adapted from (Gleason, DF *et al.*, 1966, Gleason, DF *et al.*, 1992)

1.2.4.3 Treatment

The treatment for prostate cancer depends predominantly upon the stage of the disease, histological grade of the tumour and patient choice. Treatment options available for prostate cancer management are as follows:

Active surveillance

Patients with very low-risk localized prostate cancer are initially not treated but are followed and treated with a curative intent if progression or the threat of progression occurs during follow up. It is recommended for patients with clinically confined prostate cancer with a Gleason grade of ≤ 6 and PSA levels $< 10 \text{ ng/mL}$.

Radical prostatectomy

Radical prostatectomy is a procedure wherein the prostate gland is removed from patients with low to intermediate risk of localized prostate cancer. In men with intermediate and high-risk prostate cancer an extended pelvic lymphadenectomy (removal of adjuvant lymph nodes) is also recommended (Briganti, A *et al.*, 2006).

Radiotherapy

Radiotherapy is an alternative treatment for patients who refuse surgical intervention. Major emphasis is on retention of function after treatment. Three-dimensional conformal radiotherapy (3d-CRT) is the gold standard and intensity-modulated radiotherapy (IMRT), an optimized form of 3D-CRT, is the most widely used.

Androgen deprivation therapy (ADT)

For early stage disease surgery and radiation therapy is often curative, but for locally advanced or metastatic disease initial treatment involves removing the

drive to tumour growth provided by androgens, via androgen deprivation therapy. Androgen deprivation therapy with a gonadotrophin-releasing hormone (GnRH) agonist is the mainstay treatment for metastatic prostate cancer (Huggins, C *et al.*, 1941) and may improve survival for men with locally advanced disease.

Chemotherapy

In a majority of the advanced cases, androgen deprivation therapy fails and castration resistant prostate cancer develops. Docetaxel is the most common chemotherapeutic drug in use, but newer agents like novel taxane, cabazitaxel, abiraterone, apalutamide, sipuleucel-T, and enzalutamide that have different modes of action have also been developed (Bahl, A *et al.*, 2013).

1.2.4.4 Aetiology and risk factors

- **Age:** Advancing age is by far the most well established risk factor for the development of prostate cancer (Crawford, ED *et al.*, 2003). Study of age-specific incidence curves reveals that prostate cancer risk begins to rise sharply after the age of 55 years and peaks at age 70-74, declining slightly thereafter (Gann, PH *et al.*, 2002). However, the differences in age at diagnosis, heterogeneity in geographical incidence, histology, progression, and treatment response suggest that there are additional contributory risk factors.
- **Inflammation:** Histologic evidence of inflammation commonly includes the presence of inflammatory infiltrates and proliferative inflammatory atrophy. Proliferative inflammatory atrophy is often identified adjacent to high-grade prostatic intraepithelial neoplasia. Inflammation is thought to

incite carcinogenesis by causing cell and genome damage, promoting cellular turnover, and creating a tissue microenvironment that can enhance cell replication, angiogenesis and tissue repair (Sfanos, KS *et al.*, 2012). It is likely, that inflammation promotes or encourages the outgrowth of appropriately mutated cells. Various publications describe susceptibility genes for prostate cancer that are associated with cellular defenses against inflammation and oxidative stress, and defects in their function might lead to an inability to prevent tumour formation. However, future studies in all of these areas need to elucidate the precise mechanisms that may prove useful for the development of novel strategies.

- **Genetic and epigenetic changes:** Causal hereditary genetic and epigenetic alterations are relatively uncommon in prostate cancer. Familial predisposition for prostate cancer is seen in only about 10% of the total cases (Stanford, JL *et al.*, 2001). In familial prostate cancer, 3 successive generations or 2 siblings have diagnosed prostate cancer at the age <55 years. The rest of the cases demonstrate some recurrent genomic alterations in addition to genetic and epigenetic abnormalities (**Table 1.1**). It has been proposed that about 43% of the risk of prostate cancer can be attributed to genetic influences (Michaud, DS *et al.*, 2006). Changes in the expression of genes with some other less frequent abnormalities interfere with several key signaling pathway, such as, mTOR.AKT pathway, NF-kB signaling pathway, and developmental signaling pathways (Shen and Abate Shen, 2010). However, a direct causal role of these anomalies has not been conclusively demonstrated.

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

Table 1.1

Common genetic and epigenetic alterations found in prostate cancer. Adapted from (Koochekpour, S *et al.*, 2011).

1.2.4.5 Key events in prostate cancer

Most frequent genomic alterations in prostate cancer are predominantly characterized by genomic rearrangement leading to the formation of TMPRSS2:ETS gene fusions (Demichelis, F *et al.*, 2007, Tomlins, Sa *et al.*, 2005) and deletions of *PTEN* (Yoshimoto, M *et al.*, 2012, Wang, S *et al.*, 2003) and *NKX3.1* (Asatiani, E *et al.*, 2005).

PTEN is a lipid phosphatase that controls a number of cellular processes, including survival, growth, proliferation, metabolism, migration, and cellular architecture. The generation of genetically engineered mouse models with deletion of *PTEN* has revealed that varying degrees of *PTEN* loss in combination with other genetic alterations are able to recapitulate all spectrums of human

prostate cancer, from tumour initiation to metastasis (Fraser, M *et al.*, 2012, Pourmand, G *et al.*, 2007, Mulholland, DJ *et al.*, 2012). Studies demonstrate that the role of PTEN pathway is crucial in understanding the interplay between TMPRSS2:ERG activation, PIN and prostate cancer oncogenesis (King, JC *et al.*, 2009).

TMPRSS2:ETS gene fusions were first identified in prostate cancer by Tomlins *et al.* in 2005 (Tomlins, S *et al.*, 2005). TMPRSS2 is a prostate specific, androgen responsive, transmembrane serine protease and ETS family members are transcription factors that regulate important signaling pathways involved in cell differentiation (Basuyaux, JP *et al.*, 1997, Carrere, S *et al.*, 1998). TMPRSS2:ERG gene fusion is the principle genomic alteration and most common of these fusions. TMPRSS2:ERG is a characteristic signature in approximately half of prostatic malignancies (Perner, S *et al.*, 2007, Mehra, R *et al.*, 2008, Gopalan, A *et al.*, 2009, Boormans, JL *et al.*, 2010, Stott, SL *et al.*, 2010). TMPRSS2:ERG gene fusion leads to over-expression of full length ERG (Kumar-Sinha, C *et al.*, 2008, Klezovitch, O *et al.*, 2008). However, ERG over-expression is not sufficient to induce neoplastic transformation and cooperation with other pathways and genes is necessary (Petrovics, G *et al.*, 2005). It has been shown that dysregulated expression of ERG is associated with reduced expression of the *NKX3.1* (Kunderfranco, P *et al.*, 2010). *NKX3.1* is a prostate specific homeobox gene that acts as a tumour suppressor and its loss has an important role in tumour initiation and progression to invasive disease (Abate-Shen, C *et al.*, 2008, Iwata, T *et al.*, 2010, Thangapazham, R *et al.*, 2014). Since derangement of differentiation is one of the key hallmarks of cancer and given the role of *NKX3.1*

in the control of differentiation, it is a natural target to consider for its role in prostate cancer.

1.3 *NKX3.1* Homeobox gene

In this section, I will discuss the structure and roles of prostate specific homeobox gene, *Nkx3.1* in embryonic development and prostate cancer.

1.3.1 *Homeobox* genes

Homeobox genes were first identified in *Drosophila melanogaster* and have subsequently been described in other plant and mammal species (McGinnis, W *et al.*, 1992). They are a family of genes that play an important role in embryonic development and axial patterning. They specify segment identity, namely whether a segment of the embryo will form part of the head, thorax, or abdomen, and they are all usually clustered together. Homeobox genes are found in almost all eukaryotes, and have diversified into 11 gene classes and over 100 gene families in animal evolution. Some homeobox genes are expressed in the endodermal and mesodermal layers of the gut in the embryo, whereas others are expressed throughout adult life (Beck, F *et al.*, 2002). One of the characteristics of homeobox genes is that they have a conserved 183-nucleotide sequence in a helix-turn-helix homeodomain. This domain specifically binds to DNA elements that contain a TATA motif, and may be that is the basis on which homeobox genes act as transcription factors.

The largest group is the ANTP class which includes the well-known Hox genes, plus other genes implicated in development including ParaHox (Cdx, Xlox, Gsx). EvX, Dlx, NK4, NK3 (Nkx, Msx, Enand Nanog). *Hox* genes are regulators for specification of cells in different regions of embryos, mainly through a 60 amino acids homeodomain. It is believed that *ParaHox* and *Hox* clusters are derived from an ancestral *Proto-Hox* cluster in metazoan evolution by duplication

(Garcia-Fernandez, J *et al.*, 2005). In humans there are two *NK3* genes, *NKX3.1* and *NKX3.2* on different chromosomes, generated by genome duplication at the base of the vertebrates. *NKX3.1* is a prostate-specific homeobox gene that plays a role in development and normal differentiation of the prostate. *NKX3.2* is a transcriptional repressor that acts as a negative regulator of chondrocyte maturation. It plays a role in distal stomach development and in defining the structural components of the middle ear.

1.3.2 *NKX3.1*

1.3.2.1 Genomic and protein structure

NKX3.1 (*NK3 homeobox 1*) is located at 8p21.2 and the size of the gene is 4,245 bases. The mouse *NKX3.1* gene contains two coding exons with a short 5' untranslated region (UTR) and an extensive 3' UTR that contains consensus matches for sequences involved in posttranscriptional regulation (Sciavolino, PJ *et al.*, 1997). In humans, there are at least five alternatively spliced forms of the *NKX3.1* gene, which encode variants of the N-terminal coding region upstream of the homeodomain (Korkmaz, KS *et al.*, 2000). However, the *in vivo* expression and functional significance of these alternative *NKX3.1* transcripts has not yet been elucidated. Binding-site selection experiments have identified "TAAGTA" as a consensus site for human *NKX3.1* (Steadman, DJ *et al.*, 2000), which differs from the consensus sequences identified for other homeoproteins, which typically contain a "TAAT" core (**Figure 1.5**). *Nkx3.1* contains a conserved decapeptide motif outside of the homeodomain known as the TN domain (Lints, TJ *et al.*, 1993), which is essential for interaction with members of the Groucho family of transcriptional corepressors.

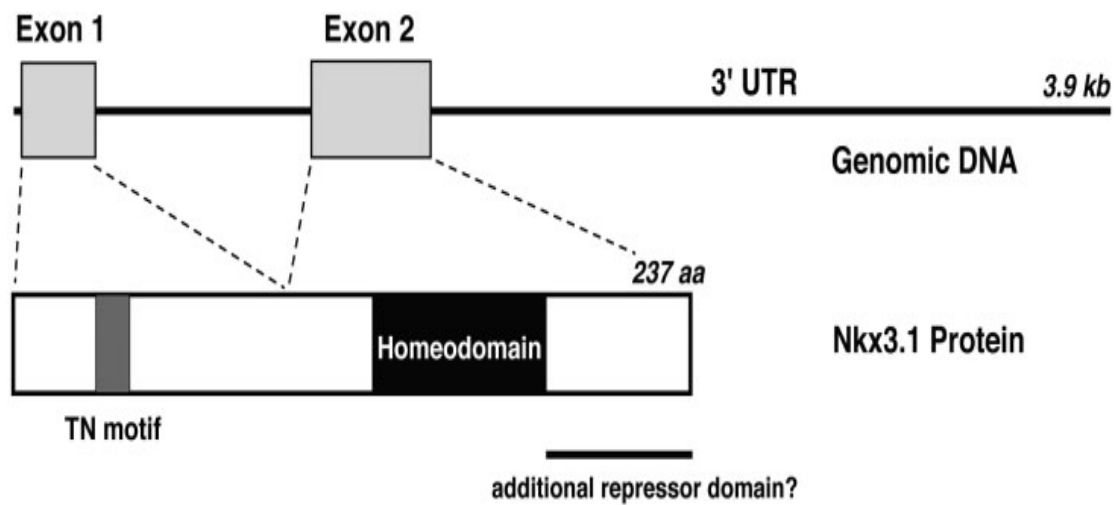


Figure 1.5

Schematic description of the *NKX3.1* genomic DNA and protein. The *NKX3.1* locus contains two coding exons, and has a very short 5' untranslated region (UTR) and a long 3' UTR. The *NKX3.1* protein contains a TN motif that potentially interacts with Gro/TLE corepressors near the N-terminus. A second potential repressor domain is located at the C-terminus following the homeodomain (Carson, JA *et al.*, 2000) (Adapted from Shen, M *et al.*, 2003).

The *NKX3.1* homeodomain forms three α -helices, the first two of which form the parallel scaffold that stabilizes the third, perpendicular helix that contacts the major groove of DNA (Zheng, SL *et al.*, 2006). Mutational studies have shown that hydrogen bonding with DNA occurs at lysine and isoleucine at homeodomain positions 46 and 47, glutamine and asparagine at homeodomain 50 and 51, and tyrosine at 54 (Sciavolino, PJ *et al.*, 1997). The stability of *NKX3.1* is influenced by post-translational modification of the homeodomain and flanking regions by phosphorylation and ubiquitination. (Ornstein, DK *et al.*, 2001).

1.3.2.2 Transcriptional activities of *NKX3.1*

It has been shown that *NKX3.1* acts as a transcriptional repressor through recruitment of Gro/TLE corepressors (Jimenez, G *et al.*, 1997). The

transcriptional repressor activities of *NKX3.1* can potentially be modulated by its interaction with other proteins, such as PDEF (prostate derived ETS factor), which is a member of the ETS transcription factor family that is expressed in the prostate and is identified as an *NKX3.1* –interacting protein (Chen, H *et al.*, 2002). However, in certain tissue contexts *NKX3.1* possesses transcriptional activator activity. *NKX3.1* interacts with serum response factor (SRF) to activate transcription from the smooth-muscle γ actin promoter (Carson, JA *et al.*, 2000, Zhang, Y *et al.*, 2008).

Androgen regulation of *NKX3.1*

Androgens influence the development, maturation and maintenance of the prostate and the prostate is not formed in the absence of androgens (Marker, PC *et al.*, 2003). Androgens also regulate both proliferation and differentiation in the luminal epithelium of the adult prostate. Both the context and cell-type dependent gain and loss of function in AR expression are implicated in the progression of prostate cancer (Shen, MM *et al.*, 2010). However, recent reports in rodent and human cell culture models indicate that prostate cancer stem cells could be AR-responsive, in spite of being AR independent (Goldstein, AS *et al.*, 2010, Oin, J *et al.*, 2012). This suggests that AR signaling could be essential for cell proliferation leading to increased tumour mass, but may not be necessary for tumour initiation.

The actions of androgens are mediated through their binding to the AR, a member of the steroid family of nuclear transcription factors. During embryogenesis, functional androgen receptors are located in the urogenital sinus mesenchyme, whereas postnatally they are found in both the mesenchyme and epithelium. The expression of AR in the mesenchyme is essential to produce

signals for prostate induction and growth. The expression of AR in the epithelium is required later for the secretory function of differentiated cell types. Although the initial appearance of *NKX3.1* expression in the prostatic epithelium precedes that of the AR, the subsequent expression of *NKX3.1* is dependent on androgen signaling, as shown in tissue recombination assays (Bhatia-Gaur, R *et al.*, 1999). The expression of *NKX3.1* in human prostate cancer cells in culture is also down regulated after depletion of androgens from the cell culture media (Prescott, JL *et al.*, 1998). Although the maintenance of *NKX3.1* expression is dependent on androgen signaling, it is not clear whether this regulation occurs directly through the *NKX3.1* promoter or whether it is the consequences of androgen signaling are indirect (Abate-Shen, CA *et al.*, 2008).

1.3.2.3 Role of NKX3.1 in prostate organogenesis

NKX3.1 is the earliest known molecular marker of the prostate epithelium and defines the initial steps in prostate formation (Bhatia-Gaur, R *et al.*, 1999). In the adult urogenital system of the mouse, *NKX3.1* is specifically expressed at high levels in all lobes of the prostate and bulbourethral glands, which are ductal tissues of endodermal origin. In contrast, *NKX3.1* is not expressed in ductal tissues that are not of endodermal origin, such as the seminal vesicle or in nonductal endodermal tissues, such as the bladder and urethra.

The prostatic epithelial buds undergo extensive ductal outgrowth and branching into the surround mesenchyme during the first 3 weeks of postnatal development (Timms, BG *et al.*, 1994). These emerging prostatic buds are marked by the expression of *NKX3.1* in the prostatic epithelium (Sciavolino, PJ *et al.*, 1997). Expression of *NKX3.1* is enriched toward the distal ends of the

outgrowing ducts, corresponding to regions of active morphogenesis (Bhatia-Gaur, R *et al.*, 1999). Before canalization of the prostatic ducts, *NKX3.1* is expressed uniformly in the epithelial cells, but later expression is restricted to the luminal compartment.

In addition to the prostate, there are severe morphogenetic and histologic defects in the bulbourethral glands, which are exocrine glands and are also derived from the urogenital sinus (Bhatia-Gaur, R *et al.*, 1999). The bulbourethral glands of *NKX3.1* homozygous mutants display greatly reduced size due to a reduction in ductal branching, as well as a nearly complete loss of mucous-producing cells.

1.3.2.4 NKX3.1 and prostate cancer

NKX3.1 is a lead candidate for the gene located within a two-megabase minimal deleted region at 8p21 (Dong, JT *et al.*, 2001, Swalwell,JI *et al.*, 2002), which is reported to most frequently be subjected to loss of heterozygosity (LOH) in prostate cancers (Emmert-Buck, MR *et al.*, 1995, He, W *et al.*, 1997, Vocke, CD *et al.*, 1996). *NKX3.1* protein expression is down regulated in a majority of human prostate cancers, in a manner that is correlated with disease progression (Bowen, C *et al.*, 2000). Complete loss of *NKX3.1* expression was observed in 20% of high grade PIN, 34% of hormone refractory prostate cancers, and 78% of metastasis (Bowen, C *et al.*, 2000). However, the remaining *NKX3.1* allele does not appear to undergo mutational inactivation (Voeller, HJ *et al.*, 1997).

NKX3.1 homozygous mutant mice develop histological features that resemble human PIN and these features become increasingly more apparent with advancing age (Tanaka, M *et al.*, 2000, Abdulkadir, SA *et al.*, 2002, Abate-Shen, C

et al., 2008). Histologic findings in these mice include perturbation of the basal cell layer, attenuation of the stroma, and heterogeneous expression of markers of epithelial differentiation (Kim, MJ *et al.*, 2002). Although aged *NKX3.1* mutant mice are highly prone to develop PIN, they do not develop invasive carcinoma.

Although *NKX3.1* loss of function is not sufficient for prostate cancer, *NKX3.1* can cooperate with *PTEN*, in suppression of prostate carcinogenesis. In particular, the combined loss of function of *NKX3.1* and the *PTEN* tumour suppressor gene in mice with compound mutations (*NKX3.1*^{-/-}; *PTEN*^{+/-}) display striking cooperativity, as evidenced by the early onset of high grade PIN lesion (Kim, MJ *et al.*, 2002). Ultimately these *NKX3.1:PTEN* compound mutant mice develop adenocarcinoma, in some cases with metastasis to the lymph nodes as well as distant organs (Abate-Shen, *et al.*, 2003). Aside from these malignant lesions, the compound mutants display no additional phenotypes compared with the single mutants, which emphasizes the prostate-specificity of the cooperativity between *NKX3.1* and *PTEN*. Prolonged exposure of *NKX3.1:PTEN* mutant mice to androgen levels that are one-tenth of normal androgen levels leads to acceleration of prostate tumourigenesis, possibly by selecting for molecular events that promote more aggressive, hormone-refractory tumours (Banach-Petrosky, W *et al.*, 2007).

In addition to localization to a prostate cancer hot spot, these lines of evidence implicate *NKX3.1* as an important prostate tumour suppressor gene.

1.3.2.5 *NKX3.1* parallel gene, *CDX1*

CDX1 is a homeobox gene and an important transcription factor in intestinal development and colorectal cancer. *CDX1* regulates differentiation of epithelial cells in the colon and is down-regulated in colorectal carcinoma (Soubeyran, P *et al.*, 1999). Because a key feature of the development of a cancer is an unbalanced program of proliferation and differentiation, dysregulation of *CDX1* may be an advantage for the development of colorectal carcinoma. Bodmer lab has studied *CDX1* extensively and has shown that it is down regulated in one fifth of the colorectal cancer cell lines and the mechanism of down-regulation is promoter methylation (Wong, N *et al.*, 2004). Selection for reduced expression of *CDX1* by methylation in CRC is likely to be associated with a reduced capacity of the cancer stem cells to differentiate and so to contribute to an increased rate of growth. It has also been shown using 3-dimensional cultures that forced expression of *CDX1* in colorectal cancer cell lines induces differentiation. For instance, a CRC cell line HCT116 does not differentiate or express *CDX1*; however, forced expression of *CDX1* in this cell line leads to reduced clonogenicity and production of differentiating crypt-containing colonies (Yeung, T *et al.*, 2010). Similarly, overexpression of *NKX3.1* in nude mice inhibits proliferation and tumorigenicity (Kim, MJ *et al.*, 2002), but no functional studies have been carried out to study the effect of *NKX3.1* in human prostate cell lines.

Since the role of *NKX3.1* in prostate development and cancer is parallel to that of *CDX1* in intestinal development and CRC, it was chosen to study its role in differentiation. The striking similarities between these two genes could provide

a general paradigm for studying the consequences of loss of function of other homeobox genes in cancer.

1.3.3 *HOXB13*

Analysis of the microarray data revealed that the expression of another homeobox gene, *HOXB13* is positively correlated with *NKX3.1* expression in prostate cell lines. Given the importance and specificity of *HOXB13* in prostate biology, further characterization of this gene in terms of its role in differentiation in prostate cancer is essential.

1.3.3.1 Role of HOXB13 in prostate development

All *HOX* genes are expressed in a tissue-specific and frequently stage-related fashion. *HOXB13* has been shown to have limited expression at the caudal extent of the spinal cord, tail bud and urogenital sinus in an androgen independent manner (Kim, YR *et al.*, 2010). The expression of *HOXB13* is abundant in the adult human prostate and its expression is localized to the ventral prostate luminal cells (Takahashi, Y *et al.*, 2004). Like *NKX3.1*, *HOXB13* is also involved in the formation and maintenance of the prostate and is required for differentiation and secretory function of the ventral prostate (Feber, A *et al.*, 2004, Takahashi, Y *et al.*, 2004). Mice homozygous for *HOXB13* loss of function mutations have shown overgrowth in all major structures derived from the tail bud (Economides, KD *et al.*, 2003) and malformation of the ducts of the ventral prostate, including complete loss of secretory proteins (Economides, KD *et al.*, 2003). The expression of *HOXB13* and AR is shown to be positively correlated in primary prostate tissue and xenograft models (Kim, YR *et al.*, 2010); however,

their expression is not mutually regulated (Jung, C. *et al.*, 2004). Interaction between *HOXB13* and *AR* is made regardless of the presence of androgens, suggesting that *HOXB13* has an important role in androgen-independent progression of prostate cancer. The phenotype shown in *HOXB13* mutant mice was similar to *NKX3.1* mutant mice and developed into swollen prostate in older mutant mice. It has also been shown that *HOXB13* regulates the expression of many *AR* responsive genes including *NKX3.1* (Kim, DS *et al.*, 2010).

The biological role of this developmental protein predominantly expressed in the prostate is not clear. In this thesis, the functional effect of *HOXB13* expression is investigated along with its correlation with the expression of *NKX3.1*.

1.3.3.2 Germline mutations in HOXB13 and prostate-cancer risk

Linkage studies have mapped a prostate cancer susceptibility locus to a 15.5Mb region on chromosome 17q21-22 (Lange, EM *et al.*, 2003, Xu, J *et al.*, 2005). Linkage analysis in combination with targeted massively parallel sequencing has identified a recurrent mutation in *HOXB13* that is associated with early-onset and familial prostate cancer. *HOXB13* G84E is associated with a significantly high risk of prostate cancer, since men with prostate cancer were more likely to carry the *HOXB13* G84E allele (carrier frequency, 1.4%) than were those without prostate cancer (carrier frequency, 0.1%) ($p < 8.5e-7$) (Ewing, CM *et al.*, 2012). Follow up studies in 5,083 prostate cancer cases and 1,041 controls of European ancestry showed that this variant was enriched in prostate cancer cases compared to controls and was associated with a 20-fold higher risk of prostate cancer ($p < 10e-8$) (Vogan, K *et al.*, 2012). However, the mechanisms by which the

HOXB13 G48E mutation might act to promote prostate carcinogenesis are unknown.

1.4 Hypoxia

Hypoxia is often present in cancers, as the demand for oxygen outstrips the supply of blood when cancers progress. The oxygen pressure is less than 5 to 10 mmHg; for example, in the normal breast the medial pO₂ is approximately 50mmHg, but in breast cancers, the pO₂ ranges from 3 to 10mmHg, depending on the haemoglobin concentration (Vaupel, P *et al.*, 2003, Vaupel, P. *et al.*, 2008). The aberrant growth of microvasculature is selected for to compensate for the low oxygen tension in the midst of the tumour mass. Cells undergo a variety of biological responses when placed in hypoxic conditions, including activation of signaling pathways that regulate proliferation, angiogenesis and death. Cancer cells may have adapted these pathways to allow tumours to survive and even grow under hypoxic conditions. Tumour hypoxia is associated with poor prognosis and resistance to radiation therapy.

Hypoxia has two opposing effects in tumour biology. On the one hand, hypoxia results in decreased proliferation, apoptosis and necrosis (Vaupel, P *et al.*, 2007). On the other, there are subclones within the tumour, which can adapt to the hypoxic stress, resulting in a more aggressive phenotype, and acquiring resistance to treatment (Vaupel, P *et al.*, 2005, Alqawi, O *et al.*, 2007). In this way, hypoxia applies strong pressure to select for cells that possess survival mechanisms in environments of low oxygen tension.

1.4.1 Hypoxia inducible factor (HIF) proteins

Hypoxia Inducible Factors (HIFs) are a family of heterodimeric DNA binding proteins, consisting of an oxygen-sensitive alpha subunit and a constitutively expressed beta subunit that is not responsive to oxygen levels. Many oxygen-

responsive genes are regulated by the HIF- α complex. Three isoforms of HIF- α have been identified- *HIF-1 α* , *HIF-2 α* and *HIF-3 α* . HIF-1 α has a very short half-life under normoxic conditions, and is continuously being synthesized and degraded. However, as the oxygen levels drop, the degradation of HIF-1 α is reduced. HIF-1 α overexpression, as evidenced by immunostaining, has been reported in a variety of cancers (including prostate cancer) and their metastases (Zhong, H. *et al.*, 1999, Lekas, A. *et al.*, 2006). *HIF-1 α* and *HIF-2 α* also play important roles in mediating the downstream effects of hypoxia on angiogenesis, tumour progression and metastasis (Rankin, EB *et al.*, 2008, Poon, E *et al.*, 2009)

1.4.2 Hypoxia and prostate cancer

HIF-1 α overexpression is clinically important because it is associated with poorer prognosis in colorectal cancer (Baba, Y *et al.*, 2010, Cao, D *et al.*, 2009). It has also been showed by the Bodmer lab that cancer stem cells preferentially maintain their undifferentiated state when exposed to a hypoxic environment and prevents differentiation of enterocytes and goblet cells by regulating *CDX1*, suggesting that the regulation of this gene is an important component of how hypoxia controls the switch between stemness and differentiation in cancer stem cells (Yeung, TM *et al.*, 2011).

Hypoxia is also a common feature of prostate tumours and has been associated with disease progression and treatment resistance (Marignol, LC *et al.*, 2008). Relapsed hormone-refractory prostate cancer cells highly express nuclear Hif1 α (Gravdal, K *et al.*, 2009), suggesting that the castration-resistant tumour cells exist in hypoxia. It has been shown that *HIF-1 α* is responsible for AR transactivation under hypoxic conditions and this process is androgen

dependent (Mitani, T *et al.*, 2011). AR negative prostate cells exhibit greater stem-like properties under hypoxic conditions (Ma, Y *et al.*, 2011). It has also been shown that hypoxia induces differentiation of AR negative neuroendocrine cells by inhibition of Notch signaling (Danza, G *et al.*, 2012). In this thesis, the functional effect of hypoxia on differentiation potential of the prostate cells is assessed using *in vitro* 3-dimensional cultures.

1.5 Mismatch Repair and prostate cancer

Combination of microsatellite instability (MSI) and studies on familial form of colorectal cancer (CRC) lead to the identification of mutations in the genes that are involved in mismatch repair (MMR). Approximately, 10-15% of sporadic CRCs and over 90% of HNPCCs are characterized by high-level of microsatellite instability. It has been shown that inactivation of the mismatch repair genes is responsible for the RER+ (replication error) phenotype in hereditary non-polyposis colorectal cancer (HNPCC) and sporadic RER+ colorectal cancer (Wheeler, JM *et al*, 2000). Though originally identified primarily in association with a predisposition to hereditary colon cancer, MMR defects have been identified in many other cancer types, including prostate cancer (Chen, Y *et al*, 2001, Beltran, H *et al*, 2013, Jarzen, J *et al*, 2013)

The primary function of the mismatch repair system is to eliminate base mismatches and insertion/deletion loops occurring as errors in the process of DNA replication. Microsatellites are regions of DNA consisting of short base pair repeats and an insertion/deletion of bases at microsatellites is referred to as MSI. MSI is a hallmark of a defect in MMR system in the cells. The MMR system is made up of 3 heterodimers: MSH2/MSH6 and MSH2/MSH3 heterodimers are responsible for recognition of base pair mismatches and insertion-deletion loops, whereas MLH1/PMS2 promotes the repair process. Lack of any of the MMR protein, mainly MSH2, MLH1 or MSH6, results in replication error (RER) and increased mutations (Peltomaki, P. *et al*, 2003). Genomic instability is caused due to elevated rates of mutations, but is not a requirement for tumour

development. However, mutations that provide a selective advantage against apoptosis or for increased growth are essential for tumourigenesis.

RER and the resulting MSI have been studied in depth in colorectal cancer where epigenetic silencing or mutation of *MLH1* and less frequently, *MSH2* or *MSH6* has been identified as the cause of the defective mismatch repair (Peltomaki, P *et al.*, 2003). However, microsatellite analysis in prostate cancer primary tissues has revealed that MSI frequency in prostate tissues is considerably lower than in colorectal cancer (**Table 1.2**). Also, the involvement of MMR proteins in prostate cancer may differ from that found in CRC, in the type of proteins, protein defects involved and the type of causative mutations. The expression levels of MMR genes are mainly studied by immunohistochemistry and hence, are not very reliable (Peltomaki, P *et al.*, 2003, Yeh, CC *et al.*, 2001). In this thesis the MSI status of the prostate cell lines is studied and the expression levels of MMR genes are analysed to determine which MMR genes are associated with MSI in prostate cancer.

	Number of patients	Instability rate (%)
Terell <i>et al.</i> 1995	40	2.5
Azzouzi <i>et al.</i> 2007	50	4
Crundwell <i>et al.</i> 1999	72	19
Egawa <i>et al.</i> 1995	66	19.7
Uchida <i>et al.</i> 1995	24	37.5
Watanabe <i>et al.</i> 1995	21	43

Table 1.2: List of different MSI rates reported in various publications. All the above studies have been carried out on prostate tissues obtained from patients.

1.6 The cell line model of prostate

There are potentially several advantages to using cell lines as a model to study differentiation. Cell lines can be readily characterized with respect to the mutational content and mRNA expression (Liu, Y *et al.*, 2006). They also represent a valuable resource that can be re-used repeatedly over time, unlike primary tissue. A meta-analysis has found cell lines to be a useful model because of inevitable stromal contamination in primary tissues (Volchenboun, SL *et al.*, 2009). An analysis of copy number variations in colorectal cancer also found a high degree of similarity between primary tumours and associated derived cell lines (Douglas, EJ. *et al.*, 2004), while epigenetic assessments of cell line pairs, derived from the same tumour but cultured separately for years, have found broad similarities between the lines (Dikomitou, O. *et al.*, 2009). There has been a debate as to whether *in vitro* studies truly reflect the *in vivo* nature of tumour growth, in the absence of an extracellular matrix and vascular supply. However, it is becoming clear that cell lines are not homogeneous and they can represent the tumour heterogeneity of the primary tissue. The cell lines have also proved to be extremely useful for carrying out functional studies in 3-dimensional culture, whereas it has not been possible yet to successfully grow and maintain the primary tumour material in 3-dimensional culture.

It has been relatively difficult to grow out cell lines out of the prostate. There are far fewer prostate cell lines as compared to the extensive colorectal cell line panel. However, a panel of 14 epithelial prostate cell lines has been compiled and used to carry out the findings stated in this thesis. These cells lines are obtained either from localized prostate cancer or different metastatic sites. Some of these

cell lines are also obtained by transforming cells obtained from a “normal” prostate.

Clearly, cell lines can serve as a useful model for studying cancer biology and can do so while providing flexibility in terms of treatment conditions and gross cancerous material available for experimentation.

Common experimental tools

In prostate and head and neck cancer cell lines, colonies have been divided into three subgroups based on their *in vitro* morphology on plastic: Holoclones, meroclones and paraclones. Holoclones are passageable from one generation to the next, can re-form all three subgroups and are tumourigenic using *in vivo* models, whereas meroclones and paraclones are unable to initiate tumours and eventually abort (Locke, M. *et al.*, 2005, Li, H *et al.*, 2008). However, this technique and terminology is no longer widely used and is not useful for determining the differentiation potential of the cell lines. To establish the differentiation potential of prostate cell lines, cells can be cultured in Matrigel™ (Richamn, P *et al.*, 1987, Clarke, RB *et al.*, 2005), an extracellular matrix-like mixture primarily made up of laminin and collagen IV that is secreted by the Engelbreth-Holm-Swarm mouse sarcoma cell line. Bodmer lab has characterized an extensive colorectal cell line panel in terms of the differentiation capacities using this technique. Each cell line gave rise to unique morphologies, but four common morphologies emerged (network, dense, lumen and intermediate). Since Matrigel™ more closely mirrors the *in vivo* environment experienced by cancer cells, assessing clonogenicity and anchorage-independent growth in a three dimensional growth matrix like Matrigel™ is likely to be more reflective of the true tumourigenic nature of

cultured cells versus soft agar or two-dimensional surface culture. Myofibroblasts or other tumour-associated cells can also be mixed into the culture matrix to assess their effects on differentiation and stemness potential of the cells. Hence, this approach was used to assess the characteristics and differentiation capacities of prostate cells.

To test the *in vivo* tumourigenecity of the prostate cell lines, the standard model is the animal xenograft. Cells are mixed with Matrigel™ and injected either subcutaneously or intraperitoneally in immunocompromised mice to assess tumour formation. NOD/SCID mice are the normal animals used, although the activities of B- and T-lymphocytes are not completely ablated. Although most xenografts are heterotopic, orthotopic xenograft protocols have been developed for certain cancers (such as pancreatic cancer) (Kim, MP *et al.*, 2009) to provide a microenvironment more like that of the original source of the primary tumour.

1.7 Aims of this thesis

Deranged and blocked differentiation is a key hallmark of cancer; and hence studying the differentiation properties of a cancer is important for understanding the basic biology of cancer and seeking new approaches to its treatment. By using a prostate cell lines panel, the experiments described herein attempt to investigate the role of NKX3.1 in regulating differentiation using 3D models. Another way to investigate the functions of NKX3.1 is to study its downstream targets. In summary, the primary aims of this thesis were as follows:

- 1.) To determine the differentiation potential of the panel of prostate cell lines
- 2.) To investigate the expression of NKX3.1 in prostate cancer
- 3.) To investigate the functional effect of overexpression and knock-down of NKX3.1
- 4.) To find the regulators of NKX3.1 using microarray expression data of the prostate cell lines
- 5.) To investigate the role of downstream targets of NKX3.1 in differentiation

Chapter 2

Materials and Methods

2.1 Reagents and Suppliers

Reagents and chemicals in this thesis were supplied from Sigma-Aldrich (UK) unless otherwise stated. Tissue culture flasks and plates were supplied from Corning (USA). All the cell lines were cultured in Gibco® cell culture media by Life Technologies.

When pre-assembled kits of reagents were used, all referenced reagents were from the named kit manufacturer.

2.2 Two dimensional cell culture

2.2.1 Cell lines

BPH-1 and BPH-1^{caftd1} were generous gifts from Professor Simon Hayward, Department of Urology, University of California-San Francisco. WPMY-1 and RWPE were purchased from American Type Culture Collection (ATCC). LNCaP_LN3 was obtained from Slav Ovtcharov, Department of Surgical Sciences, University of Oxford. PC3-NKX3.1 stably transfected cell line was a generous gift from Prof. Edward Gelmann, Department of Haematology/Oncology, Colombia University, USA. PNT1a cell line was a gift from Prof. Norman Maitland, Department of Biology, University of York, UK. All the other cell lines were obtained from the cryogenic storage of the Cancer and Immunogenetics laboratory (CIL), Weatherall Institute of Molecular Medicine. A summary of the origin and characteristics of these cells lines is given in **Table 2.1**

Cell line	Malignant/benign	Origin	Androgen sensitivity	Androgen Receptor (AR) mutation	PSA expressed
LNCaP	Metastatic	lymph node metastasis	Sensitive	T877A	Yes
LNCaP-LN3	Metastatic	LNCaP subline generated by serial passage in nude mice	Sensitive	not known	Yes
VCaP	Metastatic	vertebral metastasis	Sensitive	WT	Yes
DUCaP	Metastatic	dura mater metastasis	Sensitive	WT	Yes
PC3	Metastatic	vertebral metastasis	Insensitive	not known	No
PC3-NKX3.1	Metastatic	Derived by stably transfecting PC3 cells with <i>NKX3.1</i>	Insensitive	not known	No
DU145	Metastatic	brain metastasis	Insensitive	not known	No
22Rv1	Malignant	primary tumour-radical prostatectomy gleason grade 8 carcinoma	Sensitive	H874Y	Yes

Cell line	Malignant/benign	Origin	Androgen sensitivity	Androgen Receptor (AR) mutation	PSA expressed
M12	Derived from normal SV40T transformed cell line *metastatic in mice	derived from p69SV40T- which is established from normal epithelial cells	Insensitive	not known	No
M2182	Derived from normal SV40T transformed cell line *tumorigenic in mice	derived from p69SV40T- which is established from normal epithelial cells	Insensitive	not known	No
BPH-1	benign prostate hyperplasia-SV40T transformed	derived from a patient with benign prostate hyperplasia	Insensitive	not known	No
BPH-1_{caf}	benign prostate hyperplasia-SV40T transformed	derived by recombining BPH-1 cells with Carcinoma associated fibroblasts (CAF)	Insensitive	not known	No
RWPE	Normal immortalized with HPV18	normal epithelial cells of a patient undergoing cystoprostatectomy	Sensitive	not known	No
PNT1a	Established by immortalization of normal adult prostatic epithelial cells by SV40T	Established by immortalization of normal adult prostatic epithelial cells by SV40T	Insensitive	Not known	No

Table 2.1 Properties of the prostate cell lines held in the Bodmer lab. Cell lines derived from the same patient are identified by the same colour in column 1.

2.2.2 Culture conditions

BPH-1 and BPH-1^{caftd1} were cultured in RPMI1640 medium supplemented with 5% Fetal bovine Serum (FBS) (Invitrogen, Nivelles, Belgium) and 1% Penicillin/Streptomycin (P/S) (Invitrogen, USA). LNCaP, LNCaP_LN3, PC3, M12 and M2182 cell lines were cultured in RPMI medium supplemented with 10% FBS and 1% P/S. PC3_NKX3.1 cells were cultured in 10% FBS and 1% P/S RPMI media with 1.2mg/mL Geneticin (G418, Invitrogen, USA). DU145, DUCaP and VCaP were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% FBS and 1% P/S. RWPE cells were grown in Keratinocyte medium supplemented with Bovine Pituitary Extract (BPE) and Epidermal Growth Factor (EGF) (Life Technologies Limited). All cells were routinely grown in T75 (75 cm² growth surface) vented tissue-culture-treated flasks with 10 mL of medium.

2.2.3 Cell culture maintenance

Cells were cultured in flasks in DMEM or RPMI. Once DMEM- and RPMI-cultured adherent cells reached approximately 70-80% confluence, cells were passaged into new flasks by washing cells with 10 mL absolute phosphate buffered saline (PBSA) and detached by incubating cells with 2 mL trypsin/EDTA solution for 5 minutes (min). Trypsin was inactivated by adding 10 mL of growth medium. Subsequently, cells were harvested by centrifugation at 1,500 rpm for 5 min and resuspended in 10 mL medium for subculturing. Based on cell line growth characteristics, cell suspensions were divided into new flasks at a 1:5 to 1:10 dilution.

2.2.4 Cell counting

10 µL of resuspended cells were loaded onto a disposable cell counting chamber and inserted into a Cellometer Auto T4 by Nexcelom Biosciences, USA. 8 Images were taken by the software, in different areas of the cell counting chamber and the concentration of cells per mL was calculated.

Cells were occasionally also counted manually using a haemocytometer and a light microscope Oplympus CK2, Olympus Optical Co. UK limited. A coverslip was moistened and fixed on the haemocytomter. Equal volumes of 0.4% trypan blue stain and a well-mixed cell suspension were mixed. Trypan blue is a viability stain, hence all dead cells stain blue whereas the live cells appear colourless. 20µL of the trypan blue/cell mixture was pipetted from the edge of the coverslip and allowed to run under the coverslip by capillary action such that there would be no air bubbles. The haemocytometer was then mounted on the microscope stage and all the cells (stained and unstained) within the 20X20 small squares were counted using a 10X objective. To calculate the cell concentration per ml:

$$\text{Total number of cells/mL}$$

$$= \text{Average number of cells counted} \times \text{dilution factor} \times 10^4$$

Calculation of cell viability:

$$\% \text{ Viability} = \frac{\text{number of viable cells counted} \times 100}{\text{total number of cells}}$$

2.2.5 Cell storage and retrieval

Cells growing in culture were detached by trypsinizing and centrifuged at 1500rpm for 5 minutes. The cell pellet was then resuspended in appropriate volume of freezing media (10% dimethyl sulfoxide in FBS) so that the final concentration of the cells was 1.0e6 cells/mL. The resuspended cells were aliquoted in cryogenic vials (Corning, USA) and put on dry ice for about 2 hours before transferring them into cryogenic tanks with liquid nitrogen at -150°C for long term storage.

To thaw the cells, frozen cryogenic vials were incubated in a 37°C water bath for ~1 min. 10 mL of respective serum free media was added to the thawed cell culture and was transferred into a T75 flask.

2.2.6 Mycoplasma contamination testing

Tissue culture cell lines were regularly tested for Mycoplasma contamination using MycoAlert Assay kit, Lonza, Maine, USA. The MycoAlert assay is a selective biochemical test that detects the activity of specific mycoplasmal enzymes, which react with the MycoAlert Substrate catalyzing the conversion of ADP to ATP. By measuring the level of ATP in the sample both before and after the addition of the MycoAlert substrate, a ration can be obtained that is indicative of either the presence or absence of mycoplasma. The increase in ATP is detected using bioluminescence.

Cells for testing were in culture for at least 48 hours. A 2ml aliquot of medium from the cell line to be tested was transferred into falcon tubes and centrifuged at 1000rpm for 10 minutes. Using a 96 well plate, 100µL of positive control, 100µL of negative control and 100µL of each sample to be assessed were plated

into separate wells. 100 μ L of reagent were added to each well and then background fluorescence (reading A) was measured using BMG LABTech Luminometer (Offenburg, Germany) and FLUOstar OPTIMA software. 100 μ L of substrate were next added to each well, incubated in the dark for 10 min, and then fluorescence was measured (reading B). The cells were considered to be negative of mycoplasma infection if the ratio of reading B to reading A was less than 1. Cultures yielding a ratio between 1 and 2 were rested for a week and retested for mycoplasma contamination and those with ratio >2 were discarded.

2.2.7 Transient transfection with siRNA

The *NKX3.1* expressing prostate cell line, 22Rv1 was transfected with either a scrambled siRNA sequence as a control or 2 different *NKX3.1* siRNAs purchased from Sigma-Aldrich (siRNA-Nkx3.1-2) and Eurofins MWG Operon (siRNA-Nkx3.1-2). The *NKX3.1* siRNA was predesigned and validated by both the companies. The clone that has the greatest reduction in *NKX3.1* was selected for further study. siRNA against *HOXB13* was purchased from Santa-Cruz Biotech. siRNA mediated knockdown of *HOXB13* was carried out in 22Rv1 and PC3-Nkx3.1 cells.

2.2.8 Transient transfection of NKX3.1 in cell lines

The *NKX3.1* non-expressing cell lines PNT1a and PC3 were transiently transfected with either a full length *NKX3.1* cDNA cloned in an expression vector *pcDNA3.1* under a CMV promoter. This expression vector was a gift from Prof. Edward Gelmann, Division of Oncology, Columbia University Medical Center. The transfection was performed using Lipofectamine® 2000 Transfection Reagent as

described above. Post-transfection, the cells were subjected to 1.2mg/mL Geneticin (G418, Invitrogen, USA) selection. The vector map of *pcDNA3.1* is shown in **Figure 2.1**

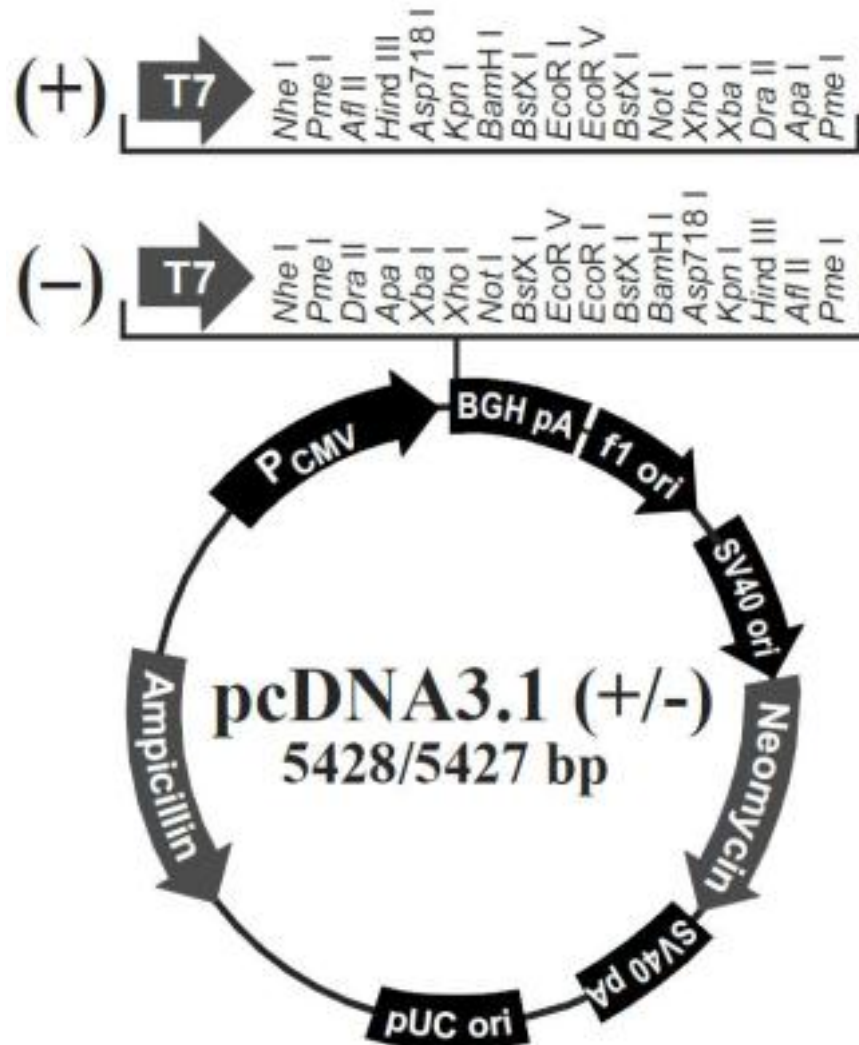


Figure 2.1

Vector map of *pcDNA3.1* full length *NKX3.1* cDNA is under control of a CMV promoter and Neomycin selection.

2.3 Cell growth analysis

2.3.1 Proliferation assays

The proliferation capacity of the cells was assessed by cell counting using Cellometer Auto T4 by Nexcelom Biosciences, USA. In brief, cell suspensions were plated in triplicate into wells of a 6-well plate to yield a final cell count of 100,000 cells per well and overlaid with 500µL of media (RPMI with 10% FBS and 1% P/S for PC3 cells and RPMI with 10% FBS, 1% P/S and 1.2mg/mL of G418, Geneticin®, Life Technologies- Invitrogen. After incubating the plate for 24 hours at 37°C in a humidified chamber, the cells were washed with PBS, trypsinized and re-suspended in fresh media and immediately counted using Cellometer Auto T4. The same process was repeated every 24 hours for 10 days. The doubling times were calculated when cells were in their exponential phase in the growth curve using Microsoft Excel.

2.3.2 Colony morphology assay in 3D

Cell suspensions were filtered through CellTrics® 20µm filters to avoid clumps of cells and obtain single cell suspension. The cell density of the suspension was determined using Cellometer and the cell suspension was diluted to obtain a cell density of 1.33e4 cells/mL.

Matrigel™ is a mixture of basement membrane proteins (including laminin and collagen IV) secreted by the Engelbreth-Holm-Swarm (EHS) mouse sarcoma cell line that was thought to mimic the constituents of extracellular matrix (ECM) (Kleinman and Martin, 2005).

Collagen I growth factor was prepared from stock by combining 1mL 10X PBSA and 1 mL sterile water with 8mL collagen I. Solution pH was adjusted to 7.4 with

5 M sodium hydroxide and sterilized using a 0.22 μm filter (Sartorius Stedium, Aubagne, France).

25 μL of Matrigel™ was spread in a 96 well plate to create a Matrigel™ bed. 75 μL of cells in complete RPMI media were added to 75 μL of 1:1 Matrigel™:collagen mix and aliquoted on top of the matrigel bed such that the final cell count in every well was 1000 cells. This mix was allowed to set at 37°C for 30 minutes. Adding collagen I stiffened the resulting gelatinous matrix and made it easier to handle the mixtures. 150 μL of media was then added to the well and changed twice in a week. The colonies were allowed to grow for up to 14 days and were then analyzed using Ziess Observer Z1 microscope and the representative images were captured using software Zen 2011 developed by Carl Ziess AG, Germany. The captured images were analyzed using FIJI sotware.

2.4 DNA methods

2.4.1 DNA extraction

DNA was extracted from the cell lines using Qiagen DNeasy Blood and tissue kit, following the manufacturers protocol. In brief, 5e6 cells were resuspended in 200µL of PBSA. Cell lysis was carried out by adding 20µL Proteinase K solution and 200µL of Buffer AL and incubating at 56°C for 10 min. Liberated DNA was precipitated with 200µL of 70% ethanol and bound to a silica spin column which was then washed by 500µL of Buffer AW1 and AW2. The bound DNA was eluted from the column with 200µL Buffer AE.

2.4.2 DNA quantification

The extracted DNA was quantified using ND-1000 Spectrometer (Nano-Drop, USA). Optical density of 1 at 260nm (OD_{260}) of nucleic acid is equal to 50µg of DNA, 40µg of RNA and 33µg of oligonucleotides. DNA quality was determined by computing the ratios of absorbances at 260nm to 280nm ($OD_{260/280}$). An $OD_{260/280}$ of 1.8 or greater indicated high quality of DNA.

2.4.3 Primer design

The primers were designed using a free online software, Primer-BLAST, National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). All the primers were synthesized by Sigma Aldrich (UK).

2.4.4 Polymerase Chain Reaction (PCR)

The total volume of PCR reaction was 50 μ L with a final concentration of 1U of *AmpliTaq* Gold polymerase (Applied Biosystems, USA), 200 μ M of each dNTPs, 0.2 μ M of each primer, 1.5mM of MgCl₂, 100ng of DNA and water. The PCR cycle consisted of 95°C annealing temperature for 15 minutes followed by 35 cycles of denaturation at 95°C (30-60 seconds), annealing at respective temperatures (**Table 2.2**, 30-60seconds), extension at 72°C (30-60 seconds) and final extension step for 10 minutes at 72°C.

PCR for the microsatellite stability test was carried out with microsatellite primers labeled with a fluorescent dye [6FAM] (6-carboxyfluorescein) at the 5' end. To detect TMPRSS:ERG fusion, reverse transcriptase PCR was performed using cDNA from all cell lines as a template.

Microsatellite	Primer sequence	Annealing temperature (°C)
Bat 25	F: 5' TCGCCTCCAAGAATGTAAGT 3'	55
	R: 5' TCTGCATTTTAACTATGGCTC 3'	
Bat 26	F: 5' TGA CTACTTTTGACTTCAGCC 3'	52.5
	R: 5' AACCATTCAACATTTTAAACC 3'	
D11S488	F: 5' AGGCAATAGAGACCCTGTG 3'	52.5
	R: 5' GATGATGAATTGTTACTGAGAG 3'	
D20S82	F: 5' GCCTTGATCACACCACTACA 3'	54
	R: 5' GTGGTCACTAAAGTTTCTGCT 3'	
TMPRSS2:ERG	F: 5' CAGGAGGCGGAGGCGGA 3'	65
	R: 5' GGCGTTGTAGCTGGGGGTGAG 3'	

Table 2.2

Primer sequences and annealing temperatures of microsatellite markers and other primers used for PCR.

2.4.5 Gel electrophoresis

DNA fragments were resolved by agarose gel electrophoresis. Agarose gels of varying percentage (normally 1-2%) were prepared by dissolving an appropriate mass of electrophoresis-grade agarose (Invitrogen, USA) (normally 0.5-1.0 g) and 10 µL SYBRSafe™ (Invitrogen, USA) DNA stain in 50mL TBE buffer. The resulting solution was poured into a mold to set, placed in a Mini-Sub Cell GT electrophoresis unit (Bio-Rad Laboratories, Hercules, California, USA) and overlaid with TBE buffer. DNA samples were mixed with 6X orange loading buffer and were loaded on the gel alongside with a 1 kilobase (kb) DNA ladder (New England Biolabs). Samples were run for 60 min at a constant 90 V. Gels were imaged under ultraviolet illumination on a Gel Doc 2000 scanner (Bio-Rad Laboratories).

To obtain higher resolution and separation of small DNA fragments the samples were run on a 1.0mm 4-20% TBE gradient pre-cast polyacrylamide gels purchased from Life Technologies- Invitrogen, USA. These gels were on XCell SureLock™ Mini Cell (Invitrogen, USA). Separation of microsatellites on 4-20% polyacrylamide gels.

2.4.6 DNA purification from agarose gel

The DNA fragments resolved through gel electrophoresis were purified from the agarose gel using Qiagen QIAquick Gel Extraction kit. The gel was observed under UV light and the DNA fragment was excised from it. The weight of the gel piece was measured. Three volumes of Buffer PB were added to one volume of the gel piece and loaded on QIAquick spin column. The column was washed with

Buffer PE. The DNA bound to the column was finally eluted using 50µL of elution Buffer EB.

2.4.7 DNA analysis

The PCR product was then given for fragment size analysis to the in house DNA Sequencing Core Facility, WIMM on an ABI-3730 48 capillary sequencing machine (Applied Biosystems, foster City, California, USA) with supplied sequencing primers. The results files were analyzed using a fragment sizing software Peak Scanner™ Software v1.0, Life Technologies- Invitrogen.

2.5 RNA methods

2.5.1 RNA extraction

RNA extraction was carried out using Qiagen's RNeasy® Mini Kit following the manufacturer's protocol. A frozen cell pellet of 1-5 million cells were thawed and 600µL of lysis buffer RLT with 1% β-mercaptoethanol was added. The cell lysates were homogenized by passing into a 21-gauge needle and 2mL sterile syringe 15-20 times. Same volume of 70% ethanol was added and then the mixture was loaded onto a RNeasy® mini column. The Column was then washed with 350µL of wash buffer RPE. To avoid DNA contamination, 10µL of DNase and 70µL of RDD buffer from the RNase-free DNase set was added onto each column for on-column DNA-digestion. The mixture was incubated at room temperature for 15 mins, followed by 2 washes with buffer RPE. RNA was then eluted with 40µL of RNase-free water and stored in -80°C. RNA stored at -80°C and was used within 10 days so that it would still be stable.

2.5.2 Reverse Transcription

2µg of extracted total RNA was reversed transcribed to complimentary DNA (cDNA) by using Omniscript™ Reverse Transcriptase Kit (Qiagen, USA). In brief, a mixture of 1µM oligo dTs (Invitrogen, USA), 4 units Omniscript reverse transcriptase, 10 units of RNase inhibitor (Ambion, USA) and 0.5mM each dNTP were added to each sample, followed by incubation at 37°C for one hour. cDNA was either diluted for PCR or stored at -20°C for future use.

2.5.3 Quantitative Real Time PCR (qRT- PCR)

qPCR was carried out in triplicates using TaqMan® Gene Expression Assay (20X FAM dye-labeled) Kit, Applied Biosystems. TaqMan® Gene Expression Assay consists of a pair of unlabeled PCR primers and a TaqMan® probe with a FAM™ dye label on 5' end and minor groove binder (MGB) and nonfluorescent quencher (NFQ) on the 3'end.

The qPCR was performed using the manufacturer's protocol. In brief, TaqMan® Gene Expression Assay (20X), cDNA, TaqMan® Universal PCR Master Mix II (2X) and nuclease-free water were mixed to a final volume of 20µL per reaction. The qRT-PCR was performed in 7500 Fast Real Time PCR Systems machine, Applied Biosystems.

Relative expression levels of genes examined in this thesis were calculated as a relative normalization of gene transcripts of the samples, or gene of interests (GOIs) to multiple house keeping genes (HKGs) by using Comparative Ct method. The HKGs were selected from a list of genes suggested by Exiqon system including *glyceraldehyde-3-phosphate dehydrogenase (GAPD)*, *Hypoxanthine-guanidine phosphoribosyltransferase (HPRT)* and *Ubiquitin C (UBC)*.

For calculation, the mean and standard deviation of the threshold cycles (Ct) were used for normalization. The number of threshold cycles of GOI and HKG were converted to raw expression values within the set of samples by using the following formula:

$$\Delta C_T = C_{T \text{ target}} - C_{T \text{ reference}}$$

The raw expression values of GOIs were normalized to the geometric mean of

raw expression values of HKGs. The standard deviation of the ΔC_T was calculated from the standard deviations of the target and the reference values using the following formula:

$$s = (s_1^2 + s_2^2)^{1/2}$$

where s_1 = standard deviation of $C_{T \text{ target}}$ and s_2 = standard deviation of $C_{T \text{ reference}}$

The $\Delta\Delta C_T$ values were calculated using the following formula:

$$\Delta\Delta C_T = \Delta C_{T \text{ test sample}} - \Delta C_{T \text{ calibrator sample}}$$

Finally, the fold change relative to the calibrator sample was calculated using the following formula:

$$\text{fold change} = 2^{-\Delta\Delta C_T}$$

2.5.4 Microarray gene expression analysis

Details of RNA submission to the microarray facilities were performed and described in (Wong 2006). Ten micrograms of rRNA of each stable transfected cell line were sent to the Molecular Biology Core Facility to the Paterson Institute for Cancer Research for gene expression microarray analysis using Human Genome U133 plus 2.0 chips following ht emanufacturer's instructions (Affymetrix, USA). In brief, total RNA was converted to double-stranded cDNA. The cDNA was then cleaned using the GeneChip Sample Cleanup Module (Affymetrix, USA). Then *in vivo* transcription was carried out to synthesise biotin-labelled cRNA using Affymetrix IVT Labelling Kit. Subsequently hybridization was carried out and

gene expression data was normalized using Robust Multichip Analysis algorithm.

A fold change of 1.5 with 95% significance level was selected the threshold for the comparison between the paired cell lines.

2.6 Microscopy

2.6.1 Antibodies for immunofluorescence

AUA-1, a mouse anti-human EPCAM antibody and biotinylated AUA1, anti-human EPCAM was obtained from the monoclonal antibody service, Cancer Research UK (CRUK), London, UK. Mouse anti-human Cam5.2 was also obtained from monoclonal antibody service, CRUK.

Mouse anti-human Vimentin antibody (clone V9) was obtained from Dako (M0725). Mouse anti-human *NKX3.1* [4H4] (ab96482), mouse anti-human AR [AR441] (ab9474) and rabbit polyclonal anti-human cytokeratin 5(ab24647) were purchased from abcam®. Mouse monoclonal α -SMA (A2547) was bought from Sigma-Aldrich. $\alpha_2\beta_1$ integrin mouse anti-human antibody (3H1472) was purchased from Santa Cruz Biotechnology, Inc.

Secondary antibodies used were HRP conjugated goat anti-mouse or goat-anti rabbit. Visualization was achieved using tyramide AlexaFluor 488 or AlexaFluor 555 (Invitrogen, USA) together with UV excitation.

2.6.2 Paraffin embedded sections

Formaldehyde fixed paraffin embedded (FFPE) blocks of normal, benign and cancerous prostate tissue were obtained from the Nuffield department of Surgical sciences at the John Radcliffe Hospital, University of Oxford. These sections were then cut into 4 μ m thick unstained sections with haematoxylin and eosin stain (H & E) section. The materials used were approved by Oxfordshire Clinical Research Ethics Committee.

2.6.3 Antigen retrieval of paraffin embedded sections

Paraffin sections with normal or cancerous prostate tissue were deparaffinised in Histoclear I for 5 minutes followed by treatment with Histoclear II for 5 minutes. The sections were then rehydrated in 100% ethanol, 95% ethanol, 70% ethanol and 30% ethanol for 3 minutes each. Slides were then rinsed with deionized water. Some slides were stained with Haematoxylin and Eosin to assess cellular morphology and others were stained for immunofluorescence. For the latter, antigen retrieval was performed by boiling slides in 1X Dako Target Retrieval solution for 15 minutes and then letting it cool at room temperature for 30 minutes. Tissue sections were then encircled on the slide using a hydrophobic ImmEdge pen (Vector labs). The slides were blocked with 10% foetal goat serum (FGS) for 30 minutes and then incubated with primary antibody.

2.6.4 Immunohistochemistry of paraffin embedded sections or cultured cells

Cultured cells were allowed to grow on coverslips in 24 well plates until they were 80-90% confluent. The cells were washed with ice cold PBS and fixed with 4% paraformaldehyde for 10 minutes at 4°C. After two washes with ice cold PBS the fixed cells were permeabilized with Triton X-100 for 15 minutes at room temperature. Cells were then blocked with 10% FGS in PBS for 30 minutes at room temperature to avoid non-specific binding of the primary antibody. The blocking step was followed by incubation with primary antibody at room temperature for an hour or at 37°C for 30 minutes depending on the antibody used. The antibodies used were *NKX3.1* (1:200), AR (1:100), Cam5.2 (1:600), Phalloidin Rhodamine (1:200), $\alpha_2\beta_1$ integrin (1:100), CK5 (1:100), α -SMA

(1:600), Vimentin (1:500), AUA1 (anti-EPCAM) (1:200). After having incubated with the primary antibody, the cells were washed twice with 0.5% PBS-Tween (PBS-T) for 5 minutes each. The cells were then incubated with either goat anti-mouse or goat anti-rabbit secondary antibody (1:1000) for an hour at room temperature in the dark after which they were washed twice with 0.5% PBS-T. The coverslips were then taken out of the 24 well plates and mounted on a slide with a drop of Vectashield containing DAPI. The slides were observed using Ziess Observer z1, Carl Ziess and analyzed with Zen 2011 software also developed by Carl Ziess. Some slides were also examined with a Ziess LSM confocal microscope. The captured images were processed using an image software, FIJI.

2.6.5 In situ staining of colonies in Matrigel

A Matrigel™ bed of was made on the bottom of a 96 well plate with 25μL of Matrigel™ and allowed to set at 37°C for 15 minutes. 1000 cells in 75μL media were then suspended in 75μL of a 1:1 Matrigel:Collagen mix and was again allowed to set at 37°C for 30 minutes. Once set, the mix was topped with 100μL of media. Colonies were observed for up to 14 days after which they were fixed *in situ* with 4% paraformaldehyde in PBS for 5 minutes at 4°C. The wells were then gently washed twice with PBS for 5 minutes each followed by a PBS-T wash for 5 minutes. To avoid non-specific binding the colonies were blocked with 10% FGS in PBS. The colonies were then incubated with secondary antibody conjugated to HRP overnight at 4°C, followed by three 5 minutes washes with PBS-T. The visual signal as amplified by incubation with tyramide 488 Alexa Fluor kit (Invitrogen, USA) for one hour in the dark at room temperature, followed by three 5 minutes washes with PBS-T. Nuclei were then stained with

DAPI and serial images were obtained as Z-sections using a Ziess LSM confocal microscope.

2.7 Fluorescent Activated Cell Sorting (FACS)

2.7.1 Fluorescently labeled antibodies

Mouse monoclonal anti-human $\alpha_2\beta_1$ Intergrin obtained from abcam®, rabbit polyclonal anti-human cytokeratin 5 purchased from Santa Cruz Biotechnology, Inc. and mouse monoclonal anti-human cytokeratin 18 obtained from monoclonal antibody service, CRUK were used as primary antibodies. These primary antibodies were unconjugated and incubated with secondary antibodies namely goat anti-mouse Alexa488 and goat anti-rabbit PE-Cy5. The secondary antibodies were purchased from Invitrogen, USA. Alexa488 and PE-Cy5 were used so that the emission spectra of these fluorophores would not interfere with each other.

2.7.2 Fluorescent Activated Cell Analysis and Sorting

1×10^6 cells were washed with FACS buffer 1% FBS and 1% sodium azide in PBS and centrifuged at 1000 rpm for 5 minutes. The resulting pellet was re-suspended with the appropriate concentration of primary antibody in 500 μ L of FACS buffer and incubated for 45 minutes. The labeled cells were washed twice with FACS buffer and incubated with secondary antibody in the dark for half an hour. The cells were then washed twice with FACS buffer and re-suspended in 4% paraformaldehyde to fix the cells. CellTrics®20 μ m filter was used to obtain single cell suspensions. The cells were analyzed using a DakoCytomation Cyan

machine.

For a live cell sort, 1×10^7 cells were incubated with primary and secondary antibodies as mentioned about. Propidium Iodide (PI) was added to the cellular suspension. Cells were sorted with a MoFlo cell sorter (Beckman Coulter). Single cells were obtained by gating out cellular aggregates. PI staining was used to exclude dead cells.

Cells were sorted using the integrated Summit data collection software. At a forward scatter gain of 1.0 and a side scatter gain of 1.0 and voltage 500V, doublets and debris were excluded with the help of pulse width vs side scatter plot. At a 488nm blue laser gain of 1.0 and voltage 750V was used to detect Alexa488 fluorescence and a 633nm red laser gain of 1.0 and a voltage of 750V was used to detect PE-Cy5. Fluorescence logarithms were plotted to identify negatively and positively staining populations.

The top and bottom 1-5% of the fluorescence distributions were sorted and collected in 1mL of serum free media for subsequent experimentation.

2.8 Statistical analysis

All the data in the graph is presented as the mean values $\pm$ SEM. Fisher's test was performed on all the 2x2 contingency tables. For all statistical comparisons, $p < 0.0001$ was denoted by ****, $p < 0.001$ by ***, $p < 0.01$ by ** and $p < 0.05$ was denoted by *. GraphPad Prism Software (La Jolla, Californiaw, USA) was used to perform all the data analysis.

Chapter 3

Characterization of prostate cell lines

3.1 Introduction

Animal models are often beneficial for understanding the characteristics and mechanisms of tumour progression. However, they present significant drawbacks like lack of appropriate models for prostate cancer, ethical issues concerning use and treatment and housing costs. While fresh prostate human tissue is the best representation of a tumour, the low accessibility, slow growth rates and survival for limited passages makes it difficult to use it as a model. Cell lines, on the other hand, are easily obtained and it has been established that cell lines retain most of the characteristics of the tumour it was derived from and also represent its heterogeneity. A panel of prostate cell lines has been created with cell lines that mimic various cancer stages, conditions of cancer progression and tissue specific metastatic sites (lymph node metastasis, bone metastasis, etc.).

Prostate tumours are often characterized and assigned Gleason grade by the pathologists by the degree of organization of the cells, with tumours showing intercellular structure and polarity termed as “well-differentiated” and tumours showing little or no organization termed as “poorly-differentiated”. Interestingly, cell lines have also been shown to have the ability to differentiate into various structures when grown in three-dimensional matrices. A large-scale study focusing on breast cancer cell lines identified four different classes of colony morphologies based on the differences in cell-to-cell contacts and cellular polarity (Kenny, PA. *et al.*, 2007). The Bodmer lab has also classified an extensive panel of colorectal cell lines into different categories based on their differentiation capabilities. The growth behavior is influenced by biochemical

characteristics, and such characteristics have relevance to tumourigenesis. An assessment of expression of differentiation markers and growth behavior could prove useful to identify novel molecules that link not only to cancer stemness or differentiation, but also to colony morphology.

Microsatellite instability (MSI) has been linked with prostate cancer progression in various studies (Beltran, H. *et al.*, 2013). The increase in incidence of replication error (RER) as the cancer progresses from being highly differentiated to poorly differentiated tumour suggests that there might be a link between mismatch repair (MMR) and differentiation.

In this part of the study, the panel of prostate cell lines was characterized in terms of expression of *NKX3.1* and *AR*, the correlation of their expression, colony morphologies in 3D and their MMR status.

3.2 Results

3.2.1 mRNA expression of *NKX3.1* and *AR* in prostate cell lines

The mRNA expression of *NKX3.1* was determined by human genome expression array data obtained using Affymetrix- GeneChip® Human Exon 1.0ST Array. 13 prostate cell lines were screened for the mRNA expression of *NKX3.1* and the mRNA expression levels are shown in **Figure 3.1**. LNCAP_LN3 and 22Rv1 cell lines express intermediate levels of *NKX3.1* mRNA, LNCaP, DUCaP and VCaP express high levels of *NKX3.1* mRNA whereas DU145, RWPE, PNT1a, M12, M2182, PC3, BPH-1 and BPH-1^{caf} are negative for *NKX3.1* mRNA expression.

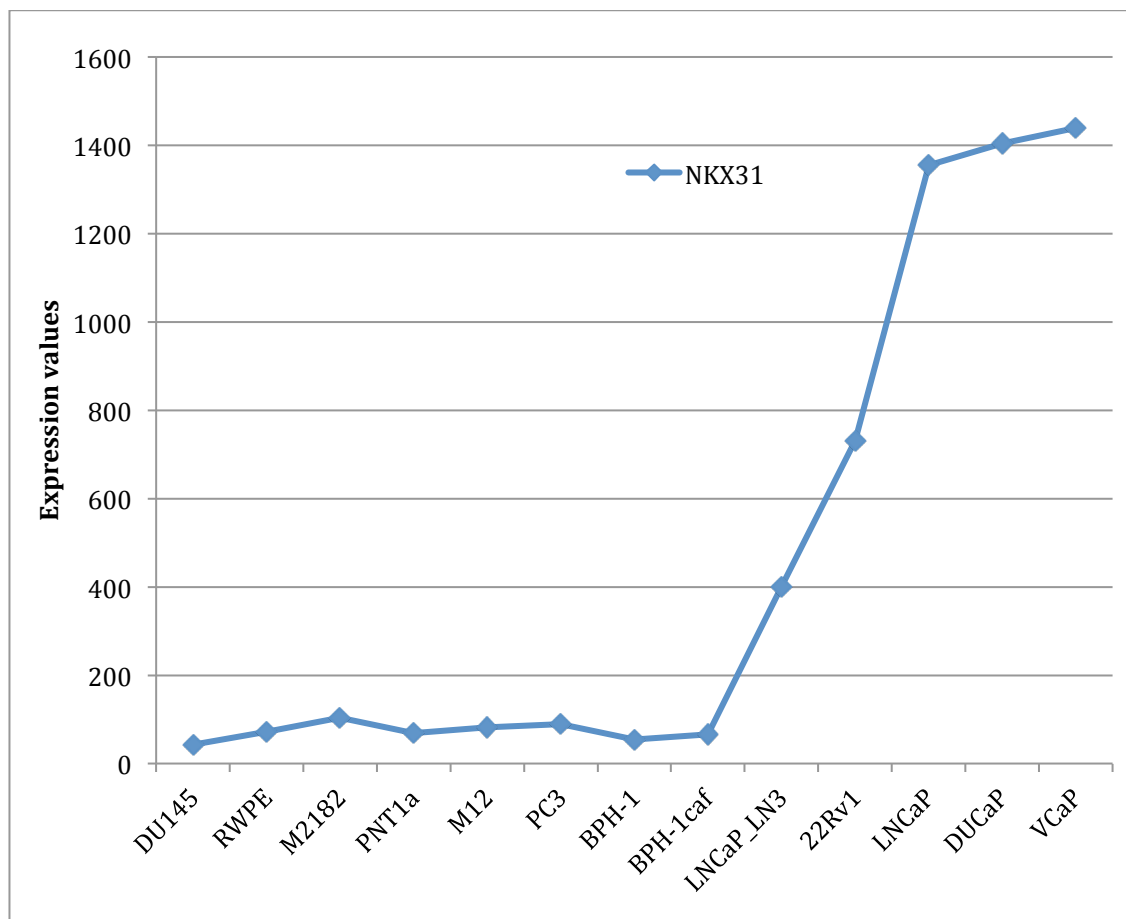


Figure 3.1

Microarray expression values of *NKX3.1* in 13 prostate cell lines.

For AR, 8 out of 13 cell lines were found to be negative and only 5 cell lines (LNCaP, LNCaP_LN3, 22Rv1, DUCaP and VCaP) express AR (**Figure 3.2**). AR expression has been lost in all of the transformed prostate cell lines, namely, BPH-1, BPH-1^{caf}, RWPE, M12 and M2182 cell lines.

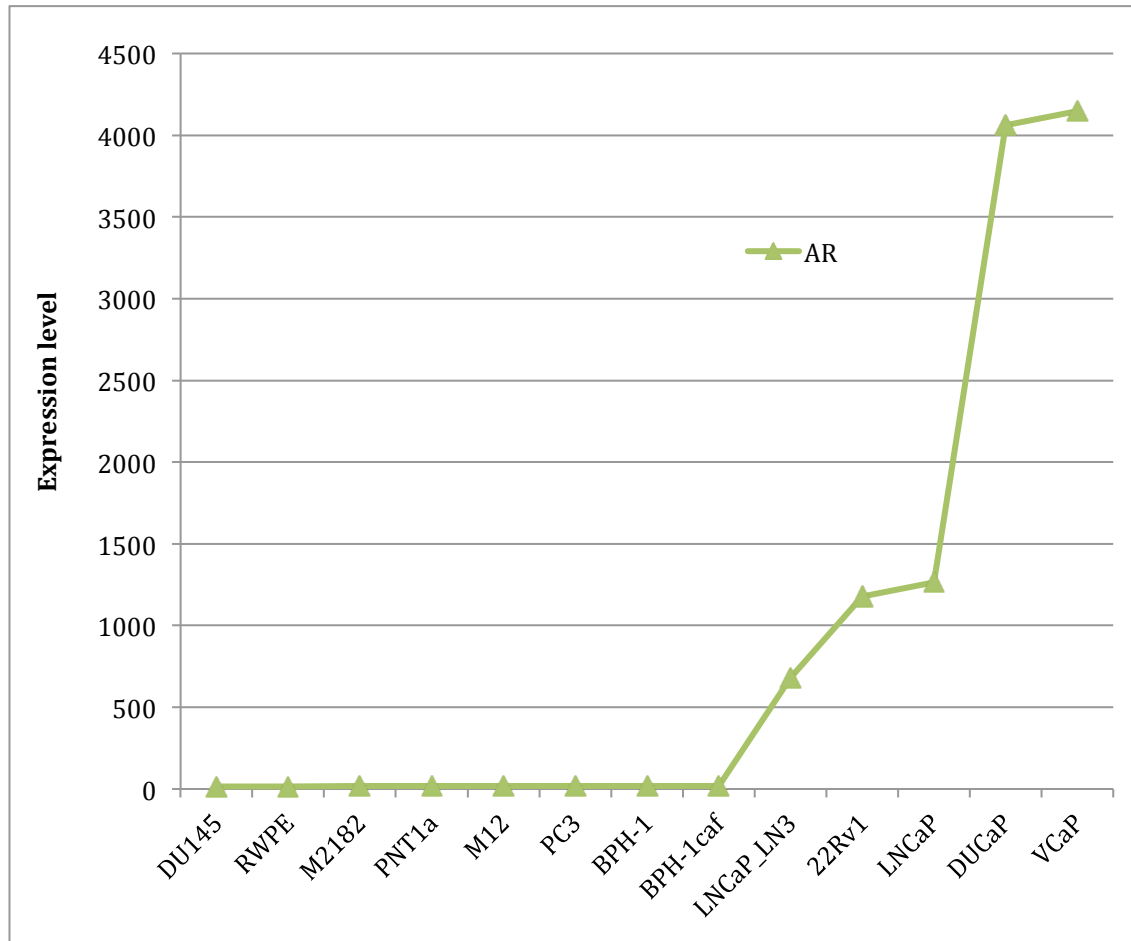


Figure 3.2

Microarray expression values of AR in 13 prostate cell lines.

Based on these expression data, the panel of prostate cell lines have been categorized in 3 different groups: low, intermediate and high. The categorization of the cell lines is shown in **Table 3.1**. VCaP, DUCaP and LNCaP are categorized as *NKX3.1* high; 22Rv1 and LNCaP_LN3 are categorized as *NKX3.1* intermediate and all the remaining cell lines are *NKX3.1* low.

VCaP and DUCaP are AR high; LNCaP, LNCaP_LN3 and 22Rv1 are AR intermediate and the rest of the cell lines fall into the AR low category.

Cell line	<i>NKX3.1</i> expression	<i>AR</i> expression
DU145	Low	Low
<i>RWPE</i>	Low	Low
<i>M2182</i>	Low	Low
<i>PNT1a</i>	Low	Low
<i>M12</i>	Low	Low
<i>PC3</i>	Low	Low
<i>BPH-1</i>	Low	Low
<i>BPH-1^{caf}</i>	Low	Low
<i>LNCaP_LN3</i>	Intermediate	Intermediate
<i>22Rv1</i>	Intermediate	Intermediate
<i>LNCaP</i>	High	Intermediate
<i>DUCaP</i>	High	High
<i>VCaP</i>	High	High

Table 3.1

Distribution of cell lines in three different categories based on their *NKX3.1* and *AR* expression level

3.2.2 Correlation of NKX3.1 and AR expression in prostate cell lines

It has been hypothesized that *NKX3.1* is associated with the AR transcriptional network by two distinct mechanisms: *NKX3.1* and AR regulate each other in a feed-forward loop, and *NKX3.1* collaborates with AR to regulate genes associated with prostate carcinoma. The mRNA expression levels from the microarray data reveal that there is a correlation between the expression levels of the two genes in the prostate cell lines (**Figure 3.3**). The regulatory loop of AR and *NKX3.1* appears to be intact in the panel of prostate cell lines.

DU145, RWPE, M2182, PNT1a, M12, PC3, BPH-1 and BPH-1^{caf} cell lines demonstrate consistency in null expression of AR and *NKX3.1*. The expression of AR in DUCaP and VCaP is almost 4 times higher than 22Rv1 and LNCaP cell lines. However, the difference in expression of *NKX3.1* is not as drastic.

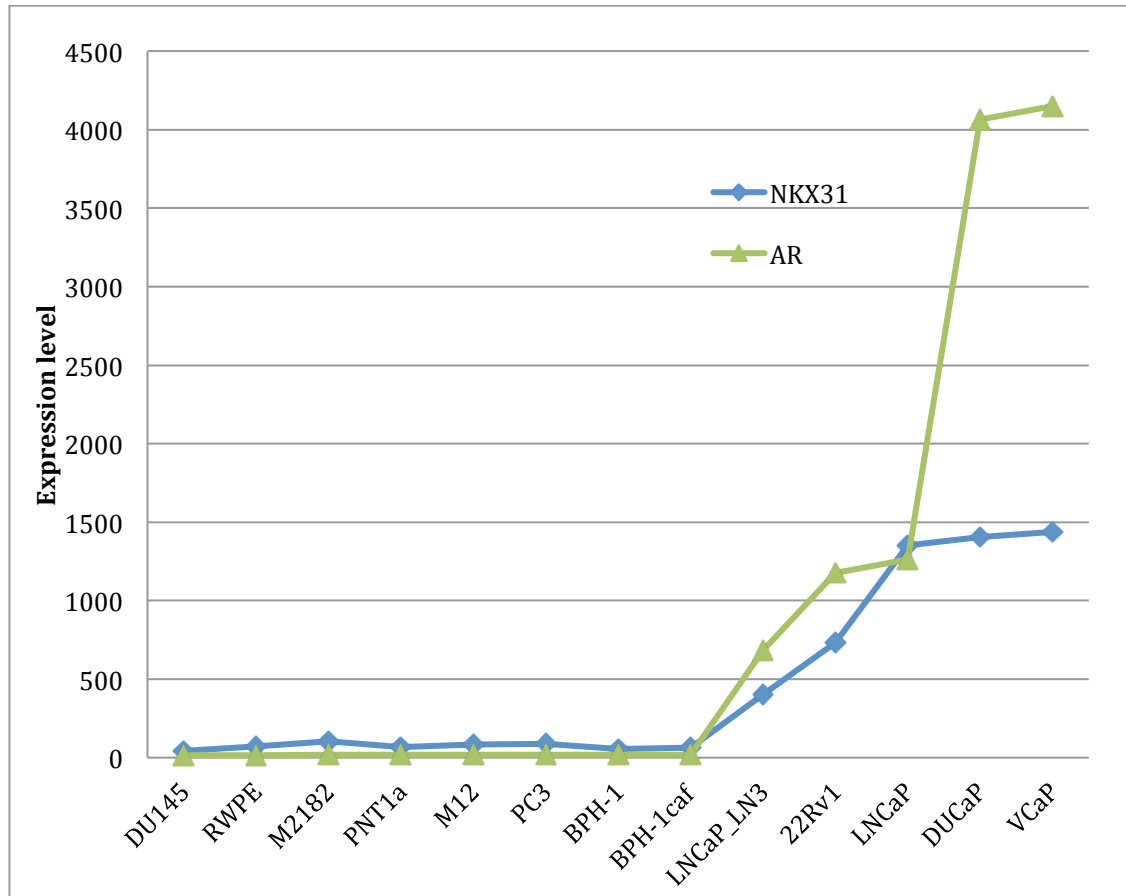


Figure 3.3

Correlation of expression levels of *AR* and *NKX3.1* in the panel of prostate cell lines.

LNCaP_LN3 is derived from LNCaP cell line by subcutaneously injecting LNCaP cells in mice. Tumours metastasised to the lymph nodes were harvested and cells were reinjected into the prostate. This cycle was repeated three times to yield LNCaP_LN3 cell line (Pettaway, C. *et al.*, 1996). LNCaP cells are non-metastatic *in vivo*, whereas LNCaP_LN3 cells are highly metastatic. Although there is a difference in *AR* expression levels in the two cell lines, the difference in expression of *NKX3.1* is more drastic and is reduced 3 fold in LNCaP_LN3 cells. The reduced expression of *NKX3.1* suggests that either the cells that metastasised in mice had reduced expression of *NKX3.1* or the expression of this gene reduced

after the cells metastasised. Hence, *NKX3.1* may have a key role to play in metastasis in prostate cancer.

3.2.3 NKX3.1 expression in normal and tumour prostate tissue

Normal and tumour tissue was obtained from patients and formalin fixed paraffin embedded on to slides. 24 different slides were obtained from 4 patients. 12 of these slides were prepared from normal tissue and 12 from prostate tumours. H&E staining of these slides is shown in **Figure 3.4**.

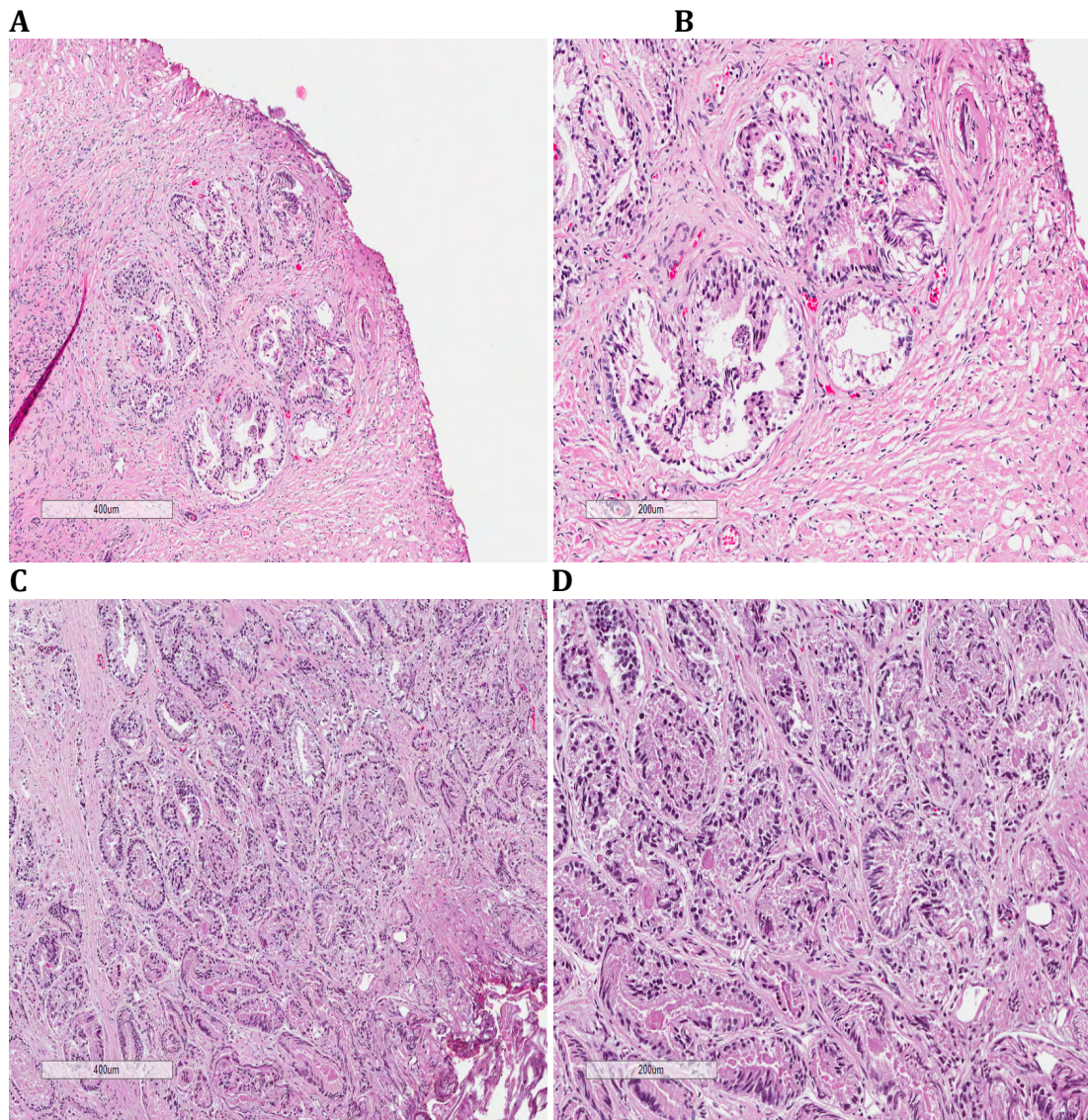
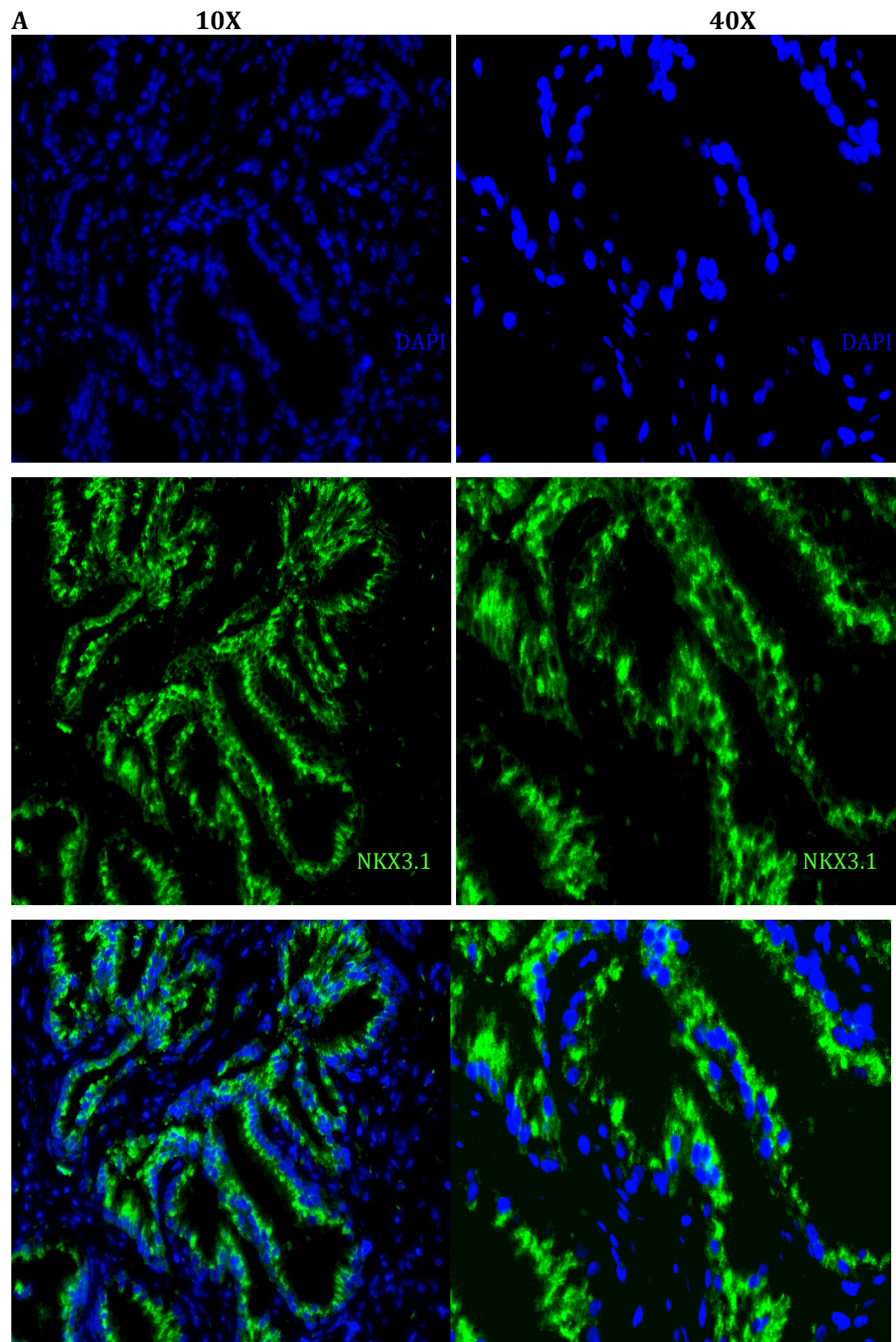


Figure 3.4

H&E staining of normal (A and B) and Grade 5 adenocarcinoma (C and D). Images A and C were captured at 5X microscopic magnification whereas images B and D were captured at 10X magnification.

Previous reports have shown that NKX3.1 is expressed in the prostate acini. NKX3.1 expression is known to be significantly reduced in prostate cancer patients compared to BPH (Aslan, G. *et al.*, 2006) and the loss of NKX3.1 is strongly associated with advanced tumour stage in prostate cancer (Bowen, C. *et al.*, 2000). It has also been reported that the expression of NKX3.1 is lost during prostate tumourigenesis in mice (Song, H. *et al.*, 2009).

The tissue samples were stained using a monoclonal mouse anti-human NKX3.1 antibody. The expression of NKX3.1 was found to be very strong in the prostate acini of normal tissue. NKX3.1 expression was mainly nuclear and perinuclear (**Figure 3.5A**). The prostate tissue obtained from a tumour and graded with a Gleason score of 5 was completely negative for NKX3.1 expression (**Figure 3.5B**). The acini appeared to be less differentiated in tumour tissue.



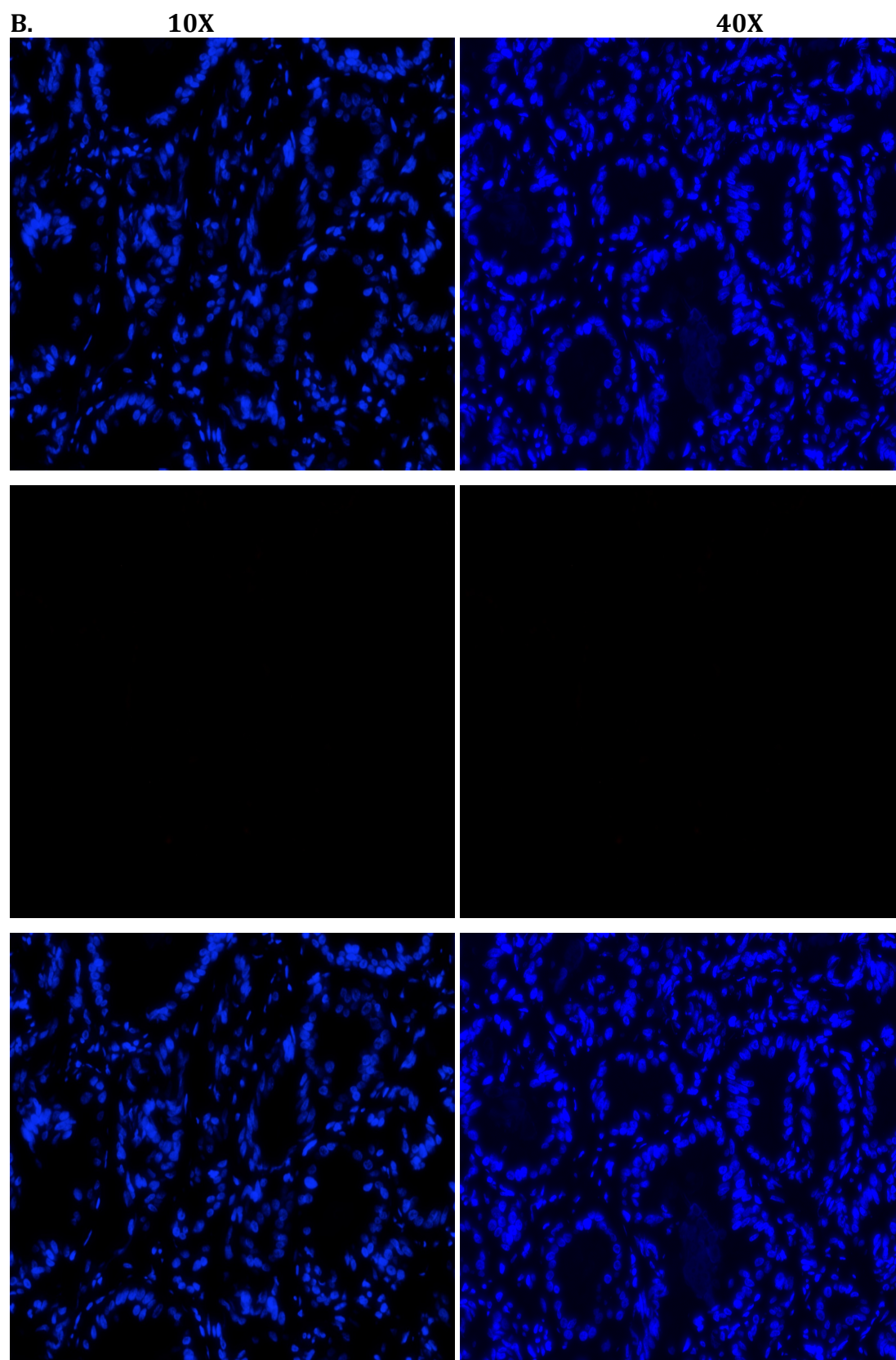


Figure 3.5

Representative images of immunohistochemical NKX3.1 staining of prostate tissues. **A.** Paraffin sections obtained from normal prostate tissues express NKX3.1 (green) and the acini. The expression of NKX3.1 is somewhat nuclear and mainly perinuclear. **B.** Paraffin sections obtained from a prostate tumour.

3.2.4 Regulation of NKX3.1 by ETS family genes

NKX3.1 expression is known to be regulated by ETS family genes. RT-PCR was performed to detect the presence of TMPRSS2:ERG fusion in the panel of prostate cell lines. Out of 13 cell lines tested, a PCR product of ERG translocation was detected only in VCaP and DUCaP (**Figure 3.6**). The expression of *TMPRSS2* is regulated by the transcriptional factor, AR. ERG translocation to form a TMPRSS2:ERG fusion leads to overexpression under the control of TMPRSS2 expression. The microarray expression data does show that ERG is overexpressed in the fusion positive cell lines, VCaP and DUCaP (**Table 3.2**). Interesting, VCaP and DUCaP are also the cell lines with highest expression of AR and NKX3.1.

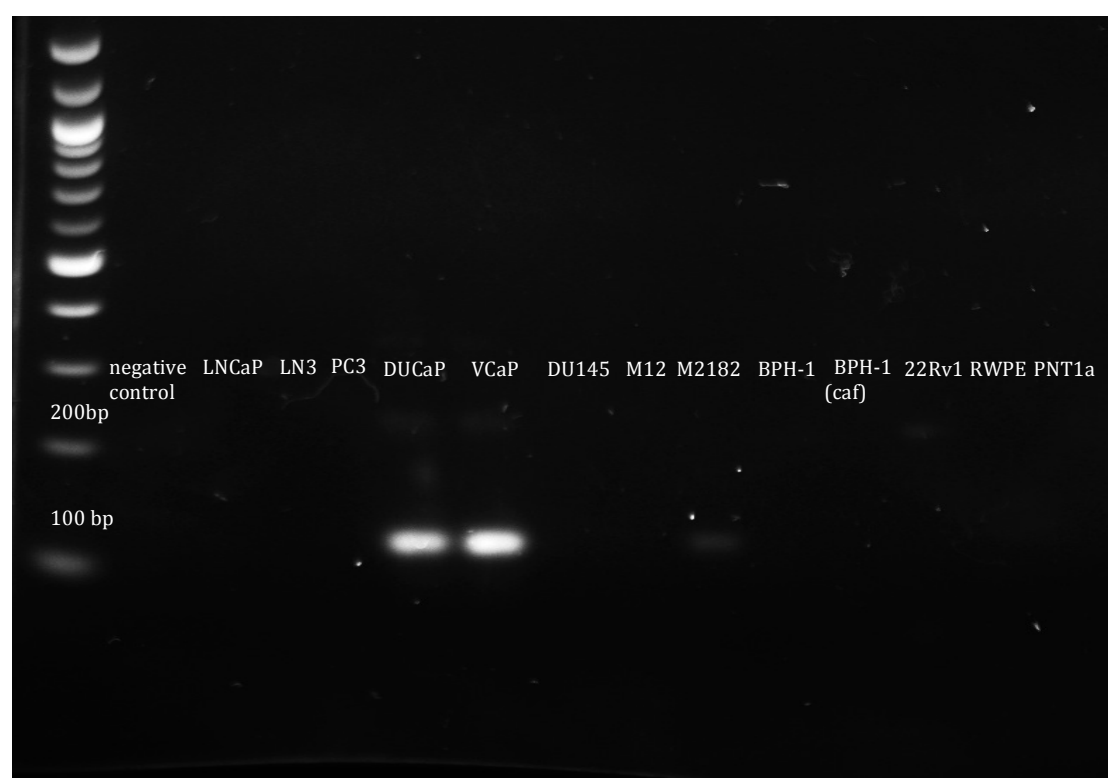


Figure 3.6

Expression of TMPRSS2:ERG (exon1 to exon4) fusion transcript detected with specific nested PCR primer sets for RT-PCR in prostate cell lines. Band sizes (bp) are indicated in white on the gel electrophoresis image.

It has previously been shown that higher ERG expression leads to suppression of *NKX3.1* expression facilitated by *EZH2* silencing (Kunderfranco, P. *et al.*, 2010). Interestingly, despite the report by Kunderfranco *et al.* describing transcriptional regulation of *NKX3.1* negatively via ERG through induction of polycomb gene *EZH2*, **Table 3.2** shows that the two cell lines (VCaP and DUCaP) with the highest levels of ERG show the lowest levels of *EZH2* and highest levels of *NKX3.1* (highlighted in pink). *EZH2* expression levels are somewhat similar to each other in the rest of the prostate cell lines. Hence, it can be said that *NKX3.1* down regulation in prostate cell lines is not because of silencing by *EZH2*.

Expression of ERG is neither sufficient to lower *NKX3.1* levels, nor is it required for the loss of expression of *NKX3.1*, because all 8 of the cell lines with low to negligible levels of *NKX3.1* do not express ERG, or have a *TMPRSS2:ERG* fusion.

	NKX31	EHF (ESE3)	ERG	EZH2	ELF3 (ESE1)	AR	TMPRSS2	TMPRSS2 :ERG fusion
LNCaP	1354.3	1435.7	19.4	161.0	44.8	1263.2	2193.6	no
LNCaP_L N3	400.9	1470.9	17.1	172.2	44.5	682.4	510.9	no
DUCaP	1403.5	1263.2	942.4	53.8	53.1	4062.9	1517.8	yes
VCaP	1439.2	1147.5	864.5	69.2	49.2	4148.9	1452.7	yes
DU145	42.3	410.1	15.3	102.5	38.1	15.4	83.9	no
PC3	89.5	20.8	17.6	166.3	49.4	17.0	14.8	no
M12	82.4	17.1	16.8	219.3	34.4	17.0	18.2	no
M2182	103.3	12.6	15.0	437.9	21.1	16.6	16.5	no
BPH-1	54.9	1291.0	23.6	282.2	50.2	17.7	18.3	no
BPH-1 ^{caf}	65.4	600.8	22.6	296.6	39.1	19.2	18.8	no
22Rv1	732.1	486.3	16.1	488.5	31.9	1176.9	150.4	no
RWPE	71.5	784.4	17.0	197.6	59.7	16.3	30.1	no
PNT1a	68.6	28.2	18.8	324.1	35.1	16.7	26.5	no

Table 3.2

Affymetrix Exon 1.0 ST microarray expression levels of ETS family genes in prostate cell lines. Expression levels of AR, AR regulated TMPRSS2 and presence/absence of ERG translocation are also shown. Gene expression levels are normalized absolute fluorescent intensities. For reference, expressions below 50 could be considered extremely low or absent.

High expression levels of ESE3 in LNCaP, LNCaP_LN3, VCaP and DUCaP cell lines correlate with high levels of NKX3.1. Kunderfranco, P. *et al.* also claimed that ESE3 activates *NKX3.1* transcription by repressing EZH2. From the expression profile of ESE3 and NKX3.1 in LNCaP, LNCaP_LN3, VCaP and DUCaP, it can be said that *NKX3.1* transcription maybe regulated by ESE3. However, the low expression levels of EZH2 in all the cell lines suggests that ESE3 may not play a role in down regulation of EZH2.

3.2.5 3D colony morphology assay as a measure of differentiation

A panel of 10 prostate cell lines was selected to assess the diversity of growth characteristics and colony morphologies in a three-dimensional matrix. The cell lines 22Rv1, LNCaP, LNCaP_LN3, DU145, PC3, PNT1a, VCaP, DUCaP, M12, M2182, BPH-1, BPH-1^{caf} and RWPE were selected because they demonstrate the diversity of prostate cell lines, thus recapitulating the heterogeneity of primary normal and cancer prostate tissue or at least an element of the normal range on variation in prostate cancers.

To characterize the differentiation capacities of the above cell lines, they were grown in a mixture of Matrigel™ and collagen I. Three-dimensional growth has been widely assessed in soft-agar, but the polysaccharide matrix does not reflect the true components of the extra-cellular matrix (ECM). Cell attachment to the ECM occurs mainly through Integrins and this attachment regulates proliferation, migration and differentiation. Matrigel™ is a rich mixture of ECM proteins and growth factors and its viscoelasticity allows for branching morphogenesis of epithelial cells. However, on its own Matrigel™ is gelatinous and soft in nature, which makes it difficult for the cells to attach. Mixing collagen I with Matrigel™ results in a stiffer matrix that could facilitate integrin interaction and enhance colony formation.

Cell lines demonstrated different growth rates when grown in Matrigel™:collagen I mix. Depending on the growth rate of the cells, cell lines were incubated in the mix along with serum-containing media for 1-2 weeks, after which they were imaged to assess the size, shape and organization of colonies. Though each cell line gave rise to unique colonies there were some common colony morphology features (**Figure 3.7**). There were five distinctive

structures that emerged- lumen, dense, spheroid, stellate and intermediate. These subgroups were characterized by intracolony organization, polarization and colony densities.

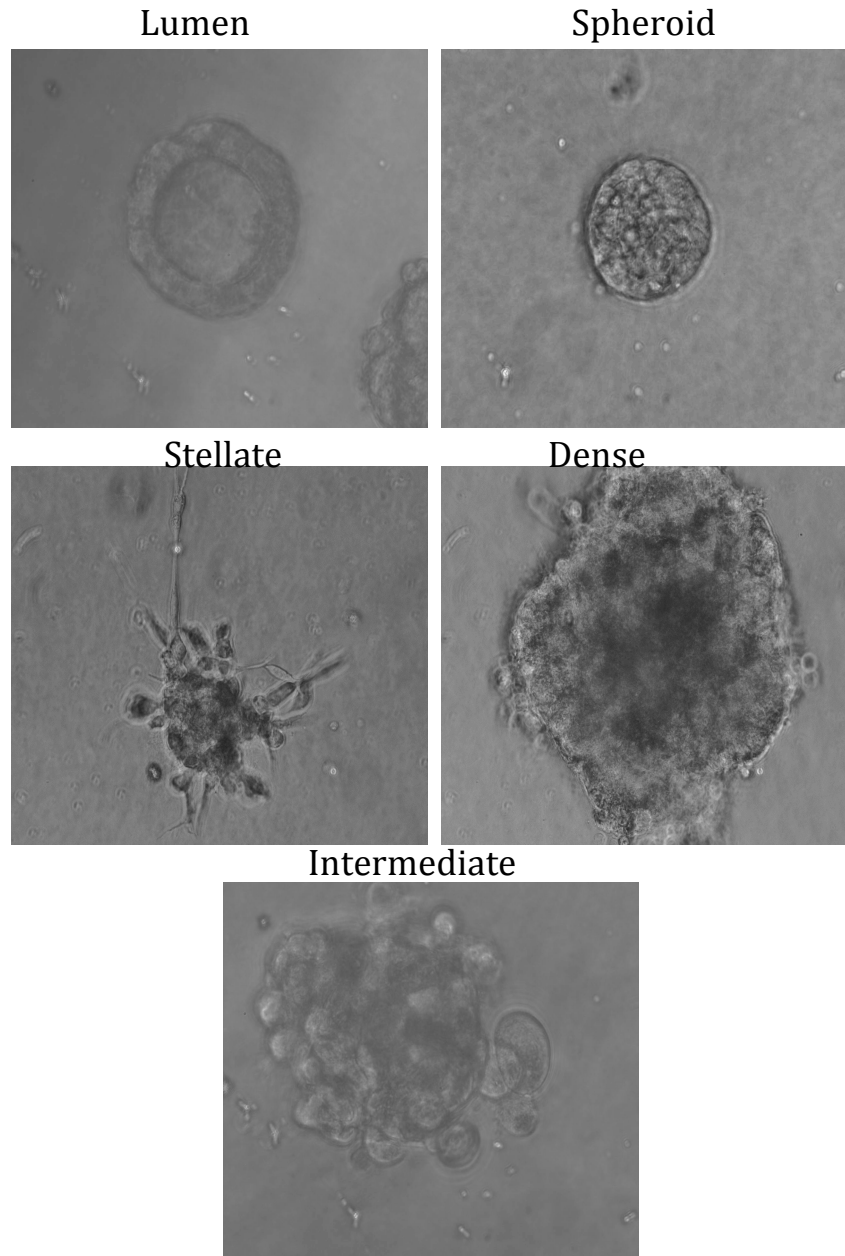


Figure 3.7

Phase contrast 10X images of 3-D growth cultures of cell lines illustrating organized and polarized lumen morphology (22Rv1), dense morphology (BPH-1), spherical morphology with tight well defined borders (RWPE), fibrous and non-spherical stellate morphology (M12) and diffused and undefined intermediate morphology (PC3).

The lumen colony morphology was characterized by polarization of cells to form a lumen in the center of the colony. The lumen and the colony had well defined borders and strikingly resembled the structure of normal prostate acini, where a layer of epithelial cells polarizes to form a hollow and release secretory proteins into the luminal space.

The spheroid colonies were the second most differentiated colonies after lumen forming colonies. These colonies had extremely well defined borders, exhibited cell polarity and high lateral cell adhesions. However, no lumens were observed in the middle of the colony.

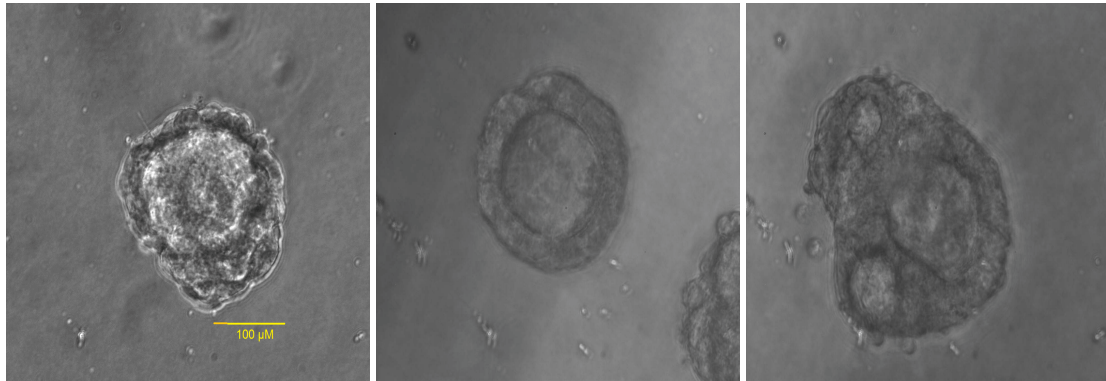
Some cell lines gave rise to dense colonies with undefined borders and densely packed interiors. All the cells in a dense colony were undifferentiated and no difference was observed in cell morphologies. There was a complete lack of cell polarity. The dense colonies tended to be the largest of all other colony types, implying higher levels of proliferation.

Intermediate colonies had somewhat loosely defined borders but did not have any stellate cell protrusions. Cell density within the colony was significantly lower than the dense colonies and showed some signs of polarity. Heterogeneity in cell morphology was also observed. These colonies tended to quite variable in size. These colonies can be categorized as more differentiated than dense colonies but less differentiated than the lumen forming colonies.

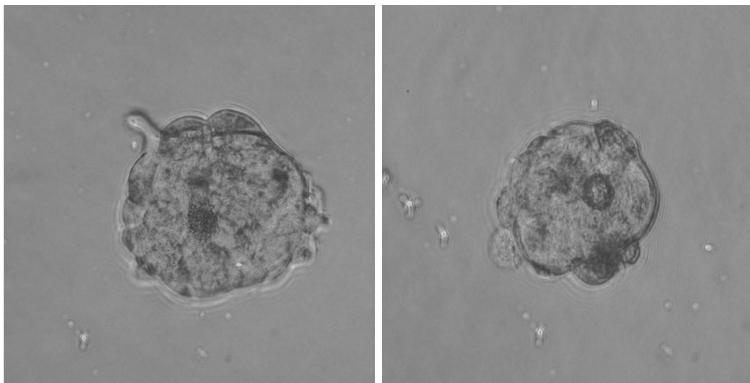
The stellate colonies had protrusions of fibrous structures formed by a network of cells. This network of cells branched out of the colony from the clonal cell of origin and did not exhibit any polarization.

Cell lines were categorized according to their colony morphologies based on their most dominant morphology (**Figure 3.8**).

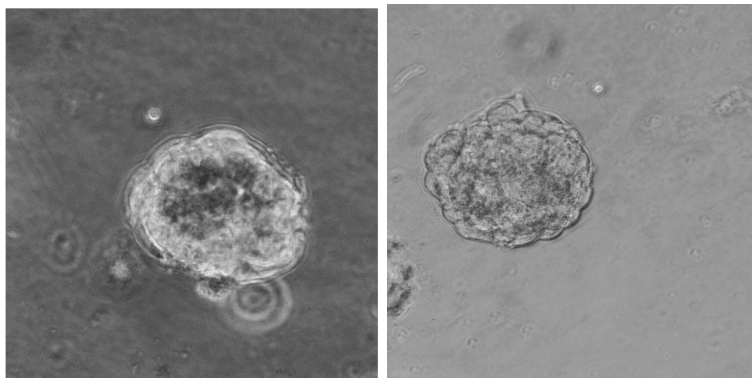
A.
22Rv1



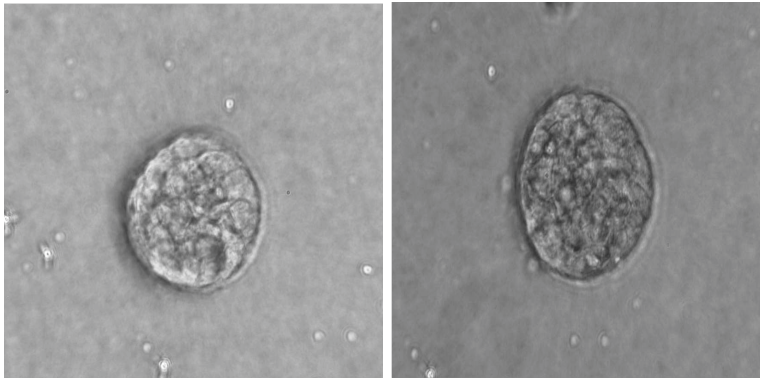
LNCaP



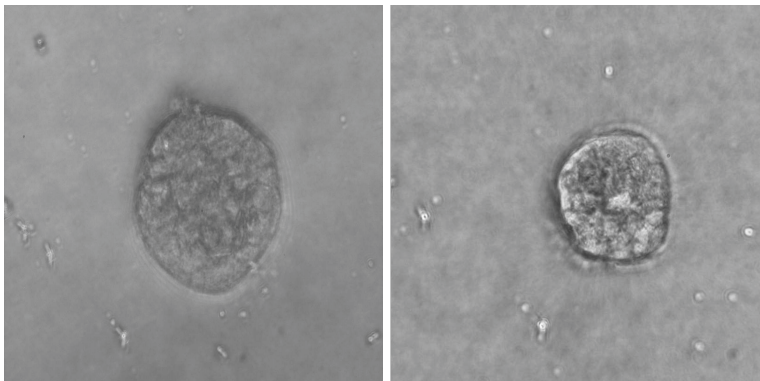
LNCaP_LN3



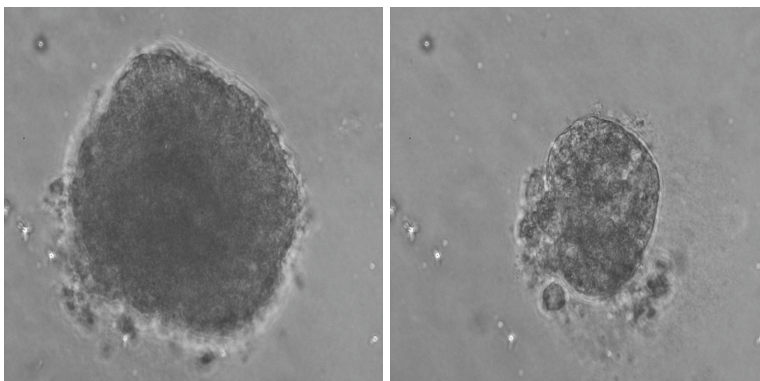
B.
RWPE



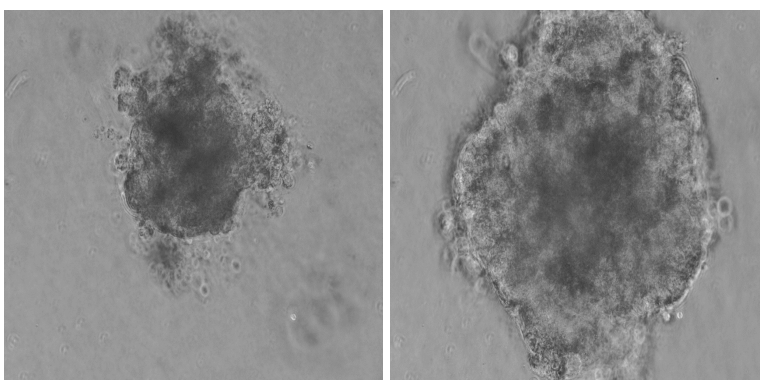
DU145



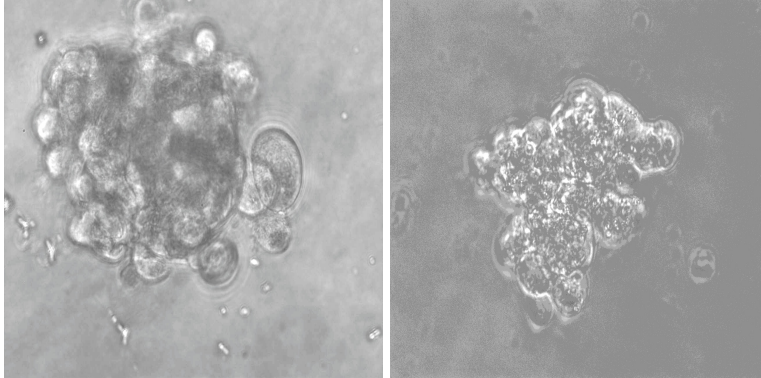
C.
BPH-1



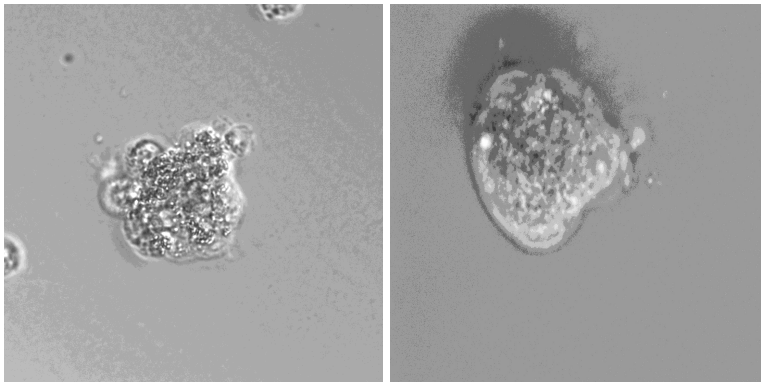
BPH-1^{caf}

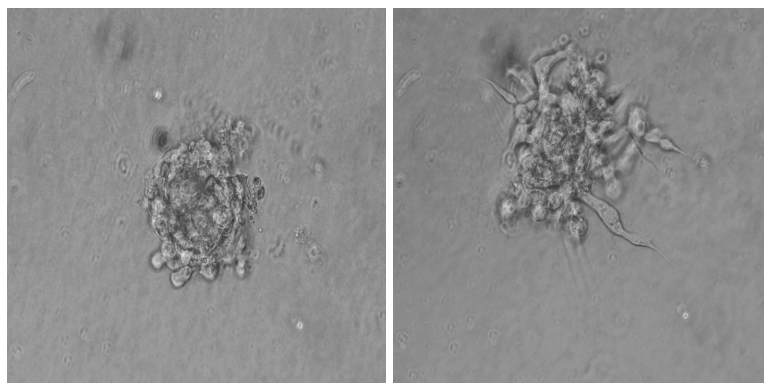
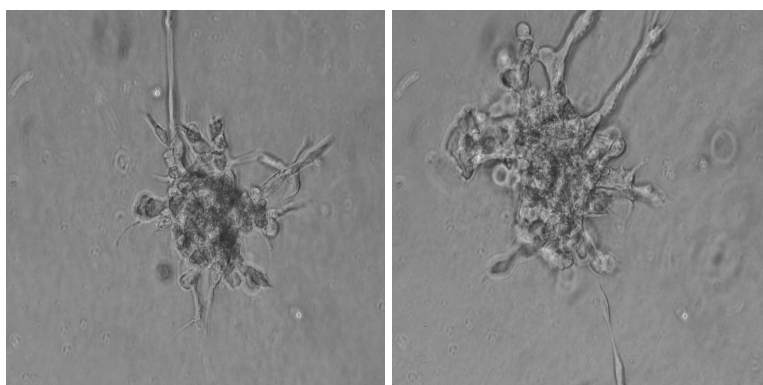


D.
PC3



PNT1a

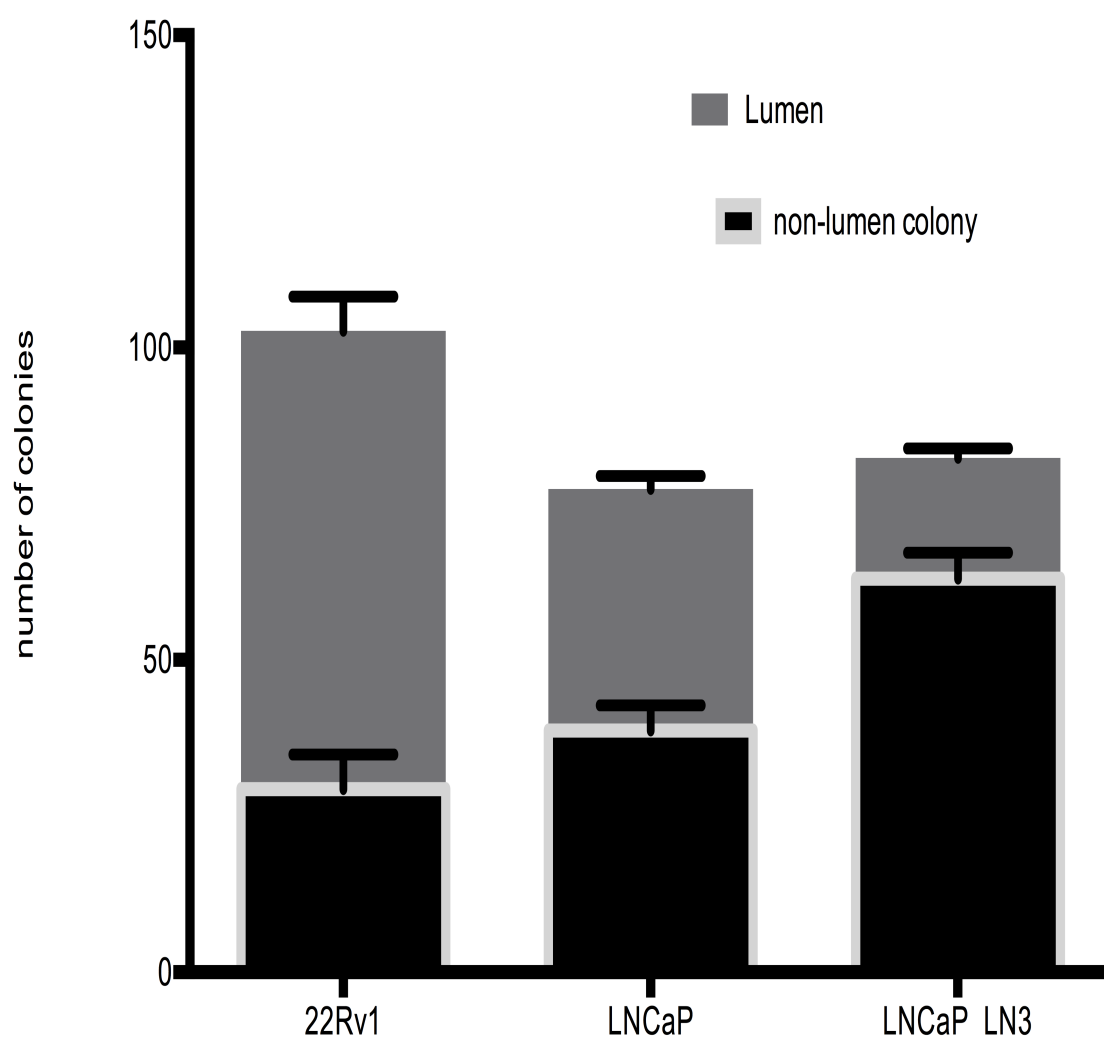


**E.
M12****M2182****Figure 3.8**

10X Phase contrast images of colonies grown in 3-D from a single cell of different cell lines. All the cell lines were classified into five different categories: Lumen (A), spehroid (B), dense (C), Intermediate (D) and stellate (E).

Although 22Rv1, LNCaP and LNCaP_LN3 all gave rise to colonies containing lumens, they showed some degree of variation in lumen size. LNCaP_LN3 cells gave rise to some lumen containing colonies, however the proportion of lumen forming colonies was much lower than 22Rv1 and LNCaP cells (**Figure 3.9**). LNCaP cells gave rise to very small lumens in the center of colony, where as 22Rv1 cells gave rise to huge lumens. Some of the colonies formed by 22Rv1 cells also had multiple lumens in one single colony. Even though the cells were filtered to obtain single cell suspensions, the multiple lumens in one colony may have been because the colony grew from more than one cell. However, there was

no significant size difference between colonies with a single central lumen and those with multiple lumens.



	<i>Non-lumen colonies</i>	<i>Lumens</i>	<i>Proportion of lumens</i>
22Rv1	29±5.50	73±5.50	71.5%
LNCaP	39±4.04	39±2.08	50%
LNCaP_LN3	63±4.16	19±1.53	23%

Figure 3.9

Proportion of lumen forming colonies in 22Rv1, LNCaP and LNCaP_LN3 cell lines. The proportion of lumen forming colonies was maximum in 22Rv1 (71.5%) followed by LNCaP (50%) and the least proportion was observed in LNCaP_LN3 (23%).

The spheroid colonies were formed by RWPE and DU145 cells. These colonies had tight and well-defined borders but did not form any lumens.

BPH-1 and BPH-1^{caf} formed dense colonies and were remarkably similar in terms of colony appearance. There was very little variation in size of the colonies formed by these two cell lines. The dense colonies were the biggest in terms of size of all other morphology categories.

PC3 cells mainly formed intermediate colonies, however about 5% of the colonies had more well defined borders than the intermediate colonies and resembled the spheroid colonies. PNT1a also formed intermediate colonies, but no spheroid like colonies were observed.

Finally, stellate colonies were formed by M12 and M2182 cell lines. The stellate protrusions out of the M2182 colonies were much longer than those from M12 colonies. However, the size of the colony in the center and the density of cells in the colony was similar in both cell lines.

Cell lines VCaP and DUCaP were also grown in 3D as single cell suspensions but because of the slow doubling time of these cells, they died off as single cells before any colonies could be formed. These cells were also plated at higher density without filtering to obtain single cells; however, no growth was observed in 3D.

3.2.6 Microarray analysis of differentially expressed genes in *NKX3.1^{+ve}* and *NKX3.1^{-ve}* cell lines

To understand the regulation of differentiation in prostate cancer, comparing the gene expression of cell lines on the basis of their differentiation capabilities as measured by *NKX3.1* expression and colony morphology may prove useful. Therefore, the microarray data were partitioned according to *NKX3.1* expression, and multiple Student's *t*-tests were performed for each gene transcript probe comparing mean expression of the *NKX3.1^{+ve}* and *NKX3.1^{-ve}* cell lines. Bonferroni correction was applied to the statistical tests in order to exclude false positives and to ensure that the identified gene transcripts actually represent those that are differentially expressed. Genes were investigated only if the corrected *p*-value was less than 0.02 and the fold change between the two morphology groups was 2.0 or larger. A detailed inspection of these genes revealed some key genes that are known to have an impact on prostate development and in carcinogenesis. At the same time, there were multiple genes found to be differentially expressed but had few, if any, known functions in the prostate.

The resulting statistical analysis identified a total of 1336 genes that were differentially expressed in *NKX3.1^{+ve}* and *NKX3.1^{-ve}* cell lines. Out of the 1336 genes, 624 genes were up regulated in *NKX3.1^{+ve}* cell lines as compared to *NKX3.1^{-ve}* cell lines (Appendix Table 1a) and 708 genes were down regulated in *NKX3.1^{+ve}* as compared to *NKX3.1^{-ve}* cell lines (Appendix Table 1b).

Out of the 624 up-regulated in *NKX3.1^{+ve}* cell lines, AR, KLK3, HOXB13 stand out because of their roles in carcinogenesis and tissue development.

HOXB13 was overexpressed 49 fold in *NKX3.1^{+ve}* cell lines with a *p*-value of 0.00047. *Hoxb13* is required for normal differentiation and secretory function of

the prostate (Economides, KD. *et al.*, 2003). Studies show that HOXB13 may have a potential role as a growth repressor in prostate (Chen, Z. *et al.*, 2013) and colorectal cancer (Akbari, MR. *et al.*, 2013), and mutation in *Hoxb13* is associated with cancer progression. HOXB13 suppresses growth of prostate cancer cells by acting as an AR repressor to modulate the complex AR signaling (Jung, C. *et al.*, 2004). However, the mechanism by which the AR suppression occurs is unknown. The involvement of NKX3.1 in this regulation, if any, will be further investigated.

Transcription factor, AR is overexpressed 104 fold with a *p*-value of 1.33e-08. These expression levels agree with the studies that prove that AR and NKX3.1 regulate each other in a feed forward loop (Tan, PY. *et al.*, 2012). PSA or KLK3 was also overexpressed 65 fold in *NKX3.1^{+ve}* cells with a *p*-value of 0.00003. PSA is a downstream target of the AR and it has also been reported that PSA transactivates AR through IGF-I signaling pathways (Liu, X. *et al.*, 2011).

FoxA1 is overexpressed 7.8 times with a *p*-value of 0.0002 in *NKX3.1^{+ve}* cell lines. Forkhead box protein A1 (FOXA1) is a transcription factor that is indispensable for the lineage-specific gene expression of the prostate. *FoxA1* acts as a metastasis inhibitor by inhibiting cell motility and epithelial-to-mesenchymal transition (EMT) in an AR-independent mechanism (Jin, HJ. *et al.*, 2013).

Since *IGF-1* is not differentially expressed and is absent from the list of up regulated 624 genes this suggests that the overexpression of AR, PSA and FOXA1 is not mediated by IGF-1 and is indeed a function of NKX3.1 regulation.

High expression of $\alpha_2\beta_1$ integrin is observed in basal and transit amplifying cells in the prostate. The $\alpha_2\beta_1$ integrin subunit is formed by non-covalent heterodimerization of α_2 integrin and β_1 integrin. The expression of α_2 integrin is

down regulated 7.4 fold with a p -value of 0.0016 and β_1 integrin is down regulated 2 fold with a p -value of 0.005 in $NKX3.1^{+ve}$ cell lines compared to $NKX3.1^{-ve}$ cell lines. The higher expression of α_2 integrin and β_1 integrin in $NKX3.1^{-ve}$ cell lines could also mean that there is a higher dimerization and consequently higher expression of $\alpha_2\beta_1$ integrin. However, this is to be established by immunofluorescence studies. Presence of $\alpha_2\beta_1$ integrin^{hi} cell population suggests that there is a subpopulation of either basal cells or transit amplifying cells in $NKX3.1^{-ve}$ cell lines but not in $NKX3.1^{+ve}$ cell lines. This means that in prostate cell lines, basal or transit amplifying cells can coexist with luminal cells only if the luminal cells have lost the expression of NKX3.1 or AR.

CD44 has been identified as a prostate cancer stem cell (CSC) marker (Maitland, NJ., *et al.*, 2008). CD44 is down regulated in $NKX3.1^{+ve}$ cell lines 8.3 fold with a p -value of 0.016. The expression levels of $\alpha_2\beta_1$ integrin and CD44 suggest that the CSC population is low in $NKX3.1^{+ve}$ cell lines as compared to $NKX3.1^{-ve}$ cell lines.

CD55 has been previously described by the Bodmer lab as a potential CSC marker in colorectal cell lines. CD55 is also down regulated 4.5 fold with a p -value of 0.0009 in $NKX3.1^{+ve}$ cell lines. Further studies need to be carried out to establish whether or not CD55 can be used as a CSC marker in prostate cell lines as well.

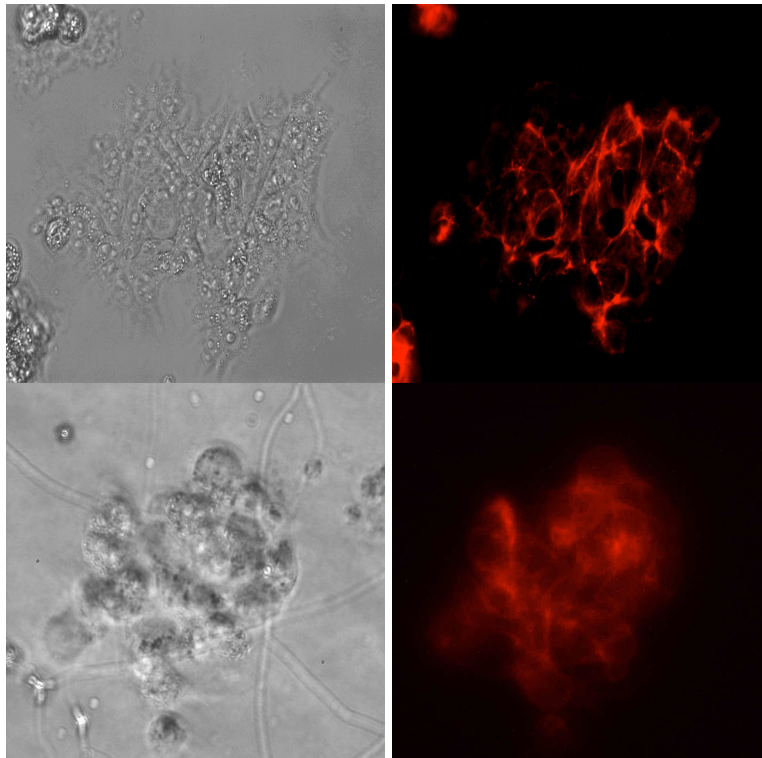
3.2.7 F-actin as a lumen marker

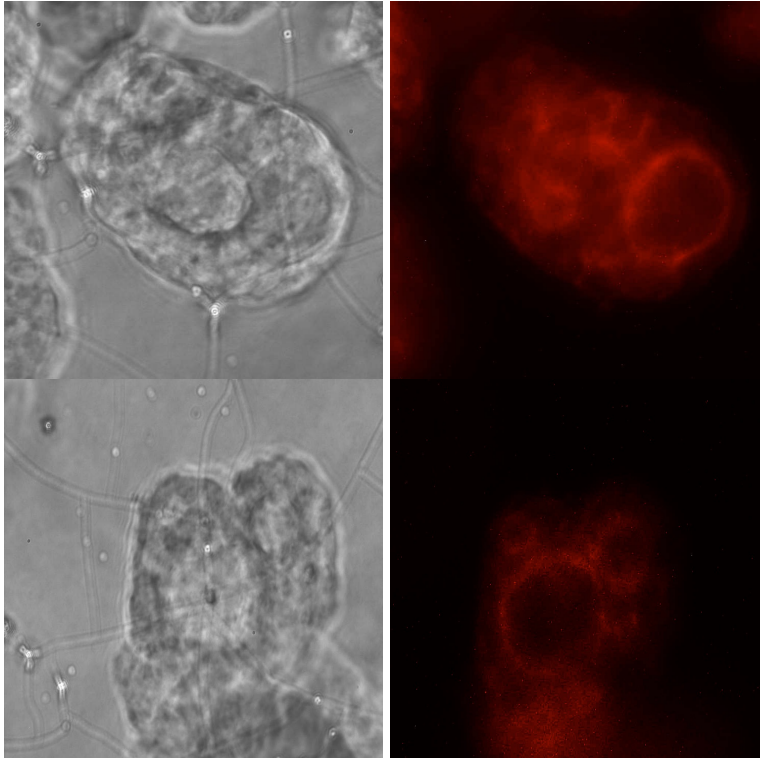
F-actin has been identified as a marker for lumens in human colon cryosections and cell lines in the Bodmer lab (Ashley, N. *et al.*, 2013). Prostate cell lines were

stained with F-actin specific fluorescent phalloidin to investigate whether there was F-actin enrichment in lumens in prostate acini, similar to that in colon.

F-actin staining was examined in 3-D colonies derived from single cell suspensions of lumen forming 22Rv1 and intermediate colony forming PC3 cell lines and grown in Matrigel™ for 10 days. PC3 colonies in 3D showed a diffuse F-actin staining and there was no indication of cell polarization. However, in 22Rv1 colonies intense F-actin staining was observed on the apical cell membranes facing the lumen but no such enrichment was observed on the basolateral membrane of the cells (**Figure 3.10**).

PC3



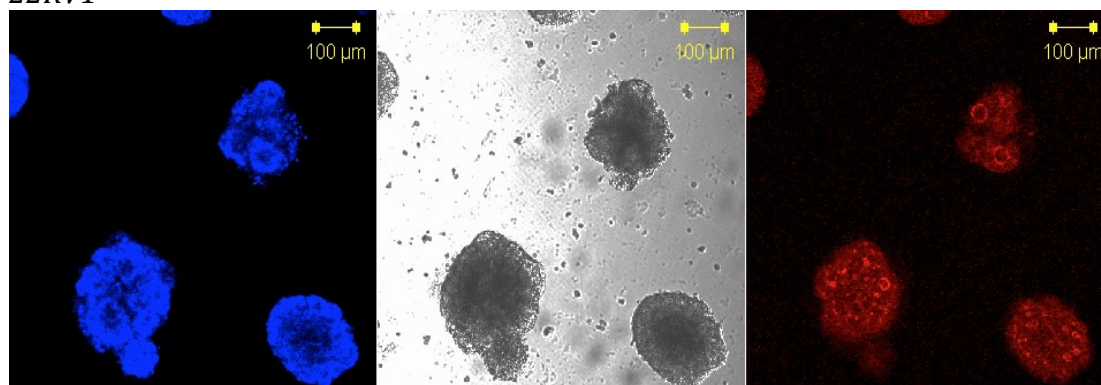
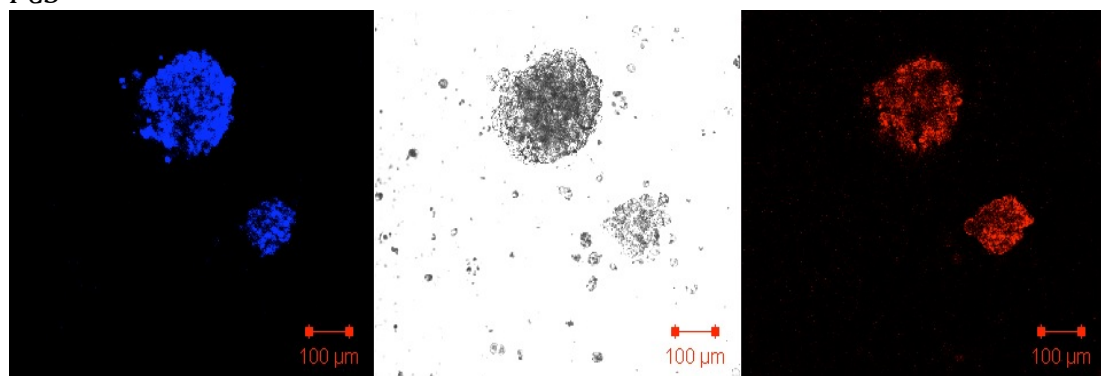
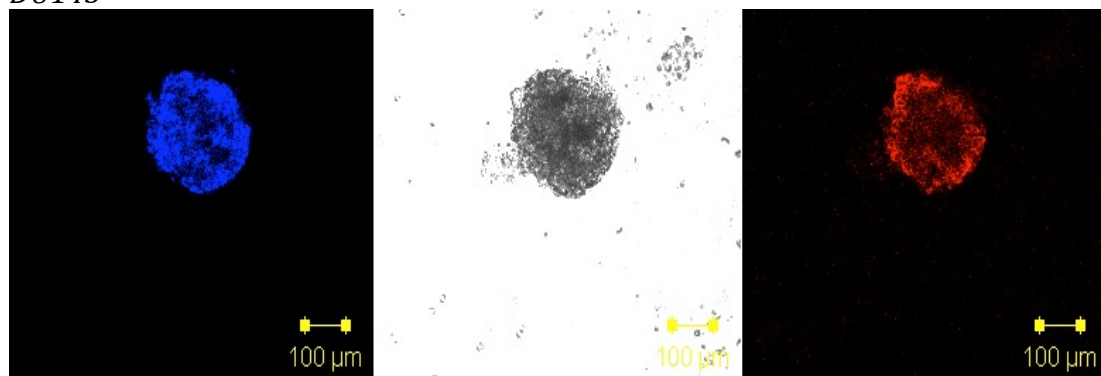
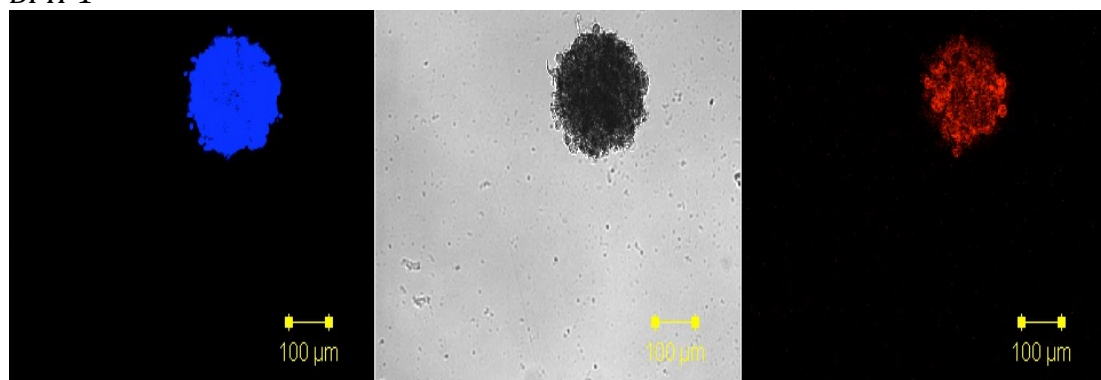
22RV1**Figure 3.10**

The localization of F-actin in a lumen forming colony (22Rv1) and an intermediate colony (PC3) by staining with phalloidin. Images were taken after 10days under 20X magnification.

Confocal Z-section images of 22Rv1, PC3, DU145 and BPH-1 colonies were taken as these cell lines are representative of lumen forming, intermediate, spheroid and dense colonies (**Figure 3.11**). 22Rv1 colonies showed enriched F-actin staining on the luminal side. All the lumens observed by phase contrast images were clearly labeled by F-actin staining as well.

Z-section images of PC3 cells do not show any cell polarization, as would be expected for intermediate colonies. In the DU145 spheroid forming cell line, F-actin staining was present on the basolateral side of the colony, which represents cell polarity and lateral cell adhesion. No lumen formation was observed either by light microscopy or F-actin staining. Finally, BPH-1 dense colonies had F-actin staining on cell membranes but no enrichment was seen.

The differences in the cell densities of different types of colonies can be seen in these Z-section images where cells are stained with DAPI. BPH-1 colonies have significantly higher cell density than 22Rv1, PC3 and DU145 colonies.

22RV1*PC3**DU145**BPH-1***Figure 3.11**

Z-sections of 22Rv1, PC3, DU145, BPH-1 cells grown in Matrigel for 10 days and stained with DAPI (blue) and f-actin (red). Images taken under 10X magnification.

3.2.8 Prostate cell lines show a high frequency of microsatellite instability.

MSI has been studied in depth in colorectal cancer where epigenetic silencing or mutation of *MLH1* has been identified as the cause of defective mismatch repair (Peltomaki, P. *et al.*, 2003). However, microsatellite analysis in prostate cancer primary tissues reveals that MSI is not a very frequent event in prostate cancer with the highest frequency reported at 37.5% in a study where normal tissue, moderately differentiated tumours and poorly differentiated tumours were analyzed.

The MSI status of the 11 cell lines was first determined using two mononucleotide (Bat25 and Bat26), and two tetranucleotide repeat markers (D11S488 and D20S82). These markers were selected as they have earlier been used extensively to analyze MSI in prostate primary tissue samples (Azzouzi, AR *et al.*, 2007) (Sun, X *et al.*, 2006). The cell line was determined to be MSI^{+ve} if there was insertion or deletion of base pairs for 3 or more of these four microsatellite markers. **Table 3.3** summarizes the MSI status of the cell lines for each microsatellite marker.

Cell line	Bat 25	Bat 26	D11S488	D20S82	MSI status
LNCap	+	+	+	+	+
LNCaP-LN3	+	+	+	+	+
Vcap	-	-	-	-	-
DUCap	-	-	-	-	-
PC3	-	-	-	+	-
DU145	+	+	-	+	+
22Rv1	+	+	+	+	+
M12	+	+	+	+	+
M2182	+	+	+	+	+
BPH-1	+	+	-	+	+
BPH-1 ^{caf}	+	+	-	+	+

Table 3.3

Microsatellite instability status for each cell line at four different microsatellite loci. The RER status is determined to be positive if the cell line is MSI⁺ at 3 or more microsatellite loci.

PC3 cells were MSI⁺ for D20S82 but negative for Bat25, Bat26 and D11S488. VCaP and DUCaP cells did not show MSI at any of the four markers. Interestingly, these two cell lines are the ones that express highest levels of *NKX3.1*. Whether there is a link between the expression of *NKX3.1* and MSI is yet to be established. Based on the criteria set for MSI status, VCaP, DUCaP and PC3 cells are MSI⁻ whereas the remaining 8 cells lines are MSI⁺. DUCaP and VCaP are cell lines derived from the same patient with metastasized prostate cancer. The results are consistent in the sense that both the cell lines yield exactly the same fragment size and show the same pattern of peaks as each other, for

every microsatellite marker. For example, DUCaP and VCaP both yield a fragment size of 122bp for Bat25, 117-119 for Bat26, 255bp and 265bp at D11S488 and 216bp and 221bp at D20S82.

M12 and M2182 are derived from same parental cell line p69SV40T. BPH-1^{caf} is a cell line derived from BPH-1 and LNCaP_LN3 is a subline derived from LNCaP. All these sets of cell lines show consistent results in terms of fragment sizes. The electropherograms of the cell lines for all four markers are shown in **Figure 3.12**.

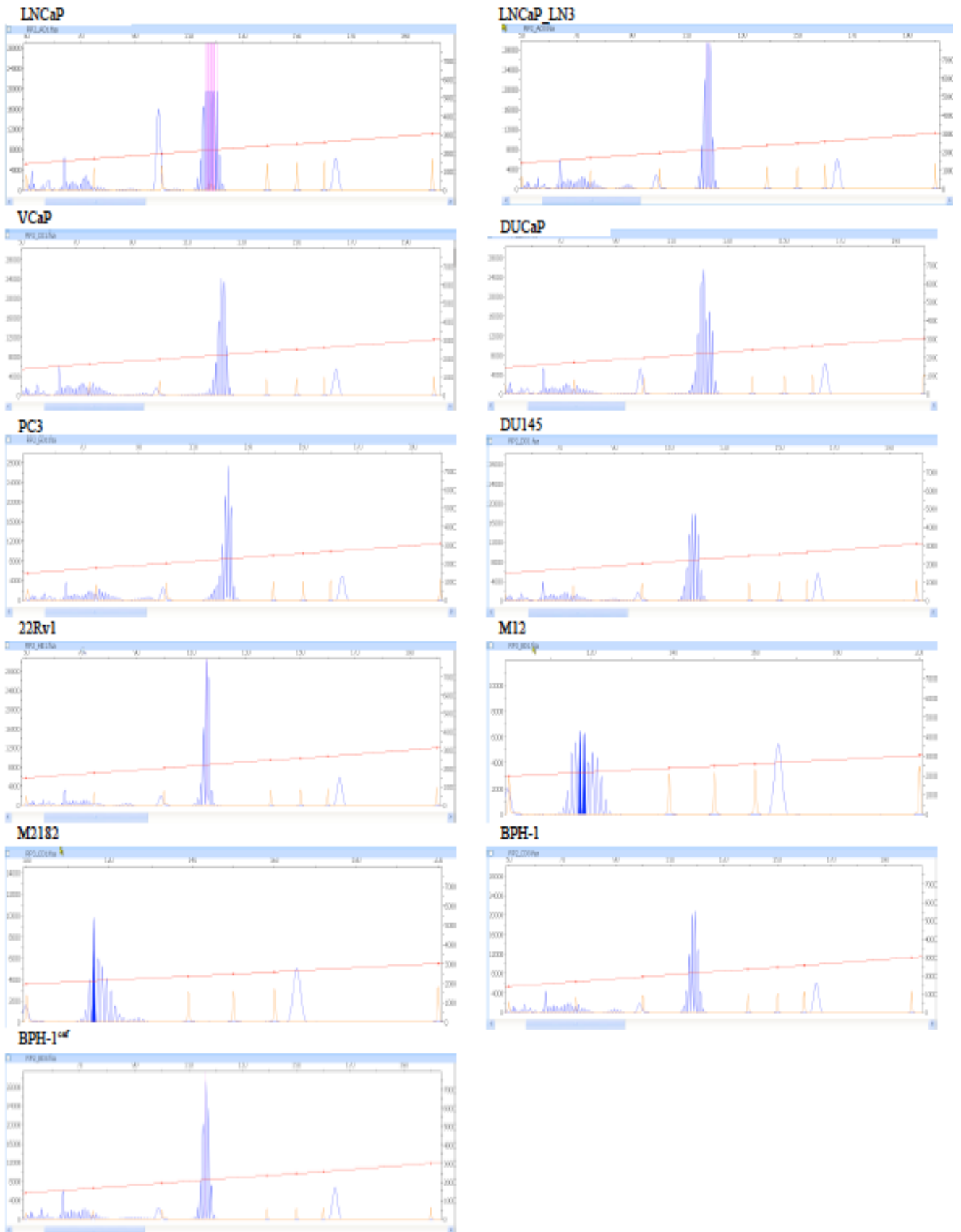
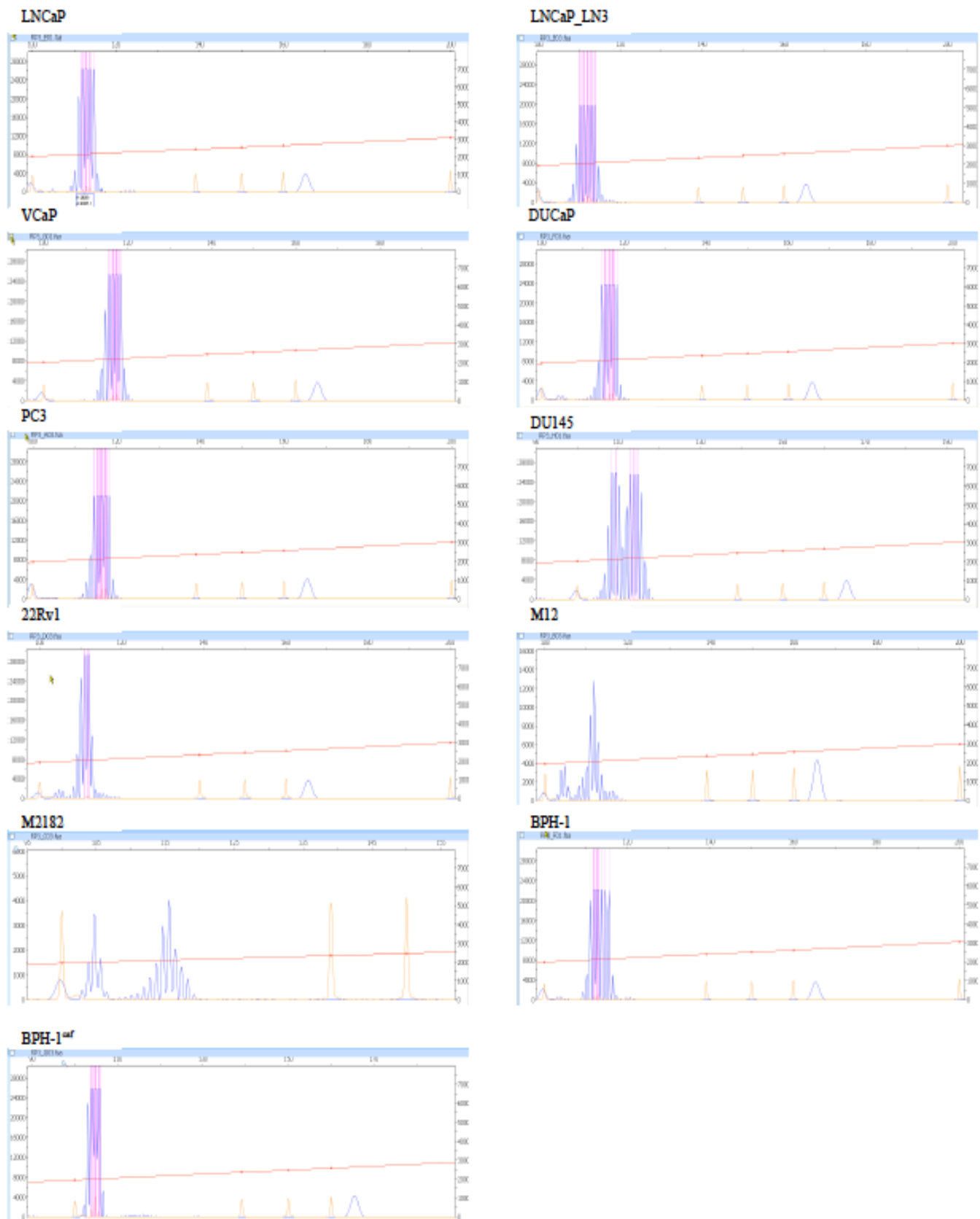


Figure 3.12A

Electropherograms of cell lines for the Bat25 marker. The size of the microsatellite for the designed primers is 121bp. Deletions in the microsatellite yield PCR products smaller than 121bp and are representative of replication error (RER).

**Figure 3.12B**

Electropherograms of cell lines for Bat26 marker. The size of the microsatellite for the designed primers is 118bp. Deletions in the microsatellite yields PCR products smaller than 118bp and is a representative of RER.

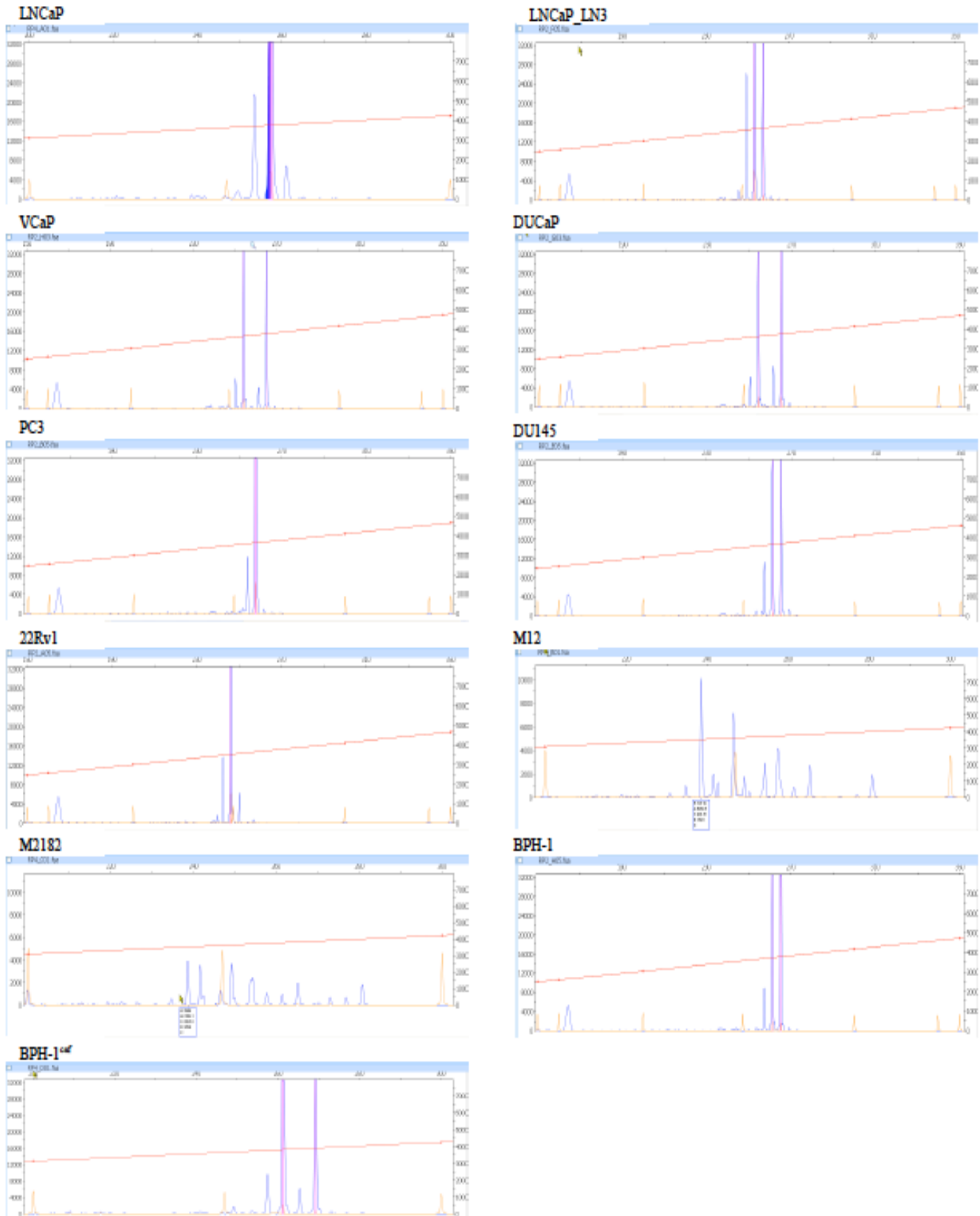


Figure 3.12C
Electropherograms of cell lines for D11S488 marker. D11S488 has (AAAG)₇₋₁₇ (GAAG)₄₋₁₄ repeats and the microsatellite size varies from 260-300 bp. Double peaks represent alleles and deletions in the microsatellite are representative of RER.

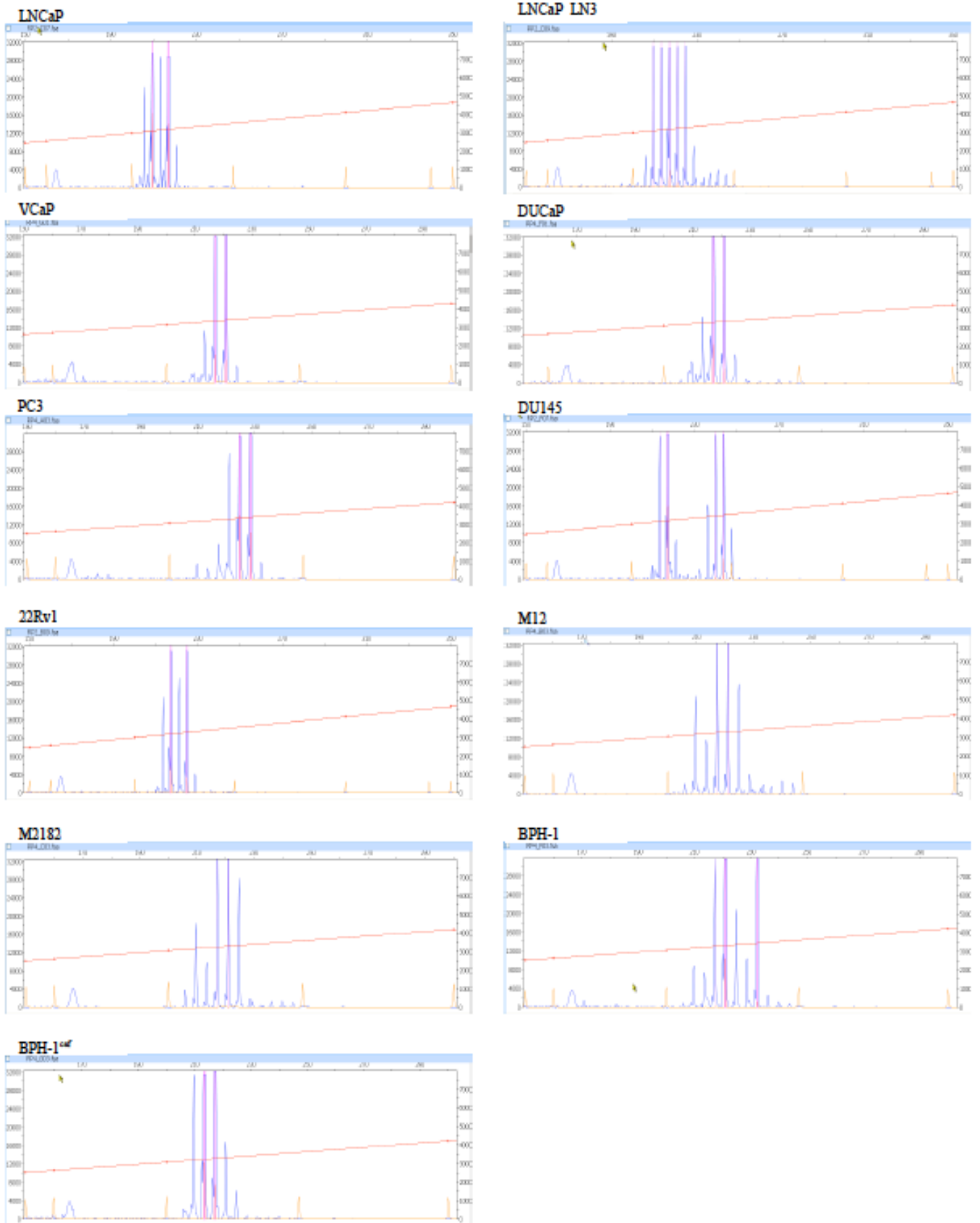


Figure 3.12D
Electropherograms of cell lines for D20S82 marker. D20S82 has (AAAG)₁₀ repeats and the size of the microsatellite is approximately 220 bp. Double peaks (VCaP and DUCaP) are alleles and multiple peaks are representative of RER.

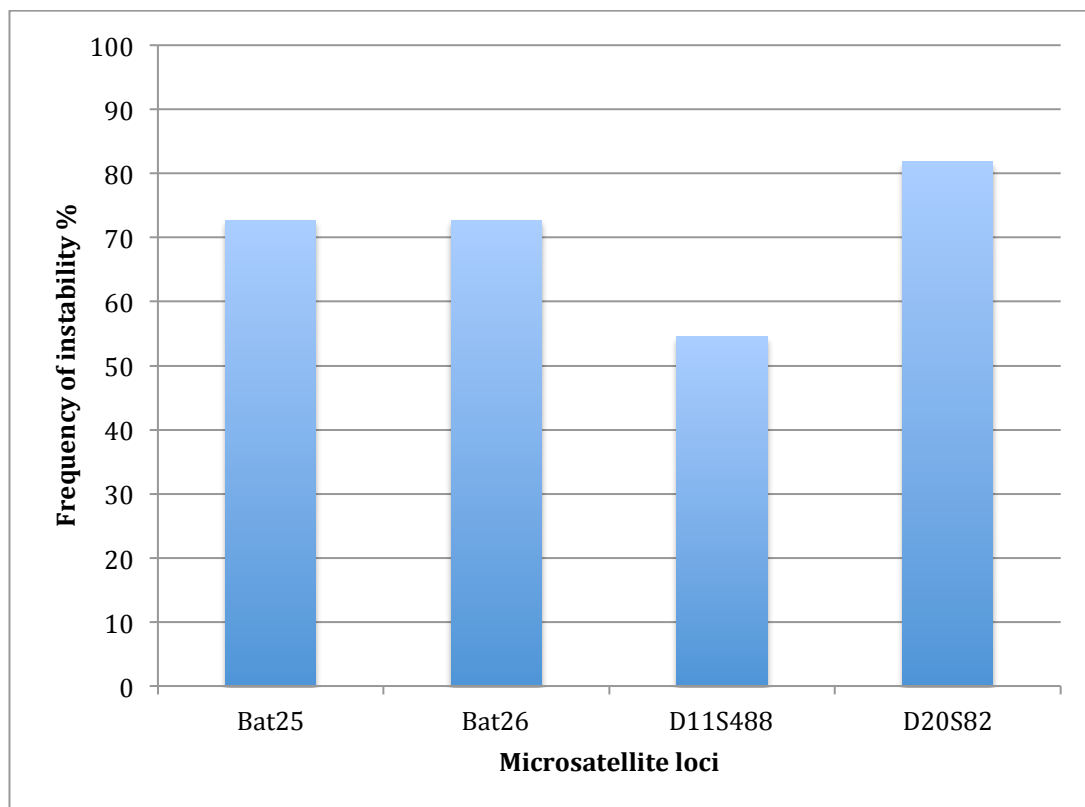


Figure 3.13

Frequency of allelic alterations at different microsatellite loci in prostate cell lines.

3.2.9 Expression of MMR genes in prostate cell lines

mRNA expression levels of MMR genes namely, *MLH1*, *MLH3*, *PMS2*, *MSH2* and *MSH3* were obtained from the microarray data of the prostate cell lines. Inactivation of MMR genes *MLH1* and *MSH2* either by mutation or hypermethylation plays a predominant role in colorectal cancer (Bodmer, W.F. *et al.*, 1999). However, in case of prostate cancer there does not seem to be any particular MMR genes that seem to be playing a role in MSI.

The expression of MMR genes was considered to be high if the non-log expression values were greater than 225 and non-log expression values less than 75 were considered low (**Table 3.4**). The MMR system is made up of 3 heterodimers. hMutS β is a MSH2-MSH3 complex that is responsible for recognition of DNA mismatch and damage. hMutL α is a MSH1-PMS2 complex and hMutL γ is a MLH1-MLH3 complex. The hMutL complexes are molecular matchmakers that act as endonucleases and are responsible for termination of mismatch-provoked excision (Li, G. *et al.*, 2008). Increased expression of some of the MMR genes could be to compensate for the lack of expression of one of the other MMR genes in the same complex or some other MMR complex.

Interestingly, all the MSI⁺ cell lines demonstrated either an overexpression or low level of expression of different MMR genes. The MSI⁻ cell line VCaP and DUCaP which are stable for all 4 microsatellite markers used had normal expression of all of the MMR genes and seems to have an intact MMR mechanism. PC3 cells were stable at 3 microsatellite markers, Bat25, Bat26 and D11S488 but unstable at D20S82. Low expression levels of *MLH1* in this cell line might have resulted in MSI to a certain degree in this cell line.

LNCaP and LNCaP cell lines have very low levels of *MSH2*. This means that the hMutS β complex in these cells is faulty in spite of normal *MSH3* levels. The lack of ability of the hMutS β complex to recognize mismatches and damage maybe responsible for MSI in

these cell lines. The expression level of *PMS2* is high in LNCaP and LNCaP_LN3 cells. DU145 also has high levels of *PMS2*.

MLH3 is downregulated in 5 out of 8 MSI⁺ cell lines, M12, M2182, BPH-1, BPH-1^{caf} and 22Rv1. M12 and M2182 are derived from the same cell line and M12 is also included in *MLH3* low category with an expression level of about 87. *MLH3* has the highest frequency of downregulation in prostate cell lines and differs from colorectal cancer where *MLH1* and *MSH2* are most commonly inactivated.

22Rv1, which is the most differentiated prostate cell line in terms of its lumen forming capacity, has somewhat higher than normal levels of *MLH1*.

	MLH1	MLH3	PMS2	MSH2	MSH3
LNCaP	202.33	118.14	299.31	32.47	179.76
LNCaP_LN3	189.33	166.16	329.46	41.19	201.18
DUCaP*	88.42	112.57	99.05	86.99	103.94
VCaP*	147.59	106.98	146.89	150.07	150.89
PC3*	51.30	111.80	166.37	148.63	94.81
DU145	155.72	107.68	235.43	117.59	118.26
M12	44.86	87.65	87.82	231.69	131.90
M2182	99.97	74.15	109.72	229.72	235.48
BPH-1	120.04	66.45	121.70	236.12	112.97
BPH-1caf	145.92	61.09	140.97	187.87	125.25
22Rv1	281.88	76.40	196.08	160.02	105.08

Table 3.4

mRNA expression levels of MMR genes in prostate cell lines. High levels of expression are marked in red and low levels of expression are marked in yellow. MSI^{-ve} cell lines are marked with an asterix.

3.3 Discussion

The expression levels of AR and NKX3.1 are strongly positively correlated which agrees with studies that suggest a feed-forward loop regulation of the expression of the two genes in primary tissue. This positive correlation is also preserved in cell lines obtained from metastasis sites (LNCaP, LNCaP_LN3, 22Rv1, VCaP, DUCaP).

In the colon, it has been shown that CDX1 is important for the polarization of intestinal cells (Keller, MS. *et al.*, 2004). The Bodmer lab has clearly shown the requirement of *CDX1* in colorectal cell lines to properly form polarized luminal structures (Yeung, T *et al.*, 2010). In the prostate cell line panel only the cell lines expressing NKX3.1 (with an exception of VCaP and DUCaP cells which did not form any colonies in three-dimensional matrix) formed lumens suggesting that NKX3.1 expression is key for cell polarization. There might a striking parallel between the functions of CDX1 and NKX3.1. Further studies are carried out and reported in the following chapters of this thesis to establish this.

Functional studies are often difficult because of a lack of *in vitro* methodologies for assessing differentiated and stemness phenotypes. The ability of cells to divide indefinitely into non-differentiated cells is called stemness. It has been demonstrated here that three-dimensional culture of prostate cancer cell lines can give rise to colonies that possess characteristics of differentiation, including the formation of prostate acini and polarized cells, and of stemness, including the formation of dense cell-to cell structures with little internal structure.

It is likely that the expression patterns intrinsic to the two colony morphologies (dense and lumen) are the major cause of the differences in colony organization. Hence, mRNA expression levels for each transcribed gene and understanding

which genes are upregulated or downregulated in response to differentiation or stemness is essential.

Out of the list of 1336 differentially expressed genes in *NKX3.1^{+ve}* and *NKX3.1^{-ve}* cell lines, AR, HOXB13 and $\alpha_2\beta_1$ integrin are chosen as genes of interest for further study. While the interaction of AR and NKX3.1 is well established, the correlation of HOXB13 and NKX3.1 has not been studied in detail.

8 out of 11 cell lines are found to be MSI^{+ve}—suggesting that MMR defect is a frequent event in cell lines derived from prostate cancer and those derived from transformed normal prostate epithelial cells. This is in contradiction to widely published data that show that clinically localized prostate cancer is microsatellite stable (Azzouzi, AR *et al.*, 2007). However, the link between the loss of expression of MMR proteins and differentiation is difficult to establish. The most differentiated cell line based on colony morphology assay in 3D, 22Rv1 is MSI^{+ve}, whereas PC3 which displays relatively more stem-like properties and has lower differentiation capability is microsatellite stable.

The striking observation from the microsatellite stability analysis is that the frequency of MSI in prostate cell lines is much higher than that of primary tumours. This suggests that tumours that are MSI positive give rise to cell lines much more frequently than those that are MSI negative. To gain more insight on this, experiments can be carried out where MMR genes are knocked down in microsatellite stable tumours and then make attempts to grow out cell lines out of the tumour.

Chapter 4

Functional role of *NKX3.1* expression in prostate cell lines

4.1 Introduction

As previously discussed, the results of the colony morphology microarray expression analysis associated the expression of the transcription factor, *NKX3.1* with the differentiated lumen colonies forming cell lines. Functional studies are necessary to conclusively demonstrate the role of *NKX3.1* in differentiation. The role of *NKX3.1* in luminal differentiation has been very well established during prostate organogenesis in various studies (Bhatia-Gaur, R. *et al.*, 1999, Kruithof-de Julio, M. *et al.*, 2013). It has been shown that knocking down *NKX3.1* in mice induces prostatic intraepithelial neoplasia (PIN) (Abdulkadir, SA. *et al.*, 2002, Iwata, T. *et al.*, 2010). Although the majority of the prostate cancer studies are carried out using prostate derived cell lines as a model, no functional studies have been carried out on the effect of *NKX3.1* expression.

We hypothesized that *NKX3.1*, a prostate specific homeobox gene might be the prostate analog of *CDX1*, an intestinal specific homeobox gene. *CDX1* has been shown to play a key role in intestinal development and maintenance of intestinal phenotype (Silberg, DG. *et al.*, 2000), and is also important for stem cell differentiation and lumen formation in colorectal cancer cell lines and primary tumours in the colon (Ashley, N. *et al.*, 2013). Extensive functional studies with *CDX1* have been carried out in the Bodmer lab. Here we show how the expression of *NKX3.1* affects the differentiation capacity of the cells using three-dimensional growth cultures and lumen marker F-actin. Interestingly, the effect of *NKX3.1* knockdown and overexpression have exactly the same effect on the differentiation capacity of prostate cell lines as does *CDX1* in colorectal cell lines. Hypoxia is another factor that regulates differentiation in prostate cancer and is known to promote stem-like properties (Bao, B *et al.*, 2012). Furthermore,

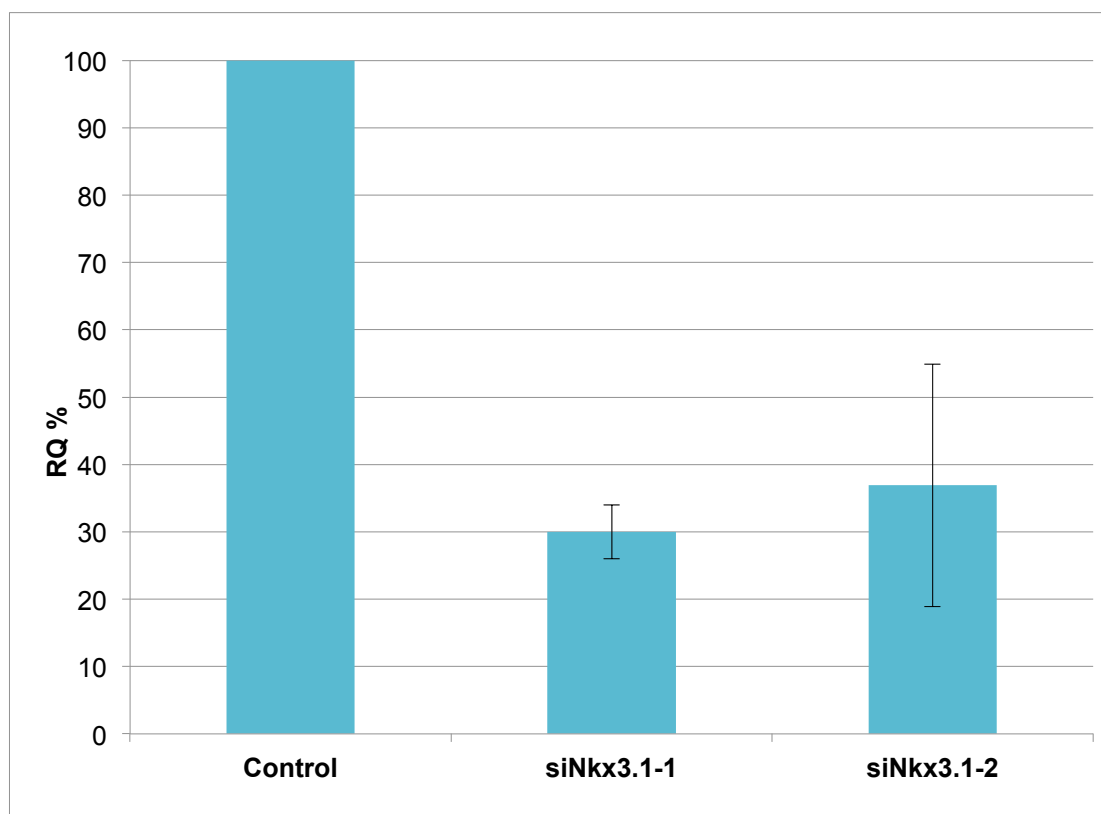
hypoxic conditions have also been proved to induce epithelial to mesenchymal transition (EMT) in various cancers (Jiang, J. *et al.*, 2011, Salnikov, AV. *et al.*, 2012). The link between hypoxia, EMT and the expression of *NKX3.1* is also established in this chapter.

4.2 Results

4.2.1 siRNA mediated knockdown of *NKX3.1* in 22Rv1 cells

NKX3.1^{+ve} and lumen forming cell line 22Rv1 was selected to study the functional effect of *NKX3.1* knockdown. The 22Rv1 cell line was transiently transfected with 2 different knockdown siRNA sequences for *NKX3.1* knockdown and with scrambled sequence as a control (designated 22Rv1-siNkx3.1 and 22Rv1-control, respectively).

NKX3.1 expression of 22Rv1-siNkx3.1 and 22Rv1-control was assessed by quantitative RT-PCR after 24, 48, 72 and 92 hours. 22Rv1-siNkx3.1 transfected with siNkx3.1-1 showed a highest reduction of 70% in message after 48 hours whereas transfection with siNkx3.1-2 showed a knockdown of 64% after 48 hours relative to the scrambled control (**Figure 4.1**). The specificity of *NKX3.1* knockdown was confirmed by the fact that there was no reduction of *NKX3.1* message or protein level in 22Rv1-control. 22Rv1-siNkx3.1 exhibited higher knockdown and hence was selected for further study.

**Figure 4.1**

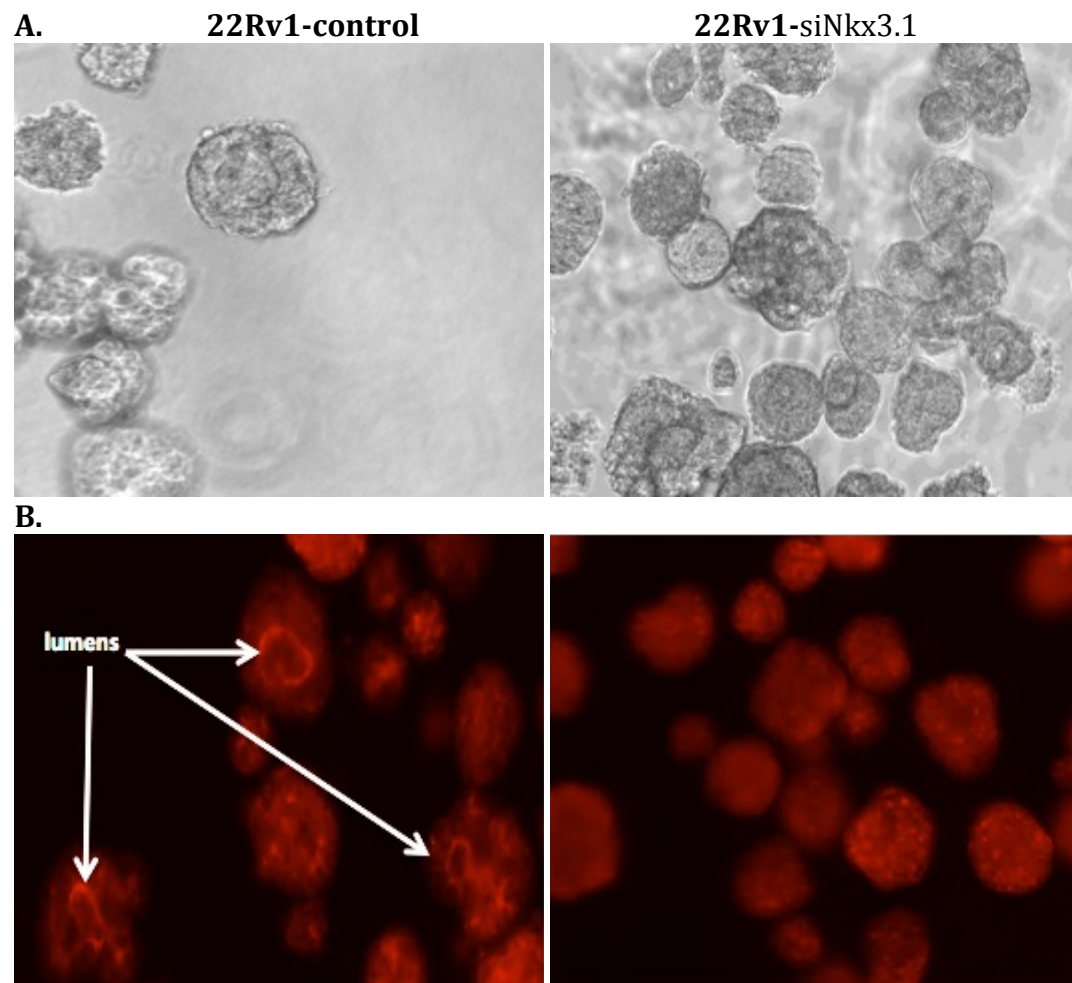
mRNA levels of *NKX3.1* after siRNA mediated knockdown in 22Rv1 cells. Maximum knockdown was achieved at the mRNA levels of *NKX3.1* post 48 hours transfection with scrambled sequence and two different *NKX3.1* siRNA sequences siNkx3.1-1 and siNkx3.1-2.

4.2.2 NKX3.1 silencing prevents differentiation of 22RV1 cells in 3D and promotes a more stem-like phenotype

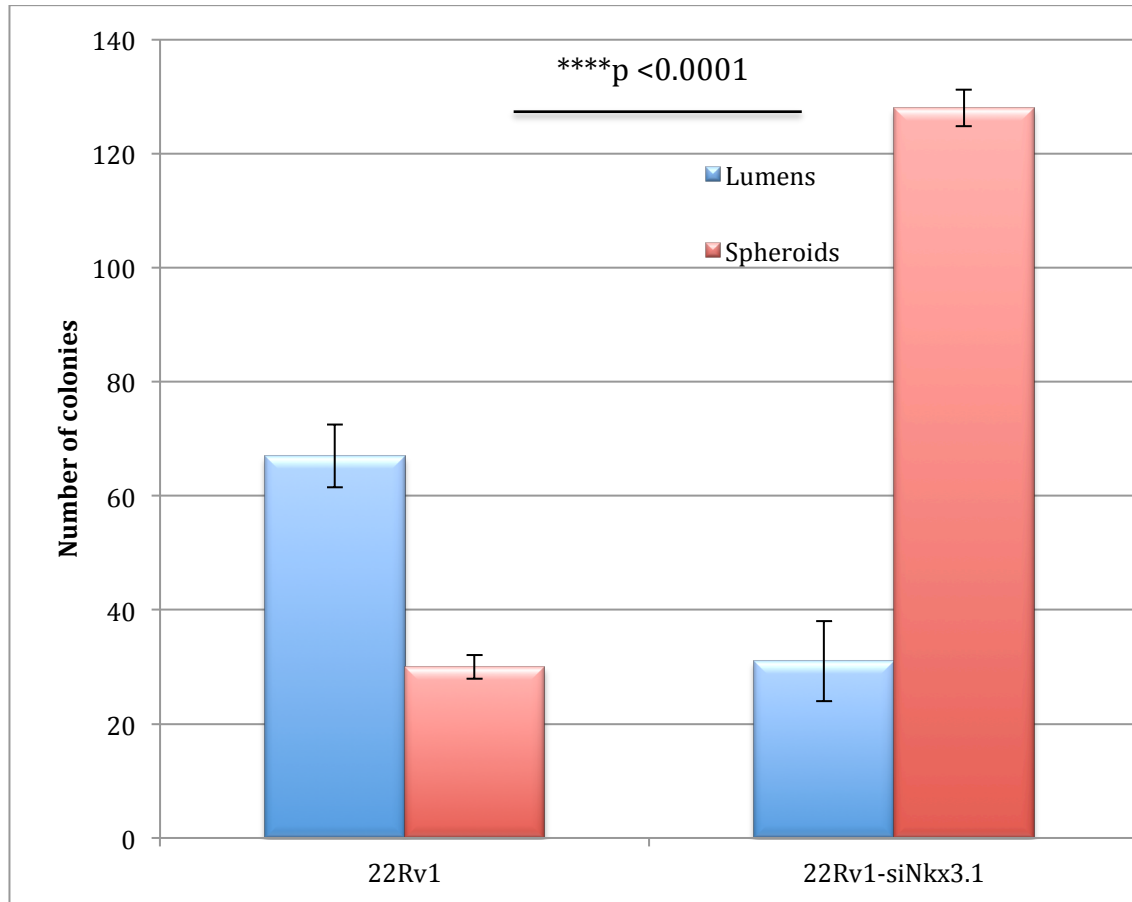
To assess the clonogenic abilities of 22Rv1-siNkx3.1 and 22Rv1-control in 3-D, these cell populations were grown in 1:1 Matrigel™:collagen mix in triplicates in 96 wells for 10 days. Cells were single sorted with 20µM cell filters and aliquots of 1000 cells were added to each well. The message level of *NKX3.1* was assessed for the first 6 days. Although the maximum knockdown was achieved after 48 hours, the initiation of clones and polarization of cells leading to differentiated lumen formation begins within 1-3 days of cell plating. Therefore the major

events that influence clonogenicity would have occurred during the period while the expression of *NKX3.1* was significantly low.

Colonies were allowed to grow for 10 days in culture, after which they were fixed with paraformaldehyde and stained with Rhodamine-phalloidin, a stain for F-actin, for visualizing lumens. The wells were photographed for assessment of number and type of colonies (**Figure 4.2** and **Figure 4.3**). The number of colonies containing lumens was counted both by F-actin staining using fluorescent microscopy as well as light microscopy. The number of lumens counted by both methods was exactly consistent in all cases.



C.



	Lumens	Non-lumen forming	Total	p-value
22Rv1	67	30	97	<0.0001
22Rv1-siNkx3.1	31	128	159	

Figure 4.2

Effect of *NKX3.1* knockdown on proportion of lumen forming colonies in 22Rv1 cell line. Phase contrast (**A**) and F-actin staining (**B**) images taken after growing 22Rv1-control and 22Rv1-siNkx3.1 in 1:1 Matrigel™:Collagen mix for 10 days. 22Rv1-siNkx3.1 cells showed an increased number of total colonies and a decrease in proportion of lumen (**C**). ****p<0.0001 Fisher's exact test comparing the proportion of lumens in 22Rv1- siNkx3.1 to 22Rv1-control

22Rv1 is a lumen forming cell line and the 22Rv1-siNkx3.1 showed a statistically significant increase in clonogenicity and interestingly, a decrease in the proportion of lumen forming colonies (**Table 4.1**). There was a dramatic decrease of 71% in the proportion of lumen forming colonies and a 64% increase in total number of colonies in 22Rv1-siNkx3.1 as compared to 22Rv-1 control cells.

Although *NKX3.1* knockdown demonstrated a striking difference in the colony morphology and cell polarization, no apparent difference was observed in the average size of the colony or the cell density within a colony. Also, quantification of lumens by both F-actin staining and bright field imaging yielded comparable results. However, F-actin staining increased the visibility of the lumens, which made it possible to notice the few number of lumens that could have been missed by bright field imaging alone.

	<i>Non-lumen forming colonies</i>	<i>Lumens</i>	<i>Proportion of lumens</i>
22Rv1-control	30	67	69.1%
22Rv1-siNkx3.1	128	31	19.5%

Table 4.1

Number of non-lumen forming colonies and lumen forming colonies formed by 22Rv1 and 22Rv1-siNkx3.1 cells.

4.2.3 mRNA expression of *NKX3.1* after transfection in PNT1a and PC3 cells

PC3 and PNT1a were used as negative controls to assess the functional effect of transfecting *NKX3.1* in *NKX3.1* negative cell lines. PC3-Nkx3.1 is a stably transfected cell line, which was a generous gift from Prof. Edward Gelamn, Columbia University, USA.

PNT1a and PC3 cells were transiently transfected with full length *NKX3.1* cDNA cloned in an expression vector *pcDNA3.1* under a CMV promoter (**Figure 4.3**).

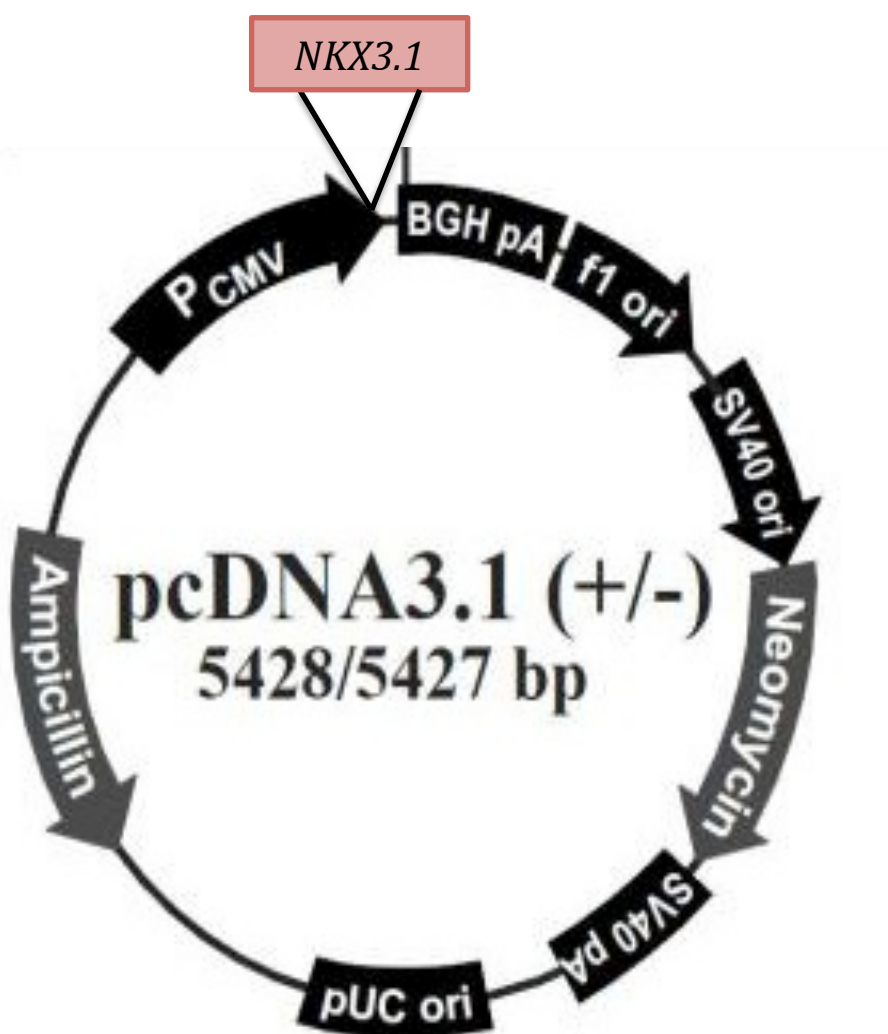


Figure 4.3

Map of *pcDNA3.1* with CMV promoter and Neomycin resistance.

As discussed in Chapter 3, PNT1a and PC3 form intermediate colonies with loose structures and no defined borders when grown in 3D. *NKX3.1* positive and lumen forming 22Rv1 cell was used as a positive control in the transfection experiments. Cells were harvested by trypsinization and aliquots of 10,000 cells were plated in each well of a 24-well plate. Cells were subsequently chemically transfected with 1µg control or *pcDNA3.1-NKX3.1* using Lipofectamine™ LTX with PLUS™ lipofection system to assess the effects of *NKX3.1* expression in these cell lines. The cells were cultured in 1.2 mg/mL Geneticin RPMI1640 media.

The transfection efficiency in PNT1a cells was evaluated by quantifying mRNA from the normal and transfected cells using qRT-PCR (**Figure 4.4**). PNT1a cells transfected with *NKX3.1* are called PNT1a-*NKX3.1* and these cells had mRNA levels of *NKX3.1* much higher than the control PNT1a cells. 22Rv1 cells were used as a positive control and hence the relative quantification of mRNA level in 22Rv1 is 1.

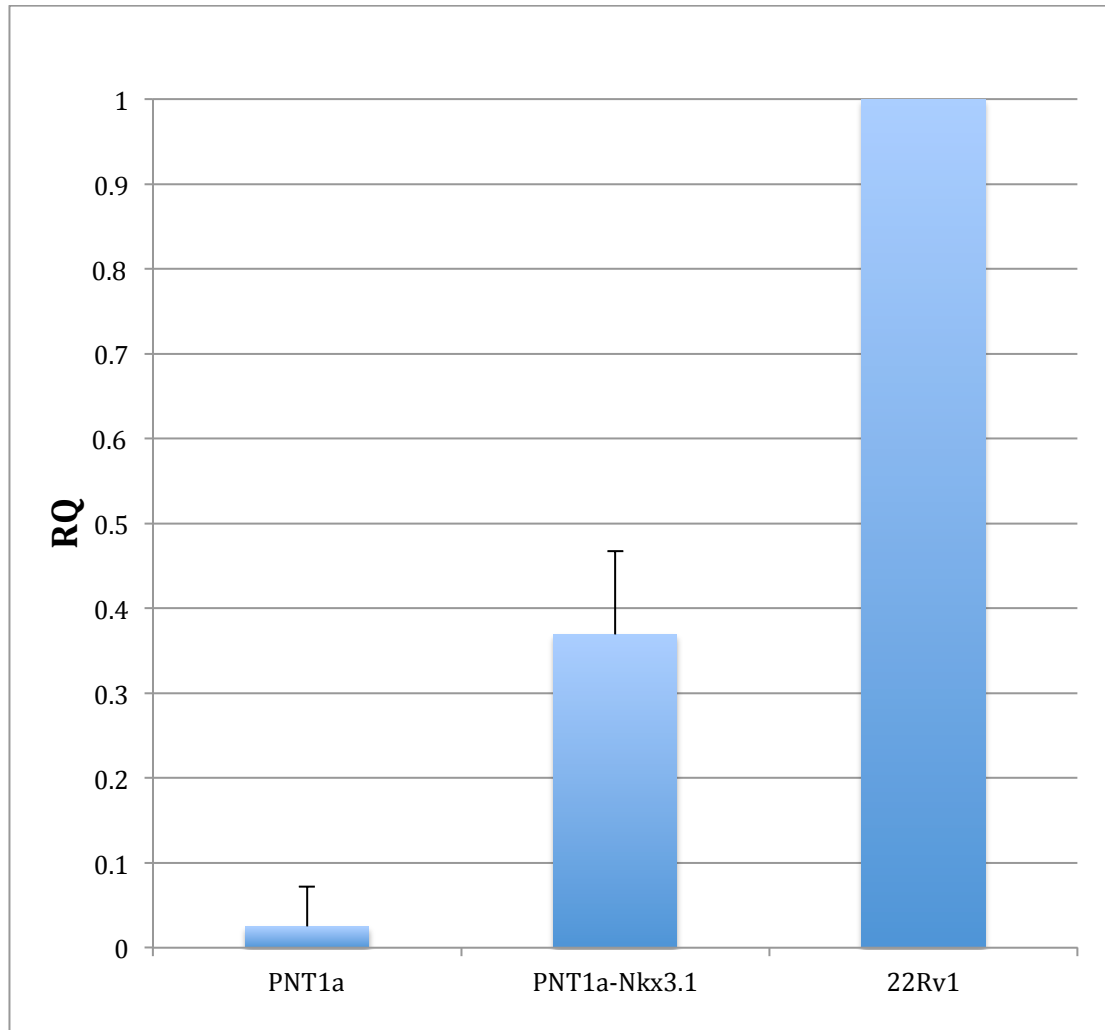


Figure 4.4

mRNA expression levels of *NKX3.1* in PNT1a cells (negative control), PNT1a cells transiently transfected with *NKX3.1* and 22Rv1 cells (positive control).

qRT-PCR was also performed on stably transfected PC3-Nkx3.1 (gift from Prof. Edward Gelmann, Columbia University) cells to quantify the expression of *NKX3.1* and determine whether that has an effect on the expression of *AR*. 22Rv1 cells were used as the positive control and the mRNA levels in PC3 and PC3-Nkx3.1 were measured in reference to the 22Rv1 cells. Hence, the relative quantification of 22Rv1 was set as 1. Interestingly, PC3-Nkx3.1 expressed high levels of *NKX3.1*. The mRNA expression level of *NKX3.1* in PC3 cells was even higher than in 22Rv1 cells. However, there was minimal or no change at all in the

expression of AR in the *NKX3.1* transfected cell line. The PC3-Nkx3.1 cell line was negative for the expression of AR. (**Figure 4.5**).

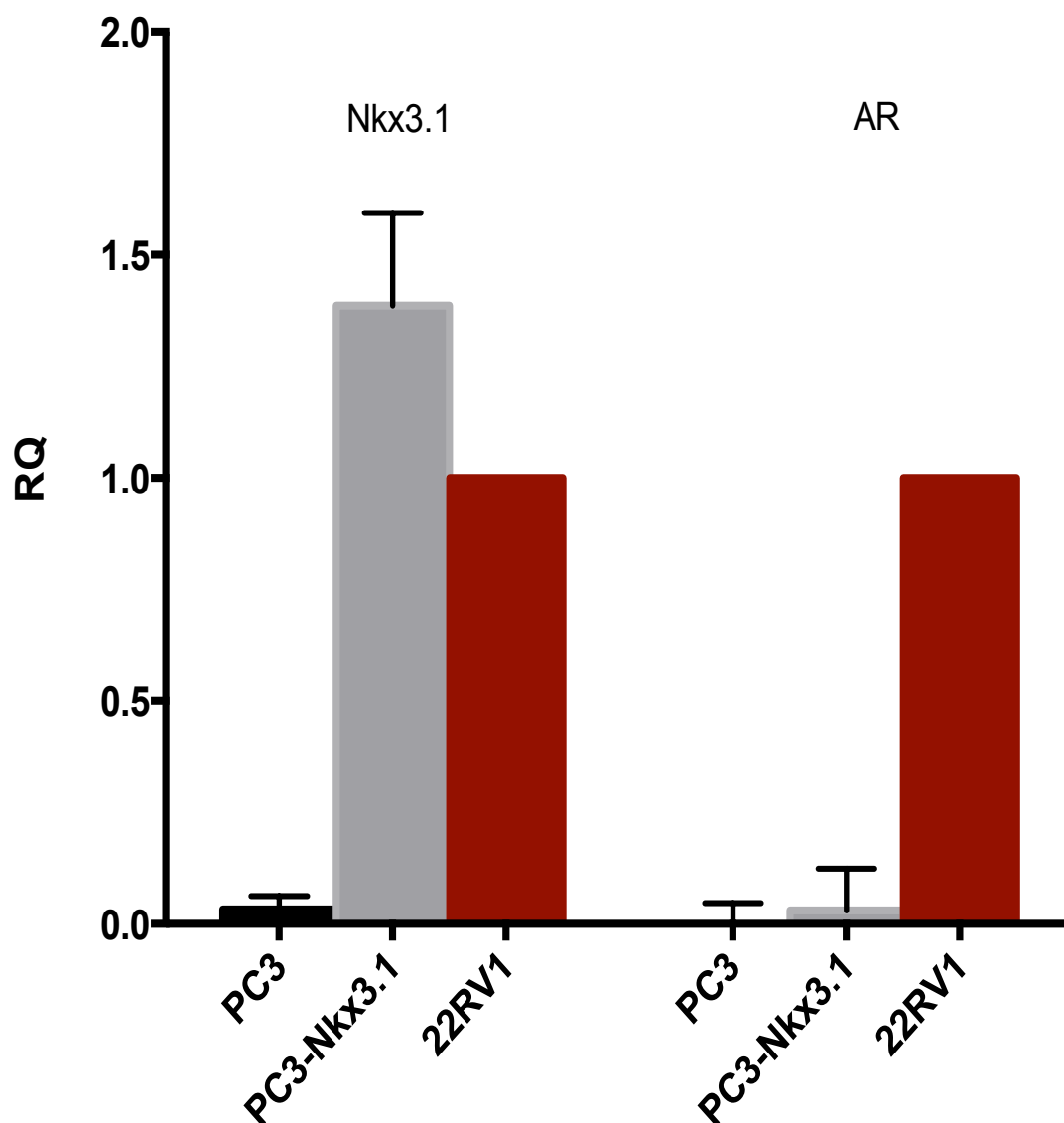


Figure 4.5

mRNA expression of *AR* and *NKX3.1* in PC3, PC3-Nkx3.1 and 22Rv1 cells. There was a dramatic increase in the mRNA level of *NKX3.1* but no change was observed in the expression of *AR* in PC3-Nkx3.1 cells compared to PC3 cells.

The stably transfected cells were stained with mouse monoclonal *NKX3.1* antibody. Strong protein expression was observed in the PC3-Nkx3.1 cell line.

PC3 cells were used as negative control (**Figure 4.6**).

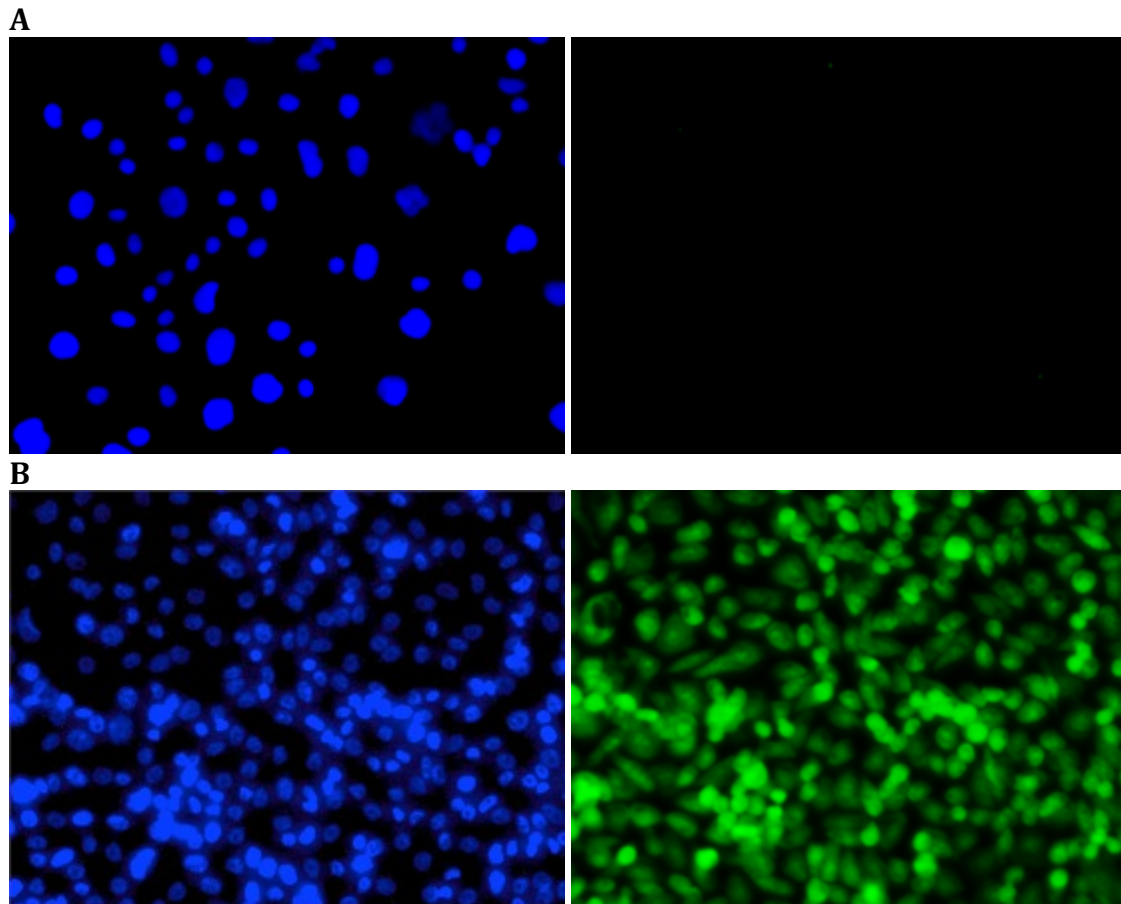


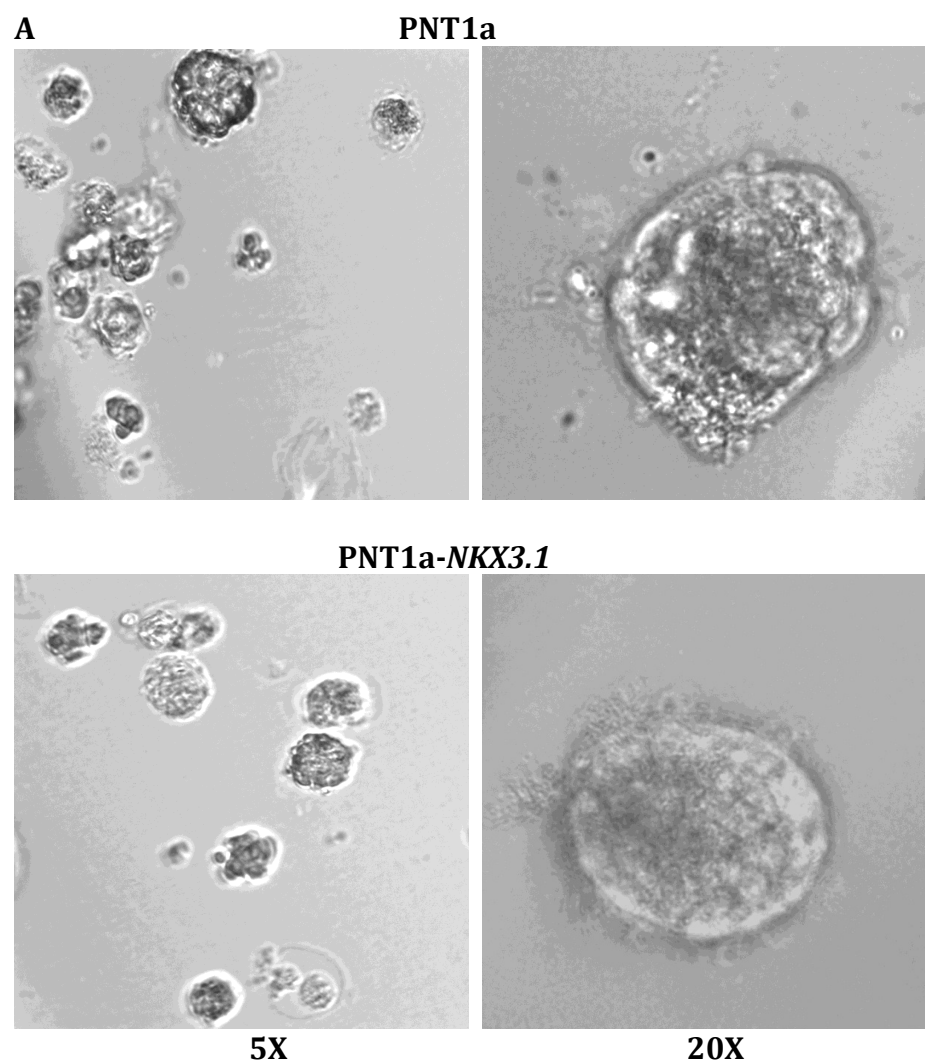
Figure 4.6

Immunofluorescent images of PC3 (A) and PC3-Nkx3.1 (B) stained with DAPI (blue) and NKX3.1 (green) under 20X magnification. PC3 cells are negative for NKX3.1 expression while strong protein expression was observed in PC3-Nkx3.1

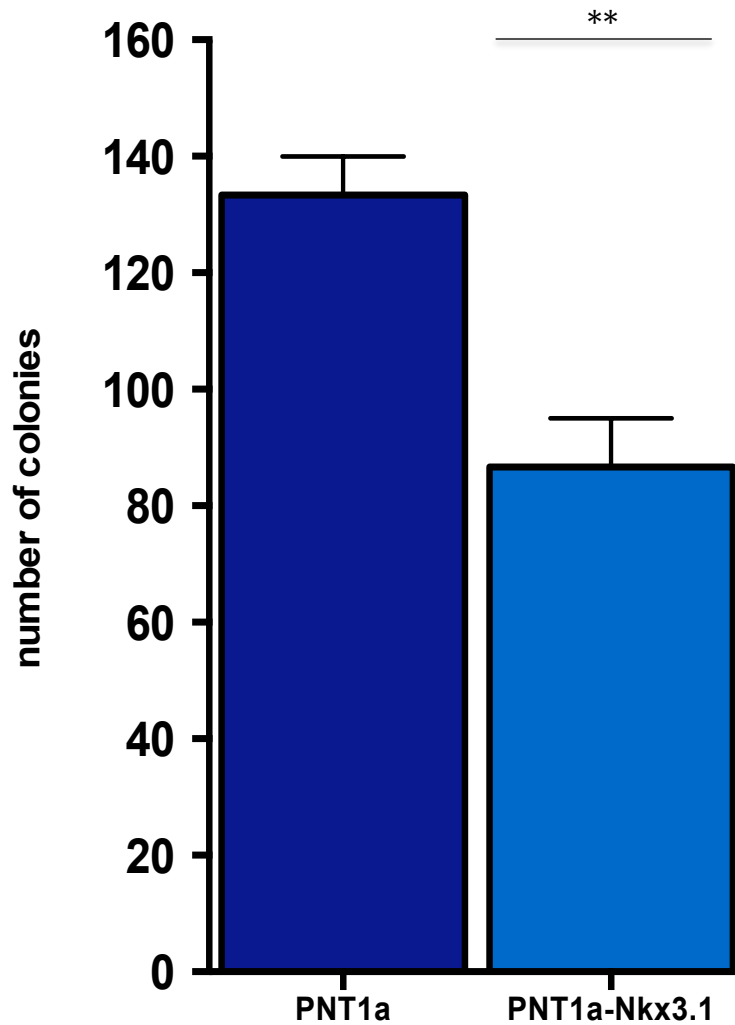
4.2.4 Effect of inducing *NKX3.1* expression on colony morphology in 3D

As discussed in Chapter 3, PNT1a and PC3 form intermediate colonies with loose structures and no defined borders. The transfected cells and the control cells were grown in triplicate in 1000-cell aliquots in Matrigel™:collagen mix in 96-well plates for 10 days. Although the transgene expression persisted for about a week, as mentioned earlier, the initiation of clones and differentiated lumens begins within hours of suspending cells in 3-dimensional culture. Major events that influence clonogenicity and differentiation would have occurred while the expression of *NKX3.1* was sustained in the cells.

PNT1a and PNT1a-*NKX3.1* cells were allowed to grow in 3- dimensional culture for 10 days and were photographed thereafter. The number and type of colonies within each well was assessed. No apparent difference was observed between the type of colonies formed by PNT1a and PNT1a-*NKX3.1* cells (**Figure 4.7A**). However, a significant decrease of 41.3% was observed in clonogenicity of PNT1a-*NKX3.1* cells as compared to the control PNT1a cells with a p-value of 0.0016 (**Figure 4.7B**).



B

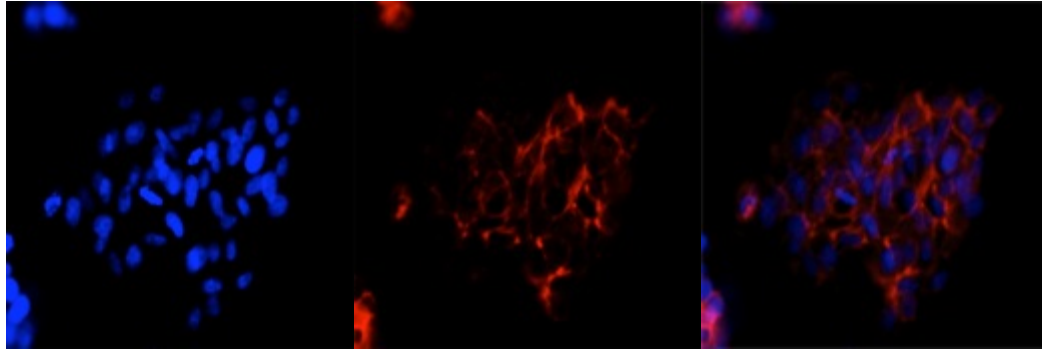
**Figure 4.7**

Effect on clonogenicity of PNT1a cells in 3D after transiently transfecting with *NKX3.1*. **A.** 5X and 20X representative images of colonies formed by PNT1a and PNT1a-*NKX3.1* cells in 3-dimensional culture. **B.** Bar graphs showing the number of colonies generated by PNT1a and PNT1a-*NKX3.1* cells.

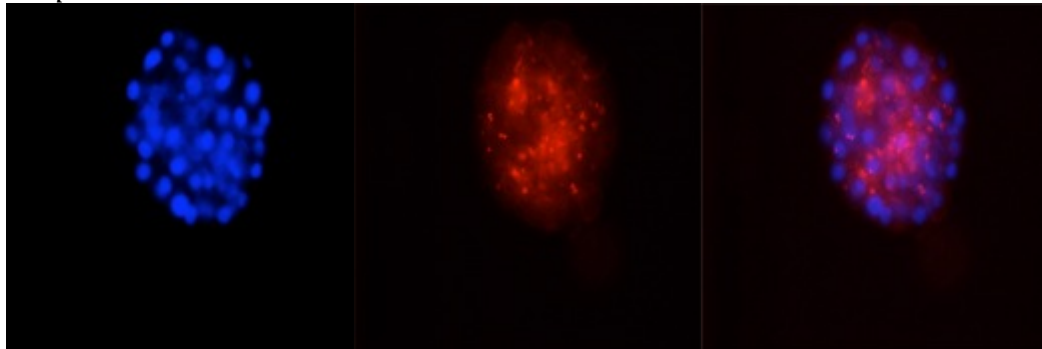
The same colony morphology experiment was carried out with PC3 and stably transfected PC3-Nkx3.1 cells. The experiment was performed in triplicate with both PC3 and PC3 cells transfected with control vector *pc*-DNA3.1 (PC3-*pc*DNA3.1). Both of these cell lines yielded comparable results and no apparent difference was observed between PC3 and PC3-*pc*DNA3.1 cells. The results shown in **Figure 4.8** and **4.9** are representative of PC3-*pc*DNA3.1. As previously shown in Chapter 3, PC3 cells mainly form intermediate colonies when grown in

3-dimensional culture (**Figure 4.8A**). Some more differentiated colonies were also observed in this cell line, which were spherical in shape (**Figure 4.8B**). However, PC3-Nkx3.1 cell line when grown in 3-dimensional culture was able to give rise to even more differentiated and polarized structures. This third type of colony formed by PC3-Nkx3.1 cells showed some level of polarization but F-actin staining revealed that there was no enrichment of F-actin staining in the luminal side of the colony (**Figure 4.8C**). Hence, these colonies were termed as “lumen-like”.

A. Intermediate



B. Spherical



C. Lumen-like

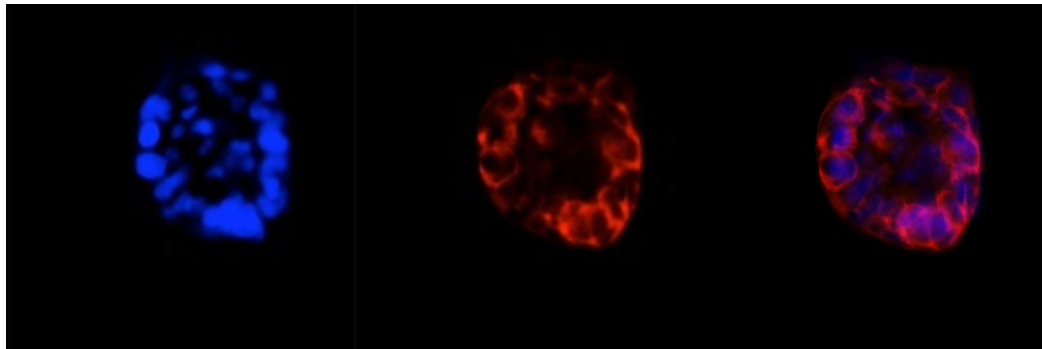
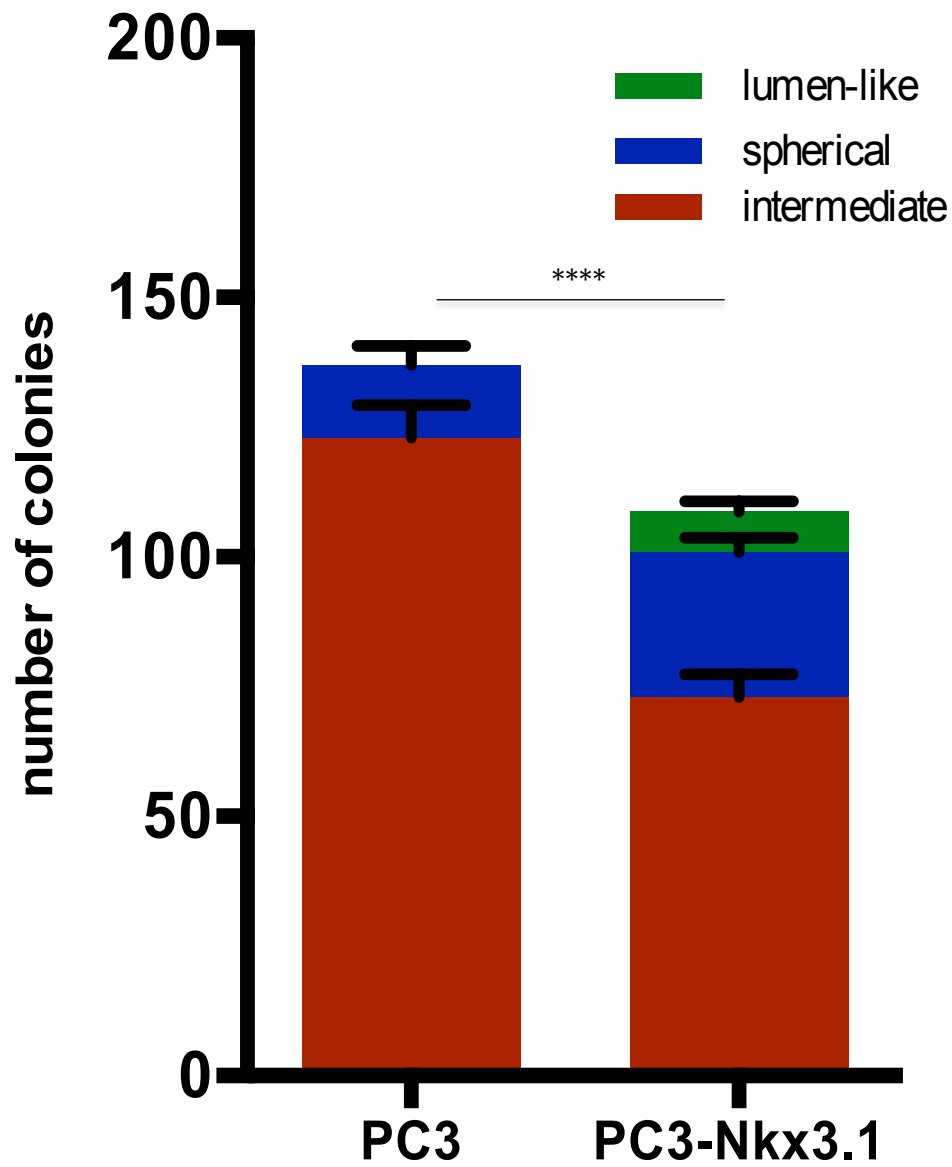


Figure 4.8

Representative images of different types of colonies formed by PC3 and PC3-Nkx3.1 cells in 3-dimensional culture. **A** and **B** are representative images of intermediate and spherical colonies found in PC3 and PC3-Nkx3.1 cell lines. **C** is a representative image of lumen-like colonies formed by PC3-Nkx3.1 cells only.

The total clonogenicity of PC3-Nkx3.1 cells as compared to PC3 cells in 3-D decreased by 26.3%. The total number of colonies formed by the two cell lines is shown in **Figure 4.9**. The number of spherical colonies in PC3-Nkx3.1 was doubled as compared to PC3 cells. 8 lumen-like colonies were observed in PC3-Nkx3.1 cells. If the spherical and lumen-like colonies were to be categorized as

more differentiated colonies, the proportion of more differentiated colonies increases from 10.2% in PC3 to 27.7% with a p-value<0.0001. The lumen-like colonies in PC3-Nkx3.1 comprised of roughly 8% of the total number of colonies. The functional effect of transfecting PC3 and PNT1a cell lines with *NKX3.1* was clearly observed by the increase in differentiation capacity and decrease in clonogenicity of the transfected cell lines.



	<i>Intermediate</i>	<i>Spherical</i>	<i>Lumen-like</i>	<i>Total number of colonies</i>	<i>p-value</i>
PC3	123	14	0	137	<0.0001
PC3-Nkx3.1	73	28	8	101	

Figure 4.9

Total number of colonies and proportion of different colonies in PC3 and PC3-Nkx3.1 cell lines.

4.2.5 PC3 cells stably transfected with NKX3.1 exhibit different cell morphologies as compared to wild type PC3 cells

While culturing the PC3 and PC3-Nkx3.1 cells, a difference in their colony morphology was observed. PC3 cell line has a heterogeneous population of cells where cells vary not only in morphology but also in size. These subcellular clones have previously been observed but not characterized in terms of the underlying differences other than their morphology (Hirokawa, Y. *et al.*, 2007).

To enhance the difference in cell morphology and make it more visible, PC3 and PC3-Nkx3.1 cells were stained with Cam5.2, which is an epithelial cell marker and Rhodamine-phalloidin, which stains F-actin (**Figure 4.10**). Cam5.2 shows cytoplasmic staining and F-actin marks the boundary of the cell. The nuclear stain used was DAPI.

The two different cell morphologies observed in PC3 cells were: round cells and spindle shaped mesenchymal-like cells. From now on the spindle-shaped cells with large nuclei will be referred to as big cells and the rounded cells will be referred to as small cells. The PC3-Nkx3.1 cells on the contrary showed a homogeneous cell population of all small round cells (**Figure 4.10**).

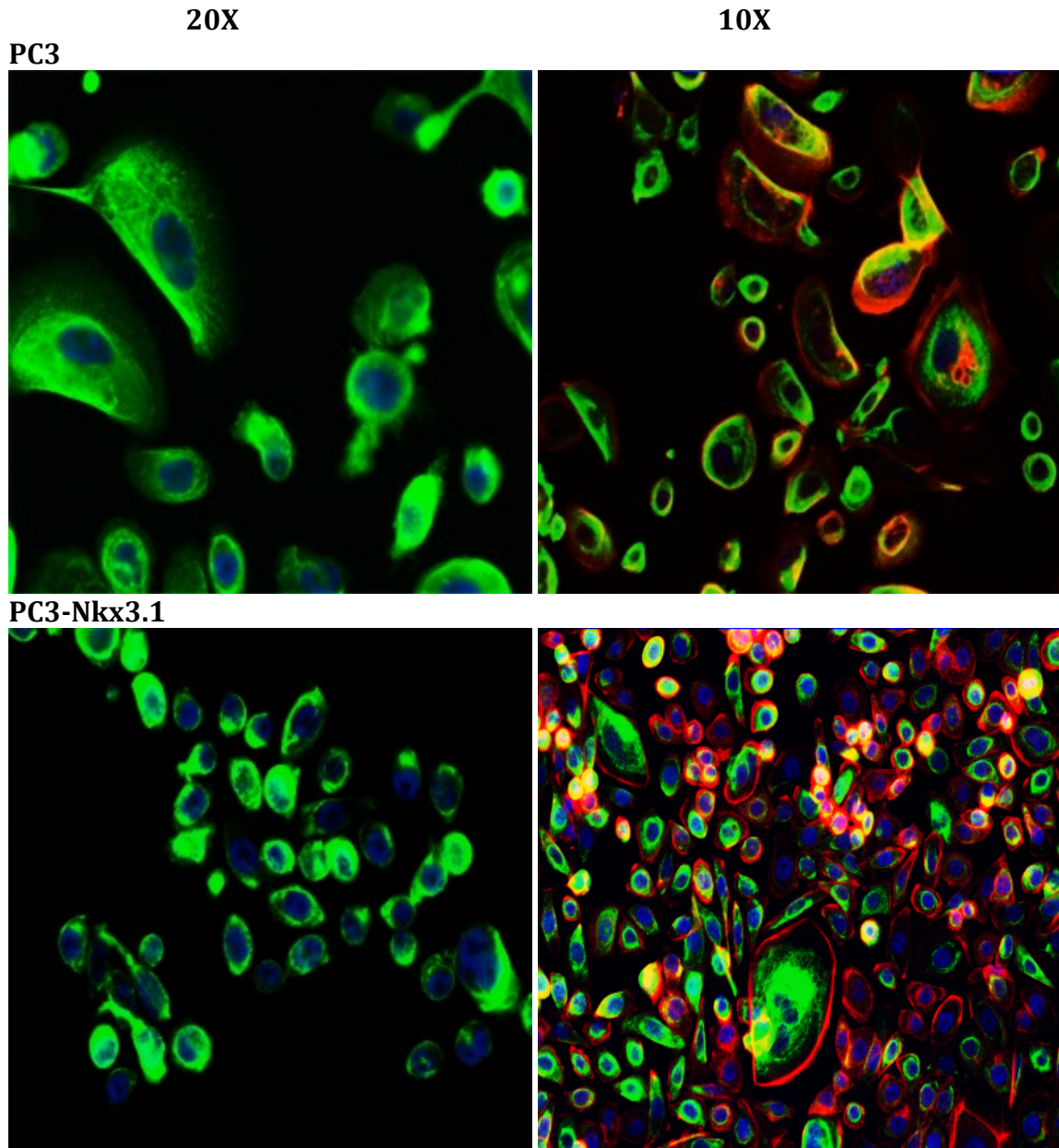


Figure 4.10

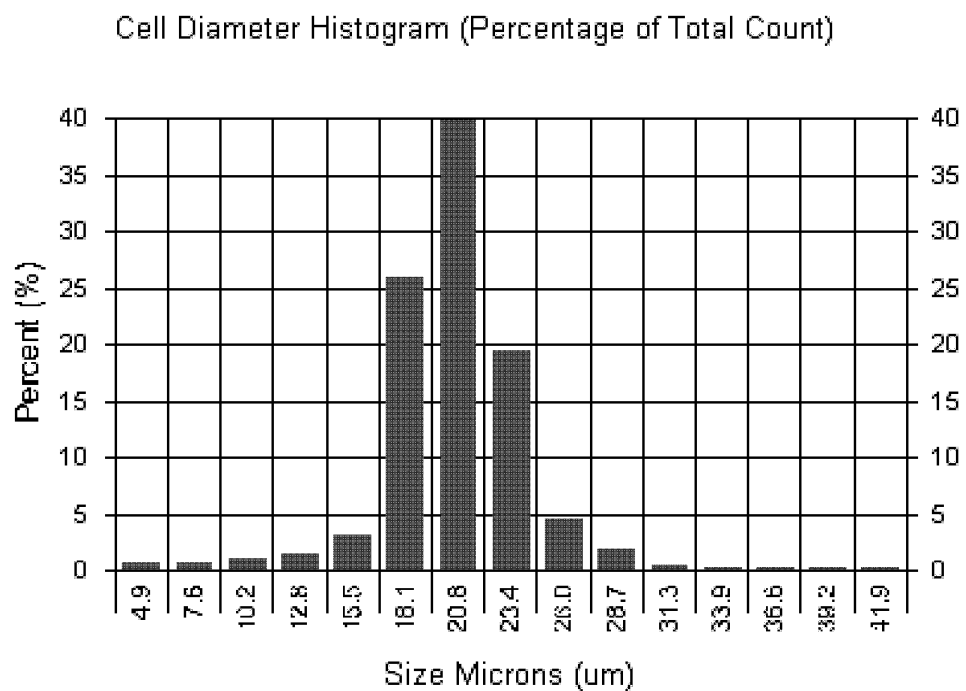
PC3 and PC3-Nkx3.1 cells stained with Cam5.2 (green), F-actin (red) and DAPI (blue).

To quantify the difference in size 1000 cells of each cell line were analyzed using Cellometer auto T4. About 40% of the PC3 cells had a mean diameter of 20.8 μ M and 18% of the cells had a diameter of 23.4 (**Figure 4.11A**). About 25% of the PC3 cells had a mean diameter of 18.1 μ M. Some cells (~5%) have a diameter as large as 26 μ M.

22% of the PC3-Nkx3.1 cells have a diameter of only 15.6 μ M, 28% have a diameter of 17.1 and 24% of the cells have a diameter of 18.6 μ M (**Figure 4.11B**). 7-8% of the cells have a diameter of 20.3 μ M, which is similar to the big cell diameter in the PC3 cell line.

A.

Mean Diameter = 20.6



B.

Mean Diameter = 17.3

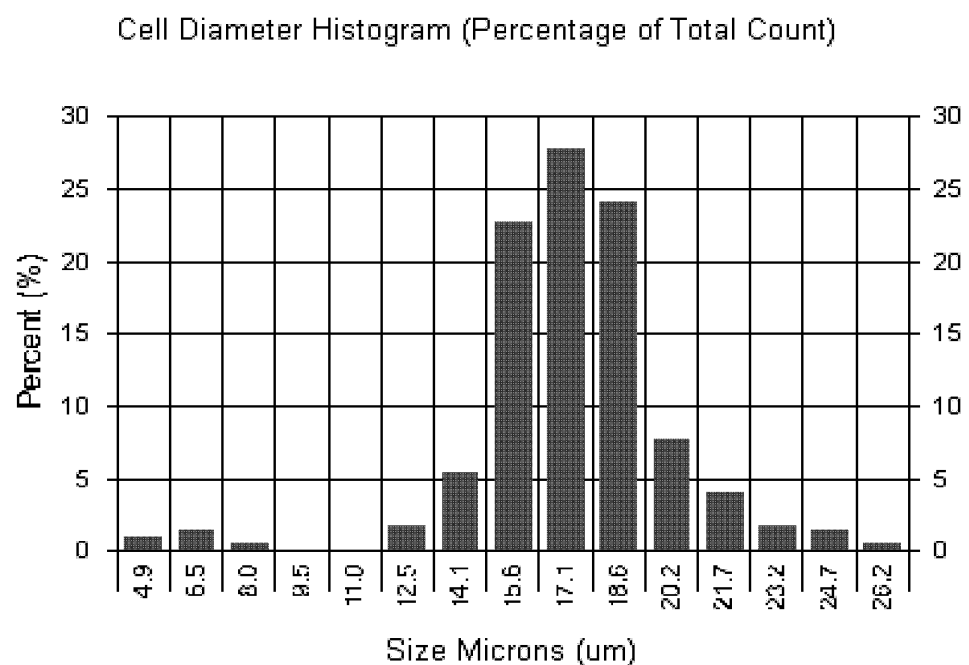
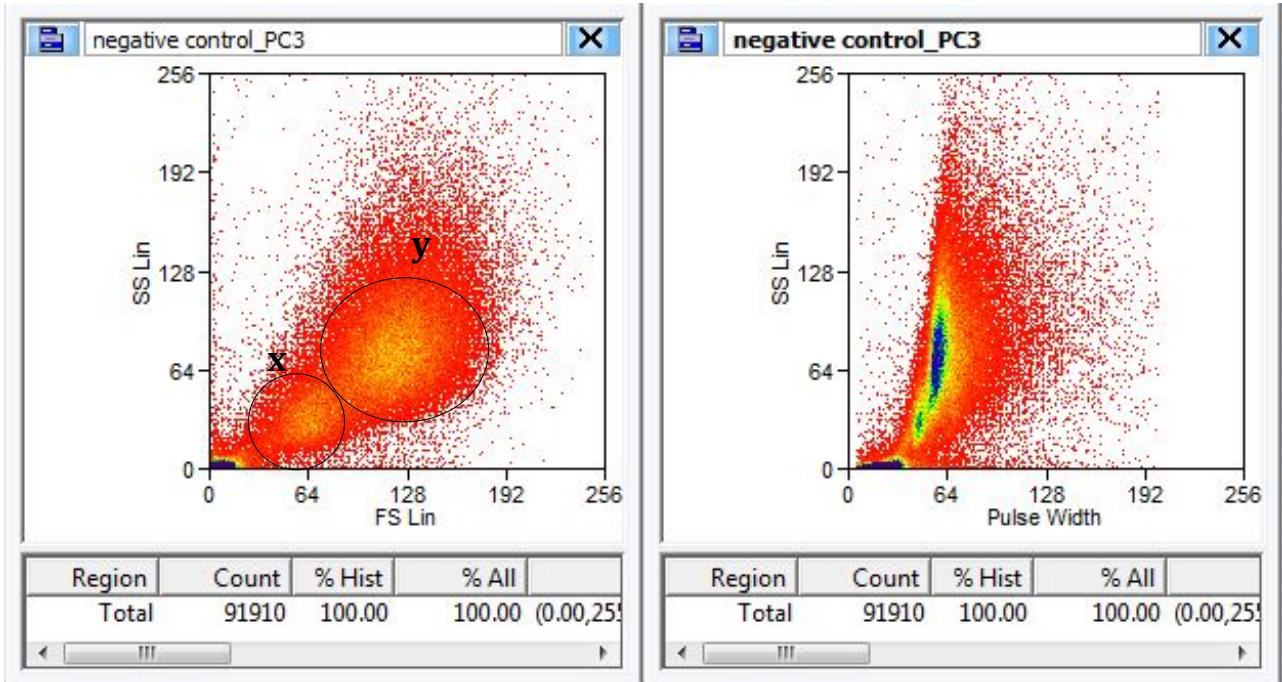


Figure 4.11

Cell diameter histograms of PC3 (A) and PC3-Nkx3.1 (B) cells obtained from Cellometer Auto T4.

Furthermore, flow cytometry was carried out on the two cell lines to sort the cells based on size. The two different cell sizes observed in PC3 cells were also observed in side-scatter versus forward-scatter plots and also in side-scatter versus pulse width. Side-scatter is indicative of the granularity of the cell, forward-scatter is indicative of the size of the cell and pulse width denotes the time taken for a cell to pass through the filter. Two different populations were observed in the side-scatter versus forward-scatter plot for PC3 cells (**Figure 4.12**). The cells on the bottom left with low side-scatter and forward-scatter are the smaller cells (marked with **circle x**), while the population on the top right with high forward-scatter is the cells bigger in size (marked with **circle y**) (**Figure 4.12**). This difference in morphology is also clearly seen on the side-scatter versus pulse-width plot with two distinct populations; one with low pulse width and side-scatter and another with higher pulse width and a wide range of side-scatter.

A.



B.

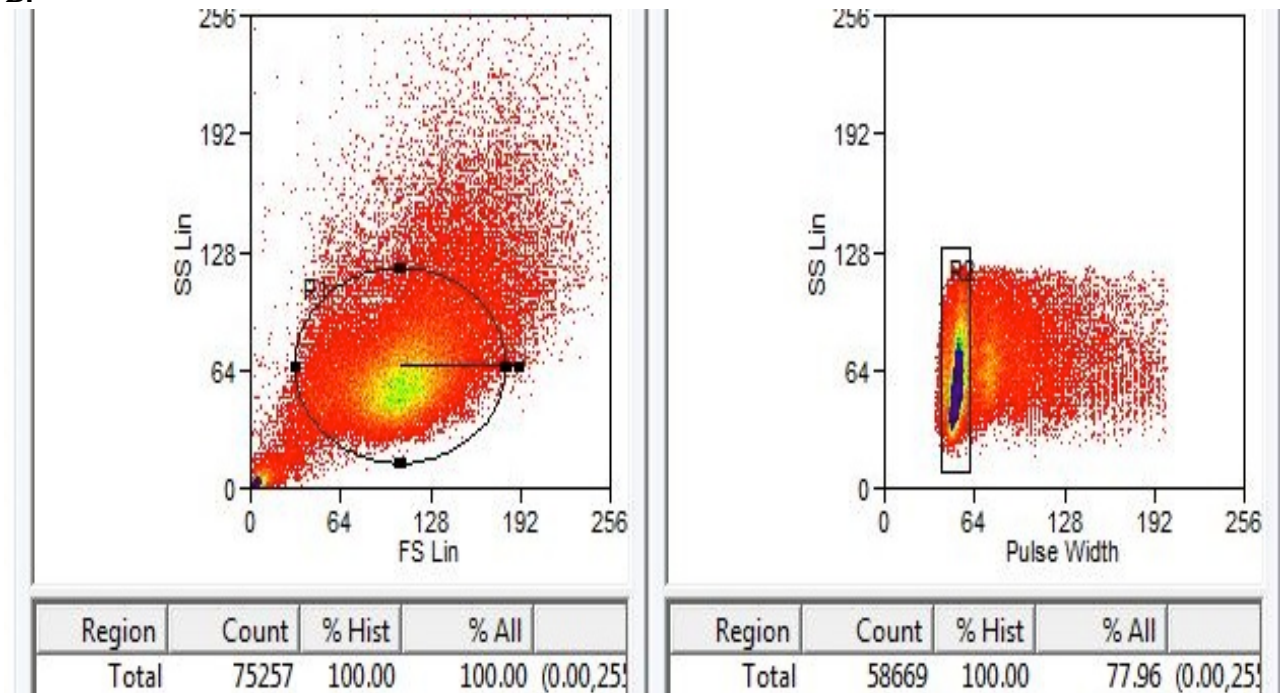


Figure 4.12

Size differentiation of PC3 and PC3-Nkx3.1 cells based on flow cytometry images generated using Summit 4.2 software. Unstained populations of PC3 (A) and PC3-Nkx3.1 (B) cells. A. PC3 cells show a bimodal distribution on the side-scatter vs. forward-sector plot. The population circled on the left (x) represents the smaller cells where as the population on the right represents the bigger cells (y). B. No bimodal distribution was observed in the PC3-Nkx3.1 cells.

To determine whether the categorization of small and big cells determined on the basis of these plots was valid or not, these cells were sorted and observed under the microscope. Although a few small cells were noticed in the big cells population, the sorted populations were more or less uncontaminated by the other cell type.

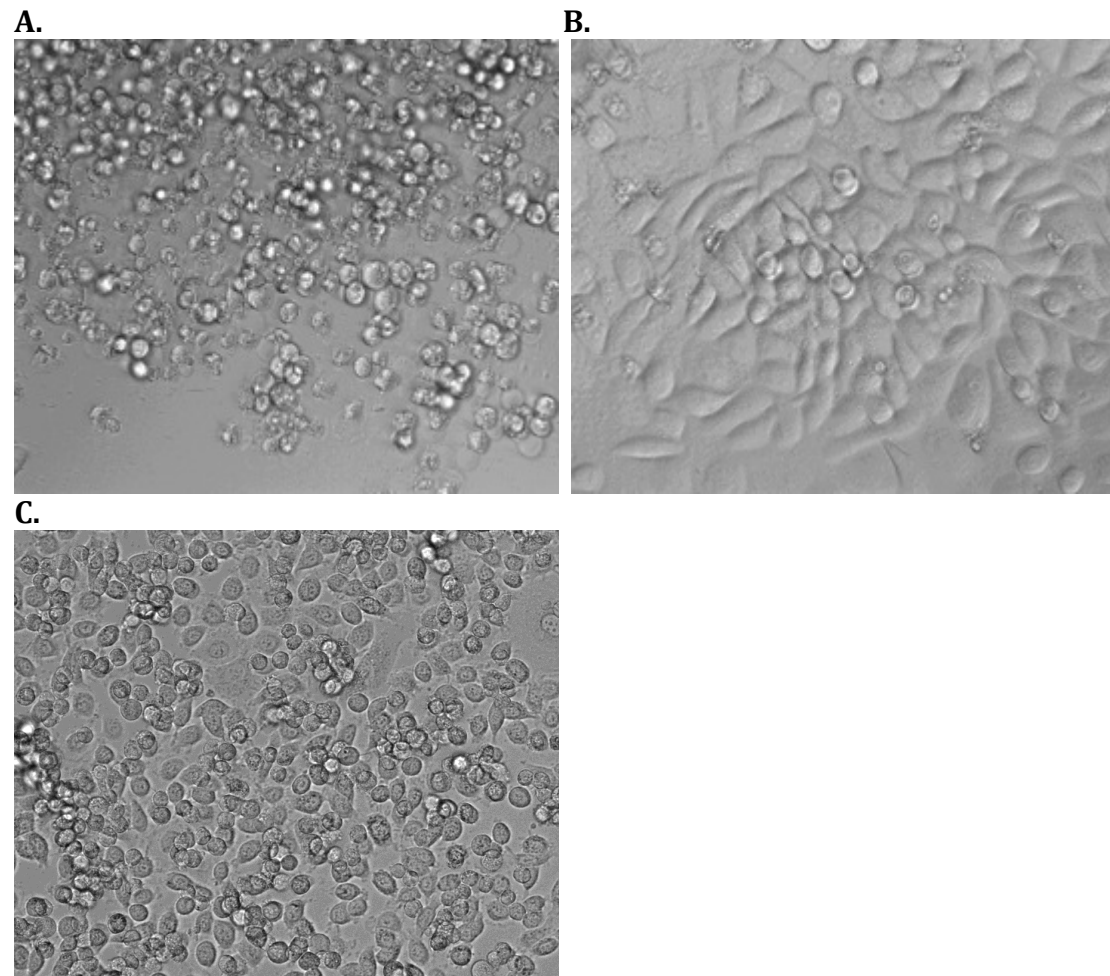


Figure 4.13
20X phase contrast images of PC3 and PC3-Nkx3.1 sorted cells. **A.** Small cells sorted from circle **x** in **Figure 4.12**. **B.** Big cells sorted from circle **y**. **C.** PC-*NKX3.1* cells sorted from gate R2 in **Figure 4.12B**.

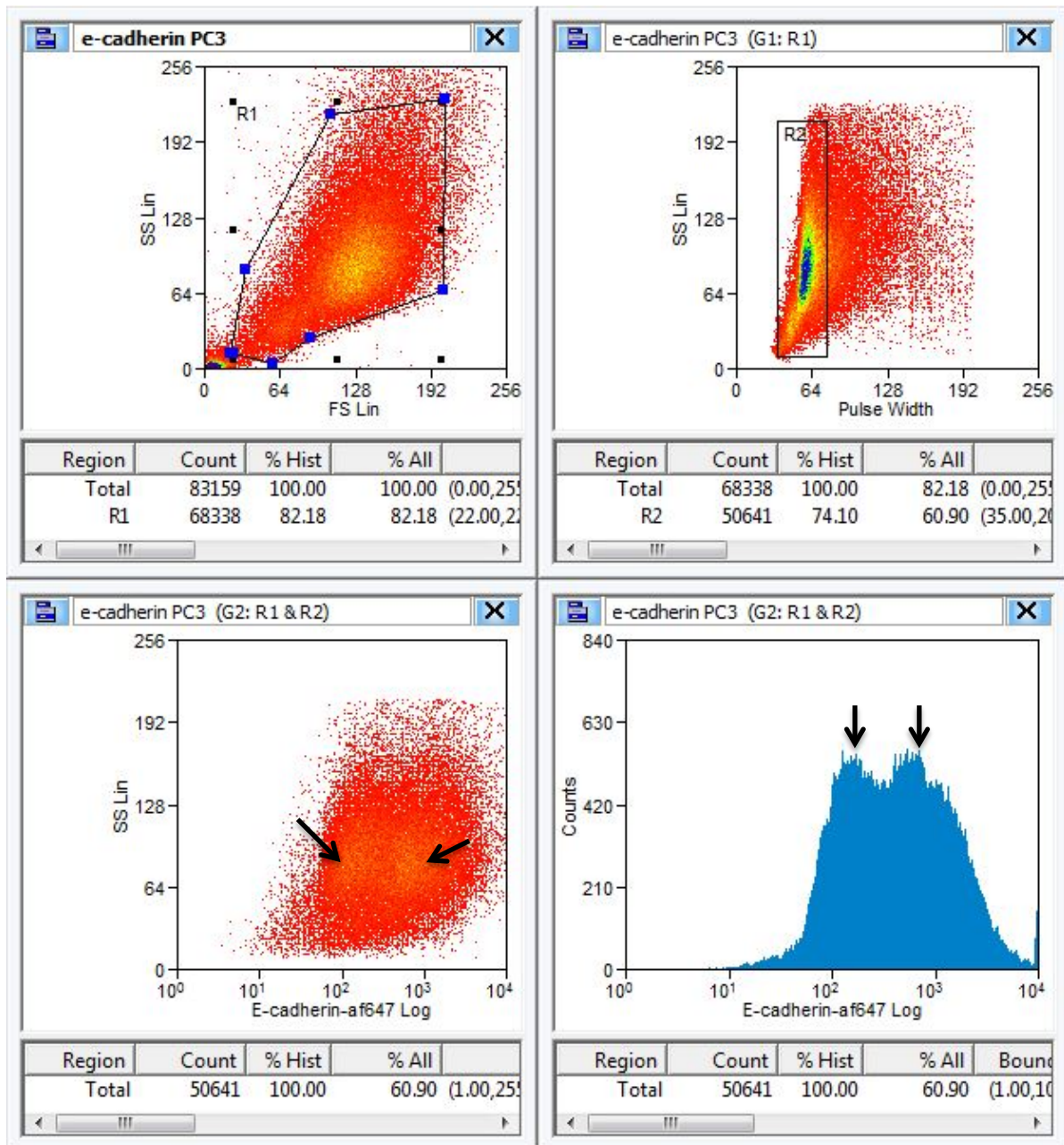
4.2.6 Expression of E-Cadherin markers in PC3-Nkx3.1 and PC3 cells

The difference in cell morphologies of PC3 and PC3-Nkx3.1 leads to two hypotheses: 1.) transfecting PC3 cells with *NKX3.1* selects for the smaller cell morphology or 2.) transfection with *NKX3.1* induces a “process” by which the heterogenous population of PC3 is driven towards differentiation or epithelial to mesenchymal transition (EMT). In canine prostate cancer, the expression of *NKX3.1* and E-cadherin is either reduced or lost (Fonseca-Alves, CE. *Et al.*, 2013). Overexpression of vimentin is also reported in canine prostatic carcinoma (Rodrigues, MM. *et al.*, 2011). While definitive evidence of physiologic EMT is still lacking in human prostate cancer, some findings indicate that EMT might be an important event in prostate cancer progression. Hence, the second hypothesis associating *NKX3.1* with differentiation and EMT was studied further.

PC3 and PC3-Nkx3.1 cells were stained with mouse monoclonal E-cadherin antibody and flow analysis was performed to check the expression level in these cell lines. Gate R2 was set on side-scatter versus pulse width graph in both **Figure 4.14A** and **Figure 4.14B** to exclude cell doublets and clumps. PC3 cells showed a bimodal distribution of E-cadherin expression with one population expressing higher levels of E-cadherin than the other (**Figure 4.14A**). This distribution is apparent in the side-scatter versus forward-scatter plot and also in the E-cadherin histogram. Interestingly, there was an enrichment of E-cadherin high cells in the PC3-Nkx3.1 cell line (**Figure 4.14B**). The bimodal distribution of E-cadherin expression seen in PC3 cells was absent in PC3-Nkx3.1 cells and majority of the PC3-Nkx3.1 cells expressed higher levels of E-cadherin. This suggests that *NKX3.1* transfection possibly drives the mesenchymal like cells in PC3 towards epithelial-like characteristics. The increase in E-cadherin

expression might be the reason for increase in polarization and differentiation of PC3-Nkx3.1 cells in 3-dimensional culture.

A.



B.

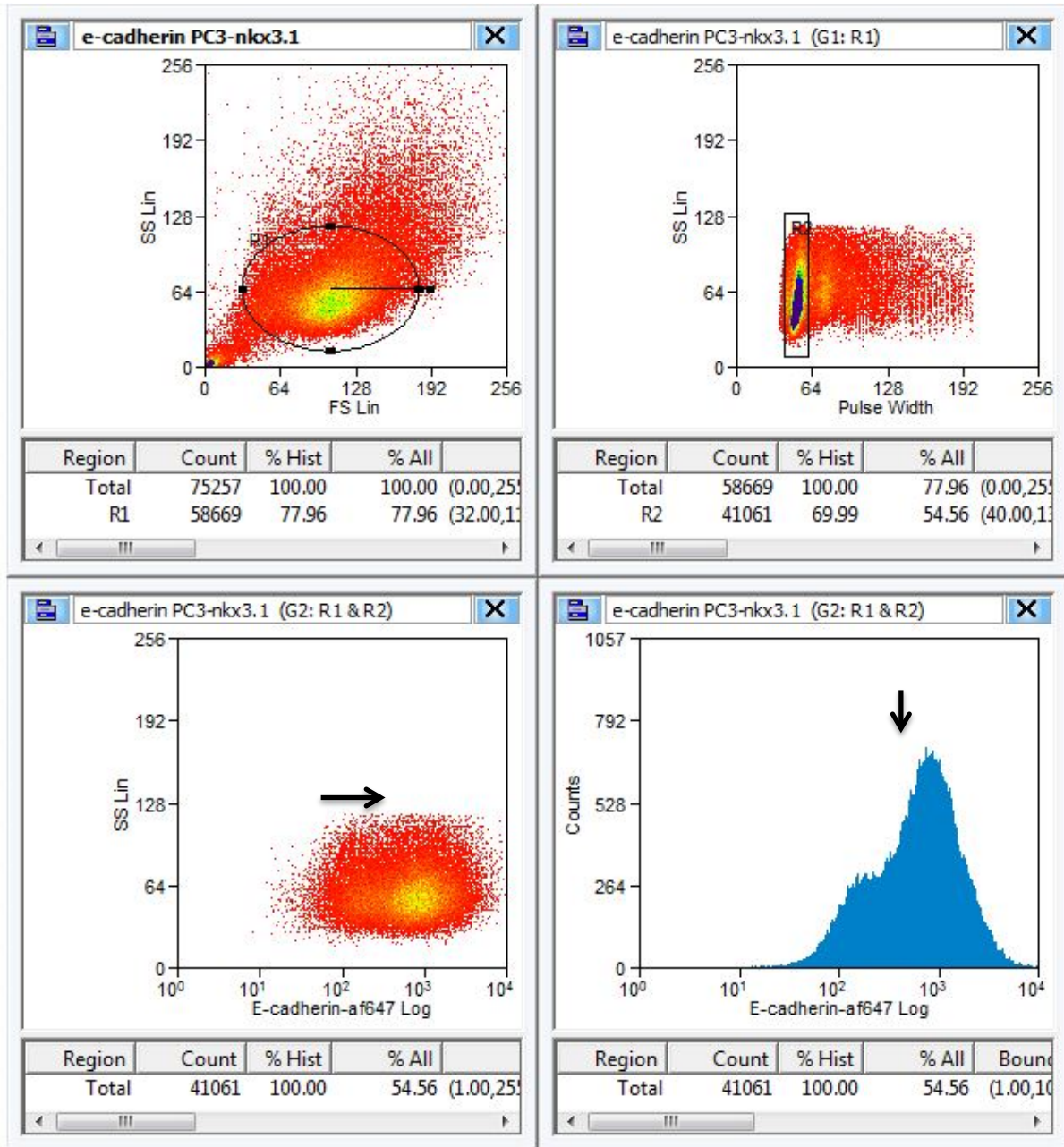


Figure 4.14

Different expression patterns of E-cadherin expression in PC3 and PC3-Nkx3.1 cells. The expression profile of E-cadherin in PC3 cells is bimodal which is clearly seen in the histogram (A). The expression profile of PC3-Nkx3.1 cells is also bimodal with an enrichment of cells with higher expression of E-cadherin. These graphs are representative images of 3 different experiments.

4.2.7 Expression of EMT markers in *NKX3.1* positive and negative cell lines

There have been no published studies establishing the role of *NKX3.1* in EMT in prostate cancer. To investigate whether or not expression of *NKX3.1* correlates with the expression of EMT markers, microarray analysis was performed on the panel of cell lines. This analysis is based on the hypothesis that *NKX3.1* expression might result in repression of EMT associated gene expressions and increased expression of some epithelial genes. Loss of E-cadherin expression is a key event in EMT along with increase in expression of the fibroblast marker Vimentin. Transcription factors ZEB1 and SNAI2 are known repressors of E-cadherin (*CDH1*) (Romero-Perez, L. *et al.*, 2013, Wang, Y. *et al.*, 2013). EZH2, also a transcription factor, has been identified as an E-cadherin repressor in breast cancer (Yu, H. *et al.*, 2012) and bladder cancer (Luo, M. *et al.*, 2013). A switch from E-cadherin to N-cadherin expression indicates EMT and has a strong correlation with prostate cancer progression (Gravdal, K. *et al.*, 2007). Fibronectin is also an important mesenchymal marker (Zeisberg, M. *et al.*, 2009). A list of differentially expressed genes in *NKX3.1* positive and negative cell lines was generated using Partek software. Expression of E-cadherin was approximately 2.5 times less in *NKX3.1* negative cell lines as compared to the *NKX3.1* positive cell lines with a p-value of 0.0002 (**Table 4.2**). N-cadherin expression, on the other hand, is 9.7 times higher in *NKX3.1* negative cell lines. The inverse correlation between the expression of E-cadherin and N-cadherin is also demonstrated in the prostate cell lines. The expression of Vimentin is increased 9 times with a p-value of 0.005 and the expression of Fibronectin is increased 7 times in the *NKX3.1* negative cell lines as compared to *NKX3.1* positive cell lines. The expression profile of all the epithelial and mesenchymal

markers (most importantly E-cadherin) in the panel of cell lines strongly suggests that *NKX3.1* expression is associated with suppression of EMT.

The expression levels of E-cadherin repressor ZEB1 are higher in *NKX3.1* negative cell lines, which also express low levels of E-cadherin. However, no differential expression was observed for SNAI1 and EZH2. The fold change was less than 2 times and the p-value was greater than 0.05, rendering the difference insignificant.

	Fold change (log values)	P-value
E-cadherin ↑	2.46	0.0002
N-cadherin	-9.65	0.0028
Vimentin	-9.03	0.0049
Fibronectin	-7.03	0.0129
ZEB1	-4.45	0.0264
Snail1	-1.04	0.7768
EZH2	-1.68	0.3458

Table 4.2

Fold change of epithelial and mesenchymal markers in *NKX3.1* positive versus negative cell lines. A positive fold change means that the gene is up regulated in *NKX3.1* positive cell lines as compared to *NKX3.1* negative cell lines and negative fold change means down regulation.

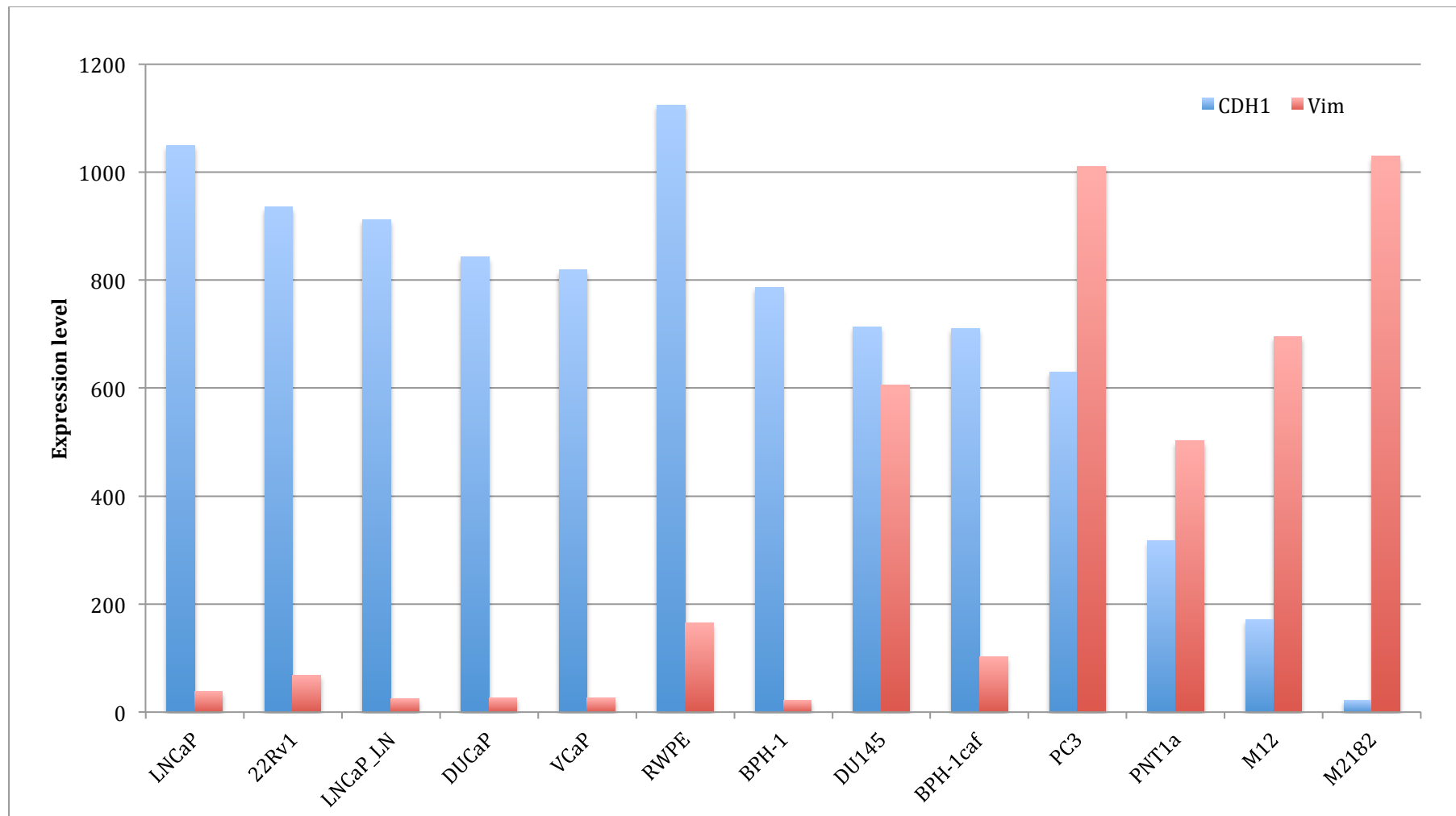


Figure 4.15
mRNA expression of *CDH1* and *VIM* in prostate cell lines.

The expression levels of the two key EMT markers, CDH1 and VIM are shown in **Figure 4.15**. All the *NKX3.1* negative cell lines express low levels of VIM and high levels of CDH1, however, some of the *NKX3.1* negative cell lines, namely BPH-1, BPH-1^{caf} and RWPE, also express low levels of VIM. BPH-1 and BPH-1^{caf} have been derived from benign prostate hyperplasia (BPH) and RWPE is derived from a normal prostate. Because of the origin of these cell lines there maybe various molecular level differences between these and cancer cell lines which may be why in spite of being *NKX3.1* negative, these cell lines still exhibit epithelial like characteristics.

DU145 and PC3 cell lines express high levels of CDH1 along with VIM. The ratio of CDH1 to VIM is more informative rather than looking at the individual expression levels of these markers. The ratio of CDH1 to VIM is shown in **Table 4.3**. DU145, PC3, PNT1a, M12 and M2182 cells have a CDH1 to VIM ratio less than 2 and hence exhibiting mesenchymal-like characteristics.

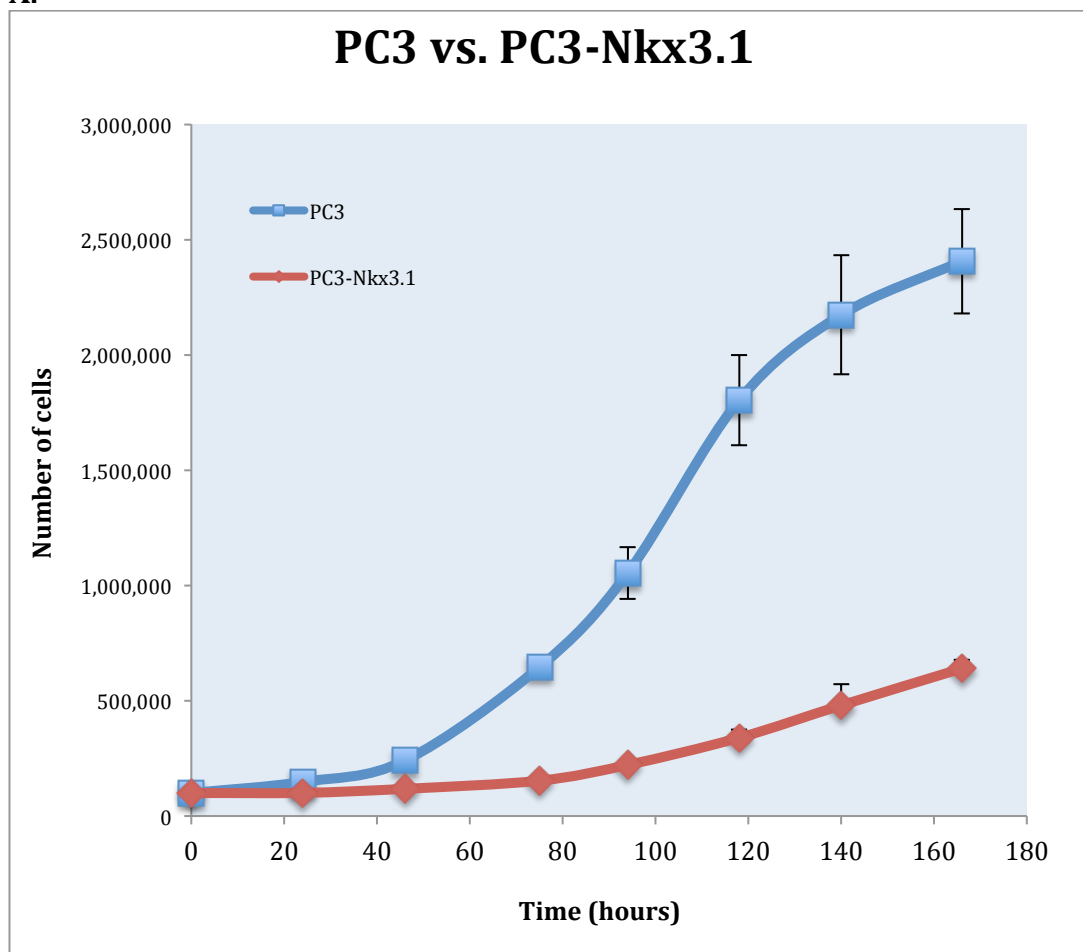
Cell line	CDH1:VIM
LNCaP	27.164
LNCaP_LN3	35.791
22RV1	13.692
VCaP	31.687
DUCaP	31.118
RWPE	6.8162
BPH-1	34.831
BPH-1^{caf}	6.9479
DU145	1.1783
PC3	0.6227
PNT1a	0.6323
M12	0.2463
M2182	0.0218

Table 4.3

The ratio of mRNA expression levels of CDH1 to VIM in prostate cell lines.

4.2.8 Growth suppression by *NKX3.1* in PC3 cells

Cell growth assays were performed on wild type PC3 cells and PC3-Nkx3.1. In vitro growth of the wild type PC3 (pcDNA3.1) cells and PC3-Nkx3.1 cells was assessed by cell counting over a period of 7 days. The exponential phase of the *NKX3.1* expressing PC3 cells was delayed by approximately 29 hours. Even for the cells in exponential phase, as shown in **(Figure 4.16)**, *NKX3.1* expression resulted in decreased cell proliferation and elongated the doubling times.

A.

B.

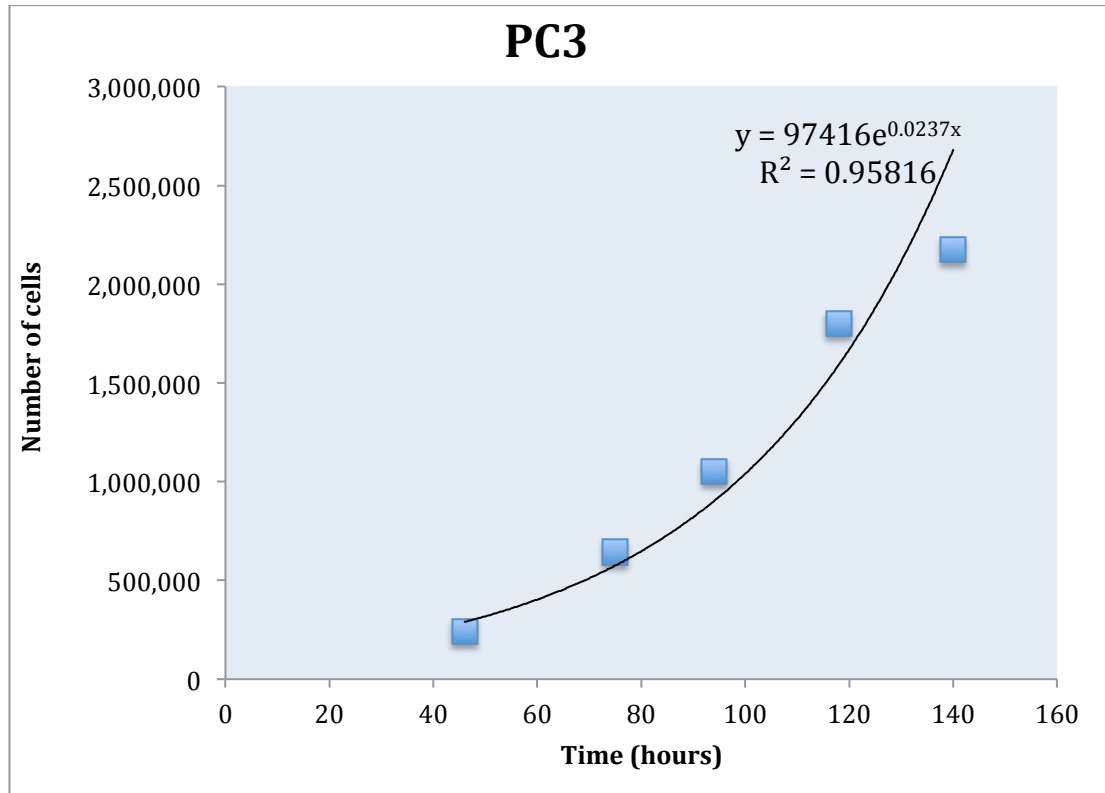
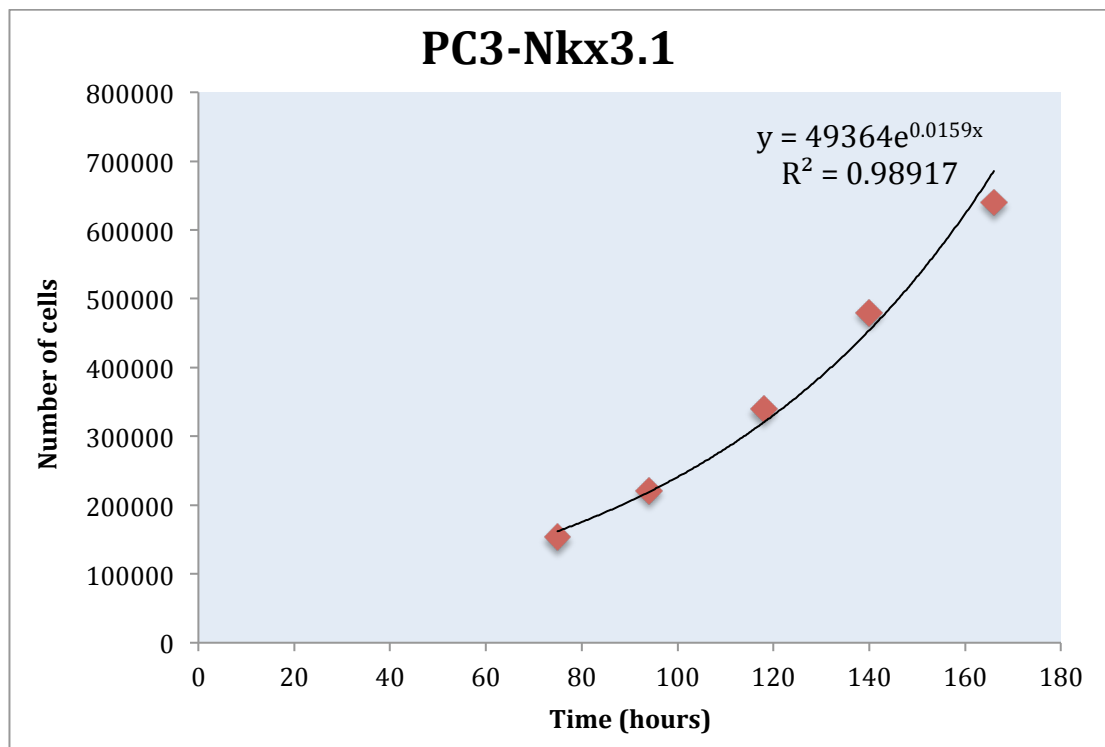


Figure 4.16

A. Growth curve of PC3 and PC3-Nkx3.1 over a period of 7 days shows that the growth rate of PC3-Nkx3.1 cells is much lower than the wild type PC3 cells. **B.** Individual plots of growth curves of PC3 and PC3-Nkx3.1 cells in their exponential phase.

The doubling times of the PC3 wild type cells and PC3-Nkx3.1 were calculated from the growth curve of the cells when they are in their exponential phase (figure 4.6B). The doubling times of both cells lines are shown in **Table 4.4**. PC3 cells expressing *NKX3.1* had a doubling time almost 50% higher than that of the parental PC3 cells.

<i>Cell line</i>	<i>PC3</i>	<i>PC3-Nkx3.1</i>
<i>Doubling time (hours)</i>	29.25	43.59

Table 4.4

The doubling times of PC3 and PC3-Nkx3.1 cells calculated based on the growth curve in the exponential phase.

4.2.9 Differentially expressed genes in PC3 and PC3-Nkx3.1 cells

Microarray analysis on the gene expression of PC3 and PC3-Nkx3.1 cell lines obtained by Affymetrix- GeneChip® Human Exon 1.0 ST array. mRNA expression levels of *NKX3.1* in PC3-Nkx3.1, as expected, was 28.204 times higher than in PC3 cells. An increase in expression of *HOXB13* in PC3-Nkx3.1 cells was approximately 8.861 as compared to control PC3 cells. *FOXA1* is overexpressed 5.398 times in PC3-Nkx3.1 in comparison to PC3 cells. These expression levels agree with the findings stated above. However, the mRNA expression levels of *CDH1* was surprisingly the same in PC3 and PC3-Nkx3.1, where the expression in PC3-Nkx3.1 was 1.1 times higher than in PC3 cells. This contradicts the protein expression levels of CDH1 in the two cell lines as observed by FACS analysis. The inconsistency in the mRNA and protein levels of CDH1 in the two

cell lines could be attributed to various reasons. It is possible that induction of expression of *NKX3.1* directly or indirectly stabilizes the CDH1 protein. It is also possible that absence of *NKX3.1* expression mis-localizes CDH1 to the cytoplasm and hence, the differential expression of CDH1 in FACS analysis of PC3 and PC3-Nkx3.1 cells.

Interestingly, the expression levels of *VIM* and *ZEB1* were much higher in PC3 cells than in PC3-Nkx3.1 with a fold change of approximately 9.681 and 9.508 respectively. This observation matches the analysis of *NKX3.1* positive and negative cell lines, where the mesenchymal markers were down regulated in *NKX3.1* positive cell lines. The reduction in expression levels of mesenchymal markers after *NKX3.1* transfection suggests that *NKX3.1* might play a role in inhibiting EMT.

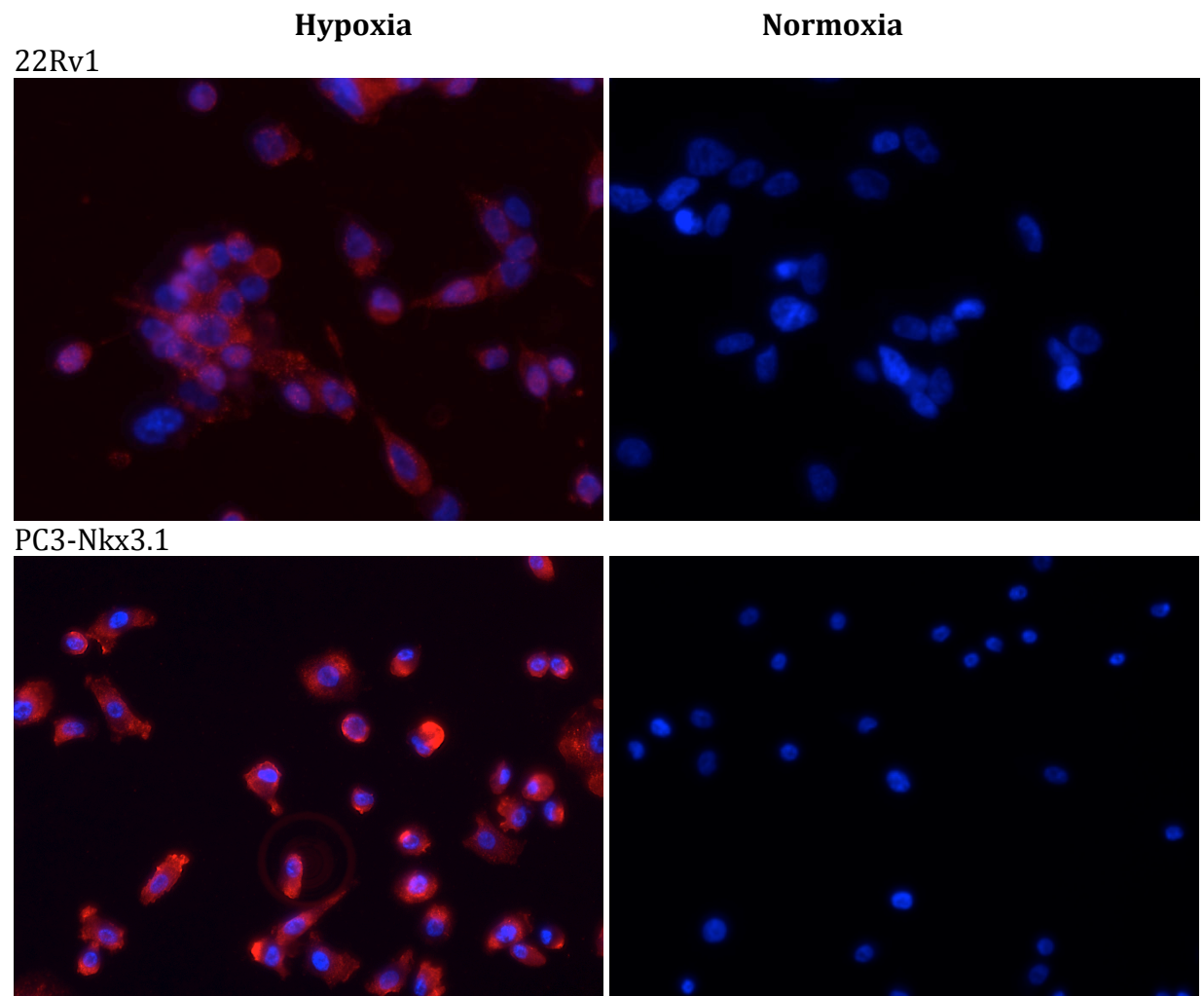
A list of other key genes up regulated in PC3-Nkx3.1 is listed in **Table 1b** (Appendix). The gene expression levels in these two cell lines was normalized with the entire panel of prostate cell lines using Partek Software.

4.2.10 Effect of hypoxia on expression of NKX3.1

HIF1a is considered to be the main molecular mediator of hypoxic effects (Dery, MA., *et al.*, 2005). It has been shown in colorectal cancer that colonies grown in normoxia express high levels of CDX1 (Yeung, T., *et al.*, 2010). Furthermore, it has been shown by Bodmer lab that hypoxic colonies show reduction in expression of CDX1. Since the role of *NKX3.1* in differentiation in the prostate has been shown to be parallel to that of CDX1 in the colon, the effect of hypoxia

on the expression of *NKX3.1* was investigated. Two *NKX3.1* positive cell lines, 22RV1 and PC3-Nkx3.1, and two *NKX3.1* negative cell lines PNT1a and PC3 were selected for this experiment.

To study the specific effect of hypoxia on differentiation, single cell suspensions were allowed to grow in Matrigel™ for 2 weeks in an hypoxic chamber (1% oxygen). Cells were also grown on plastic and stained with HIF1a mouse monoclonal antibody at 0 hour, 24 hours, 48 hours and 72 hours. HIF1a activation in hypoxic conditions was observed in all four cell lines after growing in hypoxia for 48 hours (**Figure 4.17**). Furthermore, the expression of HIF1a was mainly perinuclear and cytoplasmic. HIF1a staining in PNT1a cells was heterogeneous with some cells expressing high levels of HIF1a and some expressing low levels. The cells with smaller nuclei expressed higher levels of HIF1a and the ones with bigger nuclei expressed low levels of HIF1a.



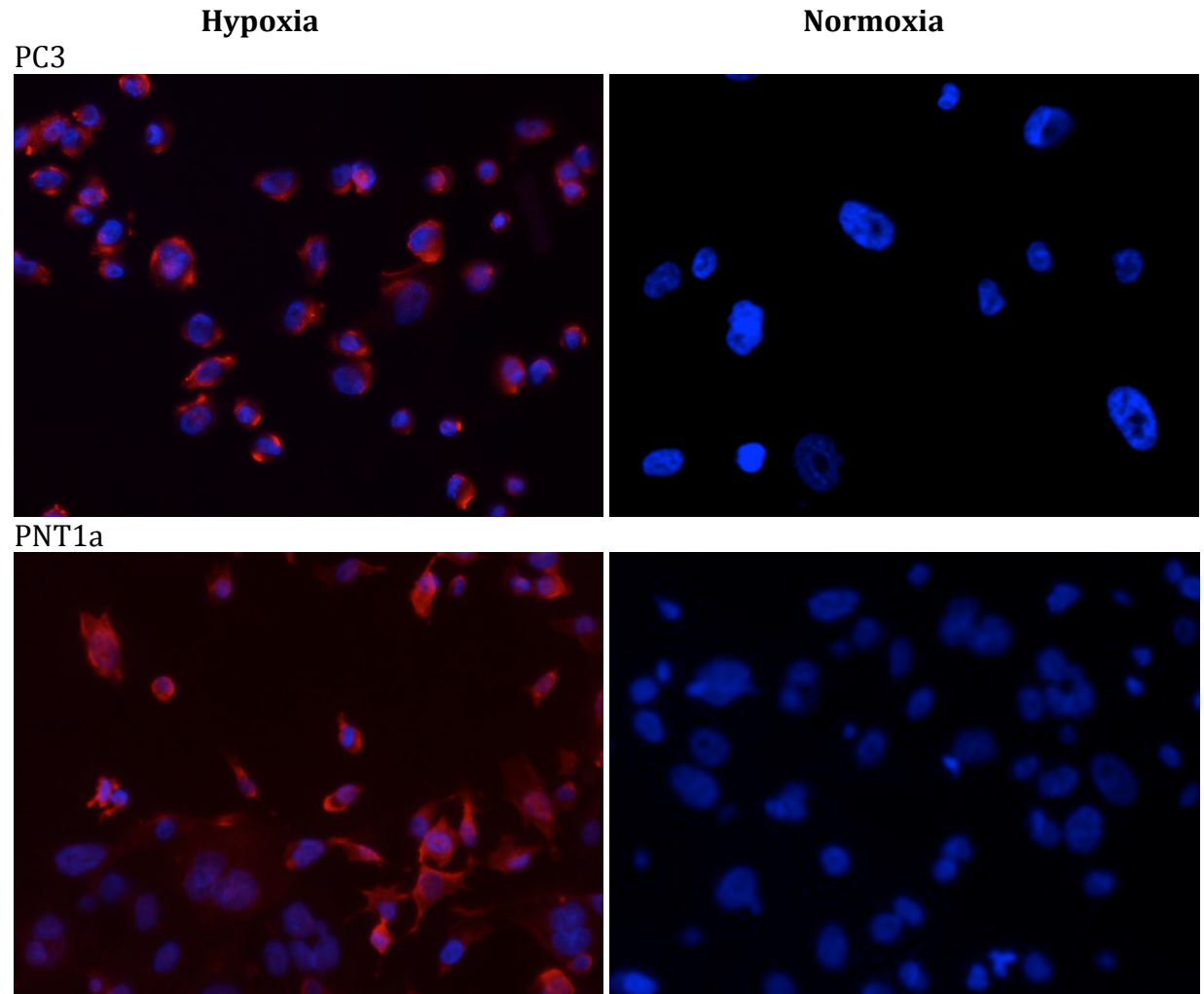


Figure 4.17

Activation of expression of *Hif1α* in *NKX3.1*^{+ve} cell lines, 22Rv1 and PC3-*NKX3.1* and *NKX3.1*^{-ve} cell lines PC3 and PNT1a after culturing cells in hypoxia for 48 hours. *Hif1α* is differentially activated in PNT1a cells cultured in hypoxia.

Furthermore, to investigate the effect of hypoxia on the expression of *NKX3.1*, PNT1a, PC3, 22Rv1 and PC3-*NKX3.1* cells were stained with mouse monoclonal *NKX3.1* antibody after 24 hours, 48 hours and 72 hours. PNT1a and PC3 cells showed no expression of *NKX3.1* in normoxia or hypoxia. There have been no published studies on the direct effect of hypoxia on *NKX3.1*, however, *NKX3.1* expression in 22Rv1 cells was increased after growing in a hypoxic environment for 48 hours.

No such increase in expression of *NKX3.1* was observed in PC3-*NKX3.1* cells (**Figure 4.18**). A slight decrease in the expression of *NKX3.1* in PC3-*NKX3.1* cell line was observed by immunofluorescence. It has been previously shown that hypoxia enhances AR transcriptional activity in LNCaP cells (Park, SY. *et al.*, 2006). However, the AR stimulatory effect is ligand dependent because this effect was abrogated when cells were cultured in androgen depleted media (Mitani, T. *et al.*, 2011). All of the above four cell lines were cultured in RPMI1640 media supplemented with 10% fetal calf serum (FCS). RPMI1640 supplemented with 10% FCS contains castrate levels of androgens (Sedelaar, J.P. *et al.*, 2009). Although, the growth media contains castrate levels of androgens, it may be that it is enough to enhance AR activity and thereby increase the expression of *NKX3.1* in hypoxic conditions. Failure of increase in expression of *NKX3.1* in PC3-*NKX3.1* cells could be attributed to the lack of AR in this cell lines.

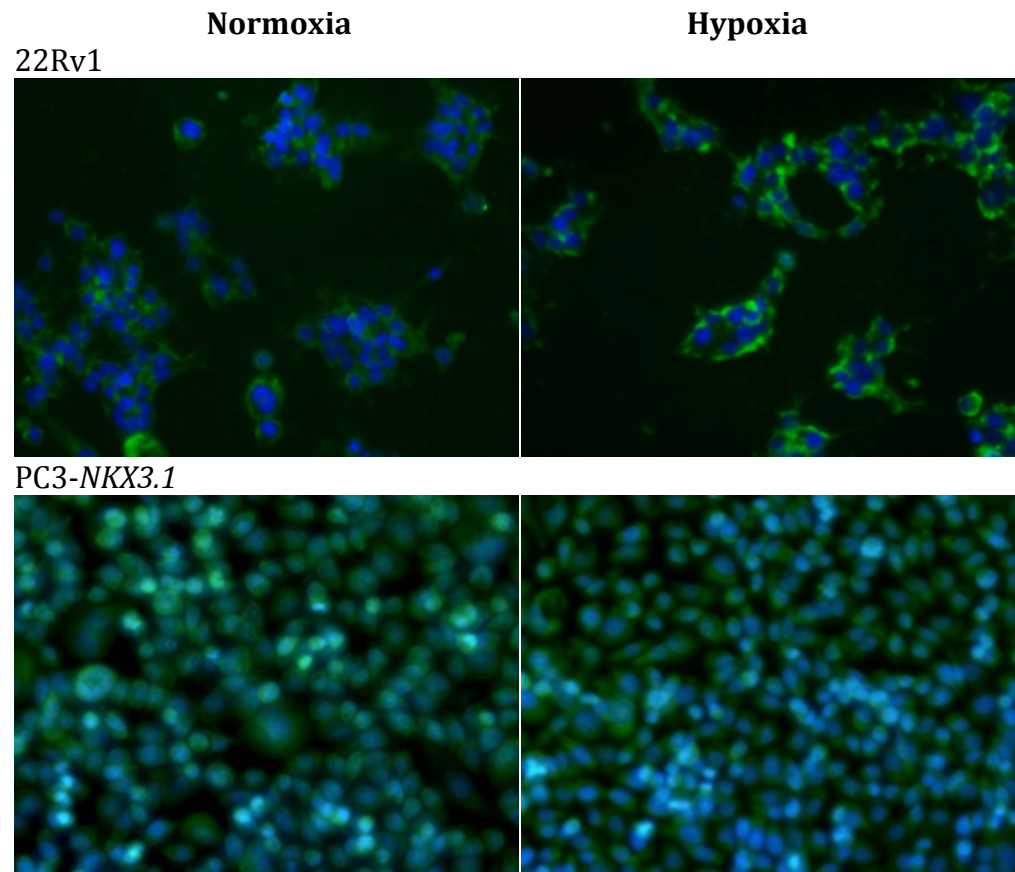


Figure 4.18

Increased expression of *NKX3.1* in 22Rv1 cells after growing them in hypoxia for 48 hours. No change in *NKX3.1* expression was observed after culturing PC3-*NKX3.1* cells in hypoxia for 48 hours.

4.2.11 Effect of hypoxia on colonies grown in 3D

Effect of hypoxia on the growth of 22Rv1, PC3-*NKX3.1* and PC3 cells was assessed by growing single cell suspensions in Matrigel™:Collagen mix under hypoxic conditions for 2 weeks. After 2 weeks of growth in three dimensional culture, the colonies were stained with DAPI and F-actin stain, Phalloidin-rhodamine.

It has previously been shown by the Bodmer lab, that growing cell suspensions of colorectal cell lines under hypoxic conditions increased clonogenicity of the colonies in comparison with normoxia. Furthermore, it has also been shown

with the use of differentiation markers that hypoxia blocks differentiation. However, in case of prostate cancer, the effect of hypoxia is a little more complicated.

PC3 cells when grown in hypoxia, showed an increase in clonogenicity and the number of colonies in each well per 1000 cells increased from an average of 120 colonies in normoxia to 197 in hypoxia (**Figure 4.19**). PC3 cells do not express AR and their growth is independent of androgens. However, no apparent difference was observed in the type of the colonies.

NKX3.1 positive and AR negative PC3-*NKX3.1* cells when grown in hypoxia showed increased clonogenicity with approximately 32% increase in the number of colonies per 1000 cells in hypoxia as compared to normoxia (**Figure 4.19**). Furthermore, the proportion of lumens decreased from approximately 10% to just 5% (**Table 4.5**). These differences in 3 dimensional growth of PC3-*Nkx3.1* cells in hypoxia and normoxia suggest that in absence of androgen regulation, hypoxia decreases the differentiation capacity of the cell line and induces stemness.

Hypoxic conditions did not have the same effect on AR and *NKX3.1* positive 22Rv1 cell line. These cells when grown in hypoxia showed an increase in clonogenicity and a slight decrease in the proportion of lumens from 59% to 51% (**Table 4.5**), although these differences are not statistically significant. The increase in expression of *NKX3.1* by transactivation of AR under the influence of HIF1a is probably counteracting the hypoxia-induced stemness.

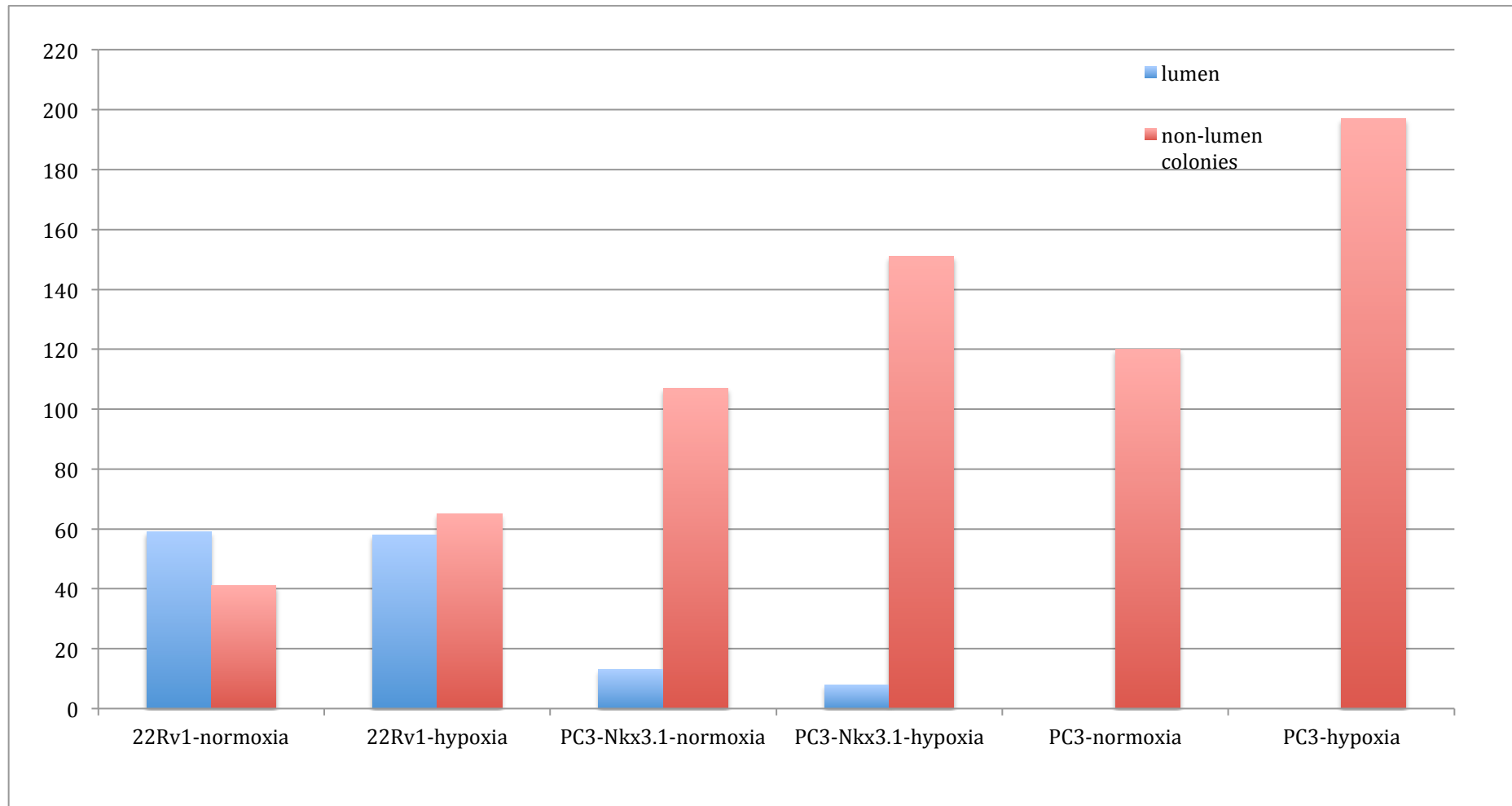


Figure 4.19

Effect of hypoxia on clonogenicity and differentiation in 22Rv1, PC3-Nkx3.1 and PC3 cells. 22Rv1 cell line, which forms differentiated colonies with lumens in normoxia, shows a reduction in clonogenicity in hypoxia. PC3-Nkx3.1 showed a decrease in differentiation and an increase in clonogenicity and non-lumen forming cell line PC3 showed an increase in clonogenicity under hypoxic conditions.

	Proportion of lumens		Total number of colonies	
	normoxia	hypoxia	normoxia	hypoxia
<i>22Rv1</i>	59	51.1	100	133
<i>PC3-Nkx3.1</i>	10.8	5.03	120	159
<i>PC3</i>	0	0	120	197

Table 4.5

Proportion of lumens and total number of colonies in 3-dimensional culture under normal and hypoxic conditions.

4.3 Discussion

While *NKX3.1* functional studies often involve knockout and over expression of the gene in mice, the question of functional effect of *NKX3.1* knockout and over expression in prostate cells lines has not been addressed extensively. Functional analysis of *NKX3.1* has been carried out in LNCaP cells but these studies were limited to proliferative differences in two-dimensional culture (Possner, M. *et al.*, 2008). Our three-dimensional studies to determine the role of *NKX3.1* in differentiation are robust and give a much better idea of *in vivo* processes. We have shown that *NKX3.1* knockdown by siRNA silencing resulted in reduction of lumen forming ability and an increase in clonogenicity of 22Rv1 cells. On the other hand, transfecting *NKX3.1* negative cell lines PC3 and PNT1a with *NKX3.1* triggered lumen formation and decreased clonogenicity of PC3 cells, and a decrease in clonogenicity with no apparent change in differentiation capacity was observed in PNT1a cells. These results strikingly parallel the findings of the effect of *CDX1* knock down and transfection into colorectal cancer cells lines reported previously by the Bodmer lab. The prostate specific homeobox gene, *NKX3.1*, and intestine specific homeobox gene, *CDX1* both have proved to be important in inhibiting cancer stemness and promoting cell differentiation.

Apart from the difference of cell growth in three-dimensional culture, PC3-Nkx3.1 cells demonstrated various characteristics that were interestingly different from the control PC3 cells. The cell morphologies of PC3 and PC3-Nkx3.1 were greatly different when observed under the microscope. PC3-Nkx3.1 cells were round shaped with a small nucleus and cell size, while PC3 has a heterogeneous cell population where some cells had a small nucleus and cell size that matched the PC3-Nkx3.1 cell morphology, and some cells had a larger

nucleus and were spindle shaped. This hinted that there was an underlying process that was stimulated by transfection of *NKX3.1*. There is ample evidence that EMT or EMT-like states occur in, and may contribute to cancer progression and metastasis (Lawrence, MG. *et al.*, 2007, Nauseef, T. *et al.*, 2011). On assessing the two cell lines for the expression of epithelial markers it was observed that E-cadherin, a key epithelial marker was expressed at higher levels in PC3-Nkx3.1 cell line than PC3 cells. This leads to two possibilities, either *NKX3.1* transfection selects for the *CDH1*^{high} cells from PC3 cell line or *NKX3.1* transfection inhibits EMT and hence the high expression of *CDH1*.

Based on the hypothesis that *NKX3.1* inhibits EMT, the panel of prostate cell lines was assessed for the expression of epithelial and mesenchymal markers obtained by microarray analysis. It was observed that *NKX3.1* positive cells lines showed an up regulation of epithelial marker, *CDH1* and a down regulation of mesenchymal markers, N-cadherin, Vimentin, Fibronectin and ZEB1 as compared to the *NKX3.1* negative cell lines. Thus, establishing a negative relationship between tumour suppressor, *NKX3.1* and EMT. The incidence of low expression of *CDH1* in prostate cell lines is much higher than CRC, where only 8 out of 95 CRC cell lines are *CDH1* negative (analysis done by Bodmer lab). Perhaps, just low *CDH1* expression is a general advantage for outgrowth of prostate cancers, which is not true for CRC, because only a minority of CRC have low *CDH1* expression.

It has been reported in various cancers that hypoxia induces EMT (Jiang, J *et al.*, 2011, Salnikov, A. *et al.*, 2012, Zhang, L *et al.*, 2013,). Next, we focused on the association, if any, between *NKX3.1* and hypoxia. It has been shown in prostatic cell lines that TWIST1 plays a key role in EMT and is under tight regulation by

NKX3.1 (Eide, T. *et al.*, 2013). The effect of hypoxia on colorectal cell lines suggests that hypoxic conditions support self-renewal of embryonic stem cells (Chen, HF *et al.*, 2010) and inhibit differentiation of adipocytes (Yun, Z. *et al.*, 2002). Our hypoxia study suggests that the effect of hypoxia on prostate cells is regulated by the function of AR. In other words, the effect of hypoxia is highly dependent on the presence or absence of AR. In absence of AR expression, hypoxic conditions block differentiation and promote stem-like properties, which is reflected by the increase in clonogenicity, and decrease in the lumen forming capacity of the cells. On the contrary, AR positive cells in hypoxic conditions show transactivation of AR in presence of androgens. Due to this the expression of AR regulated gene, *NKX3.1*, is increased. It may be that due to the increase in expression of differentiation regulator, *NKX3.1* the induction of stemness by hypoxia is somewhat negated.

Our results show how careful analysis of the relationship between colony morphology and the expression of markers of differentiation such as *NKX3.1*, using a panel of prostate derived cell lines, can provide valuable insights to the controls of differentiation and stemness of cancer cells. This approach can now serve as a step to better characterization of these processes at the molecular level, which will eventually make a major contribution to developing new targets for treatment and more specific approaches to determination of prognosis.

Chapter 5

HOXB13- a downstream target of NKX3.1

5.1 Introduction

The microarray assessment of differentially expressed genes in *NKX3.1* positive and negative cell lines revealed the gene *HOXB13* to be potentially linked with *NKX3.1* and differentiation. *HOXB13* is known to co-localize with AR and regulate the cellular response to androgens, however its expression is androgen independent in the prostate, thus confirming that *HOXB13* is regulated by *NKX3.1* and not AR (Kim, SD *et al.*, 2010). *HOXB13* encodes a transcription factor that belongs to the homeobox (hox) gene family. Hox genes are a family of transcription factors that are major regulators of animal development. Genes of the homeobox family are highly conserved among vertebrates and essential for embryonic development. *HOXB13* and other hox genes form a gene cluster on chromosome 17 in the 17q21-22 region.

This gene, unlike *NKX3.1* is not prostate specific and is known to play a key role in fetal skin development and cutaneous regeneration (Komuves, LG, *et al.*, 2003). In the prostate gland, *HOXB13* is highly expressed from the embryonic stages to adulthood. A lot of recent studies are focused on germline mutations in *HOXB13* and its implications in prostate cancer risk. It has been shown that *HOXB13* mutation (G84E) increases risk of early onset, familial prostate cancer (Stott-Miller, M *et al.*, 2013, Witte, JS. *et al.*, 2013,). It has also been shown that *HOXB13* expression levels are decreased in colorectal cancer cell lines (Akbari, MR *et al.*, 2013) and in urinary cancer (Marra, L. *et al.*, 2013).

HOXB13 is known to be required for normal differentiation and secretory functional of the ventral prostate in mice (Economides, KD. *et al.*, 2003). Inducing *HOXB13* expression in PC3 cells resulted in significant inhibition of *in vitro* and *in*

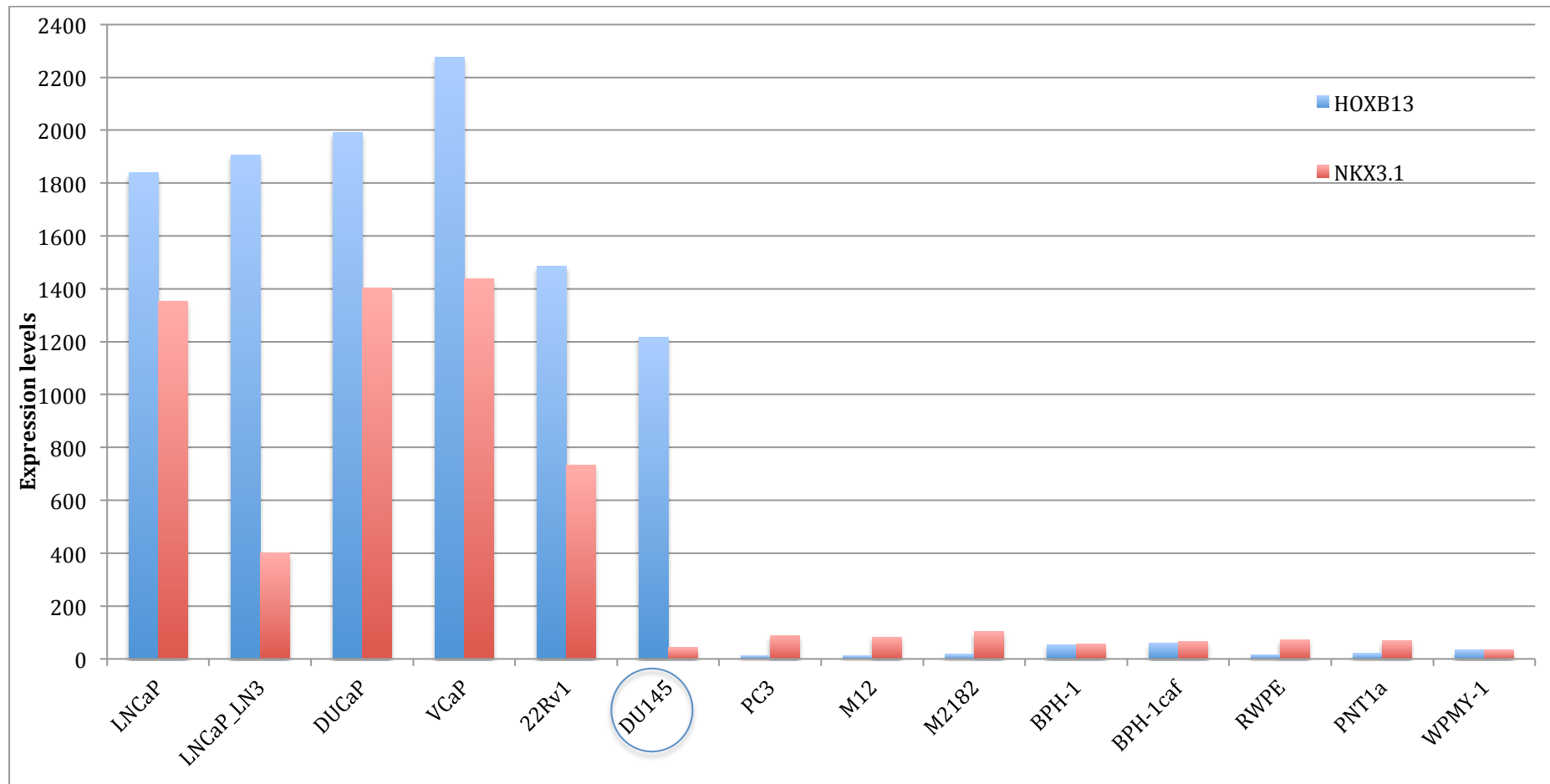
vivo cell growth (Jung, C. *et al*, 2004). It has also been shown that in AR expressing LNCaP cells, *HOXB13* promotes androgen independent growth (Kim YR. *et al*, 2010).

We found that the mRNA expression levels of *HOXB13* positively correlate strongly with *NKX3.1* expression in all the prostate cell lines with the exception of DU145. The function of *HOXB13* in normal cell growth and tumourigenesis in human prostate is not been explored in detail. Hence, its role in colony formation in 3 dimensional cultures was explored along with regulation of the gene by *NKX3.1*.

5.2 Results

5.2.1 Expression levels of *HOXB13* and its regulators, *NKX3.1* and *FOXA1*

HOXB13 mRNA expression levels were examined by analysis of microarray data obtained on an Affymetrix U133 plus 2 platform. Non-log expression values of *NKX3.1* and *HOXB13* in the panel of prostate derived cells lines are shown in **Figure 5.1**. Interestingly, the expression of *NKX3.1* and *HOXB13* showed a tight positive correlation, where all the *NKX3.1* positive cells lines also expressed *HOXB13* and all the *NKX3.1* negative cell lines were *HOXB13* negative with an exception of DU145 cell line, where in spite of no *NKX3.1* expression, the cells still express *HOXB13*. This leads to suggest that the expression of *HOXB13* in DU145 is driven by a transcription factor other than *NKX3.1*. LNCaP_LN3, in spite of expressing much lower levels of *NKX3.1* than LNCaP, expresses the same level of *HOXB13*.

**Figure 5.1**

mRNA expression levels of *HOXB13* and *NKX3.1* derived from microarray data of the prostate cell lines. All the *NKX3.1* positive cell lines also expressed *HOXB13*. Whereas, all the *NKX3.1* negative cell lines were also *HOXB13* negative with the exception of DU145, which in spite of being *NKX3.1* negative expresses *HOXB13*.

FOXA1, which also appeared on the list of differentially expressed genes in *NKX3.1* negative and positive cell lines, is known to be a direct regulator of *HOXB13* (McMullin, RP. *et al.*, 2010). The mRNA expression levels of *FOXA1* and *HOXB13* in all prostate cell lines are shown in **Figure 5.2**. The expression of *HOXB13* and *FOXA1* is positively correlated in all the cell lines. PC3 and BPH-1^{caf} do express some level of *FOXA1*, but in absence of *NKX3.1* expression this may not be enough to induce the expression of *HOXB13*. The high expression of *HOXB13* in DU145 cells in spite of low expression of *NKX3.1* could be explained by the high expression of *FOXA1*.

For all the other cell lines, combination knock down and transfection experiments with *NKX3.1*, *HOXB13* and *FOXA1* are essential to establish the key regulator of *HOXB13*.

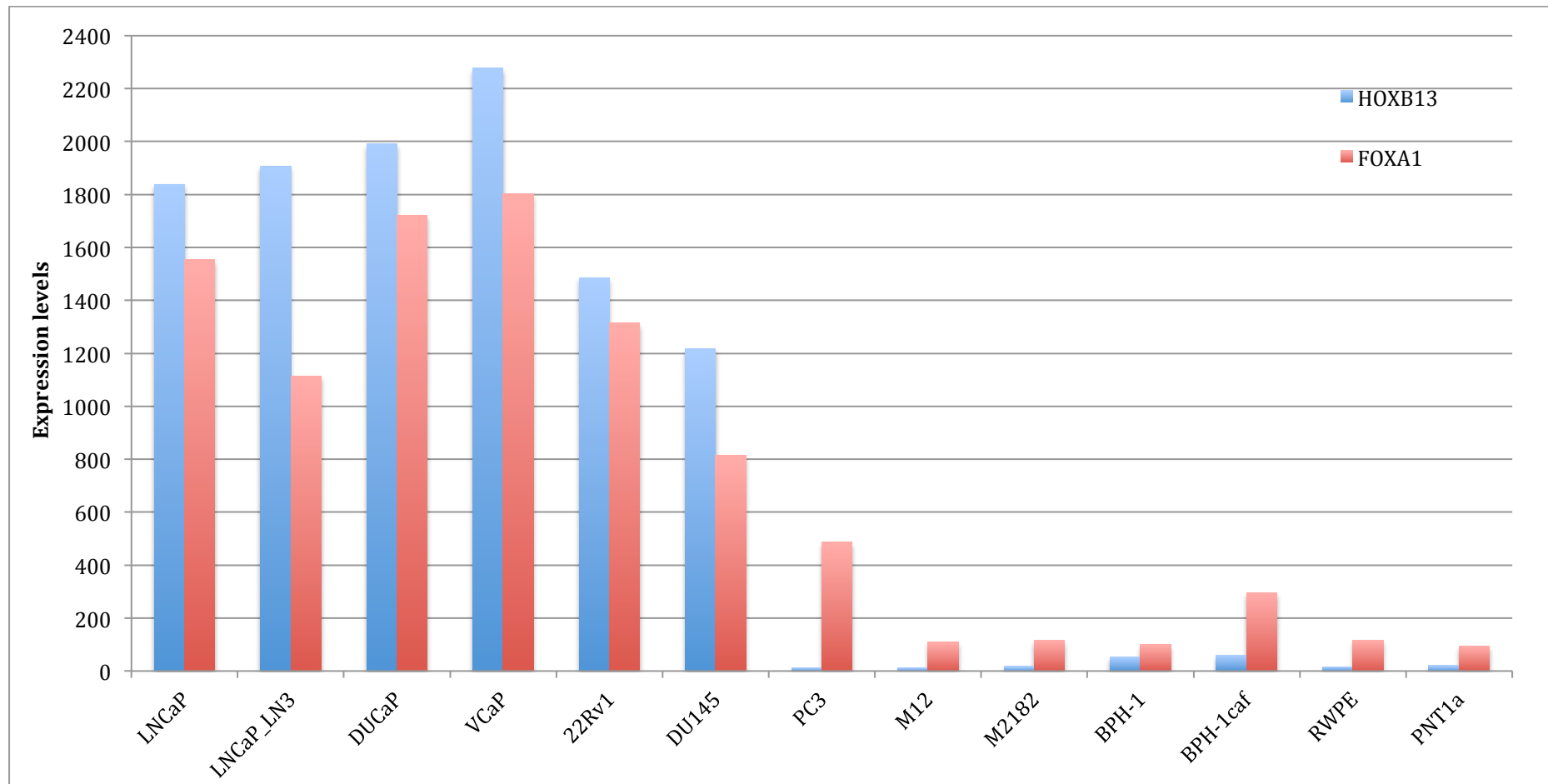
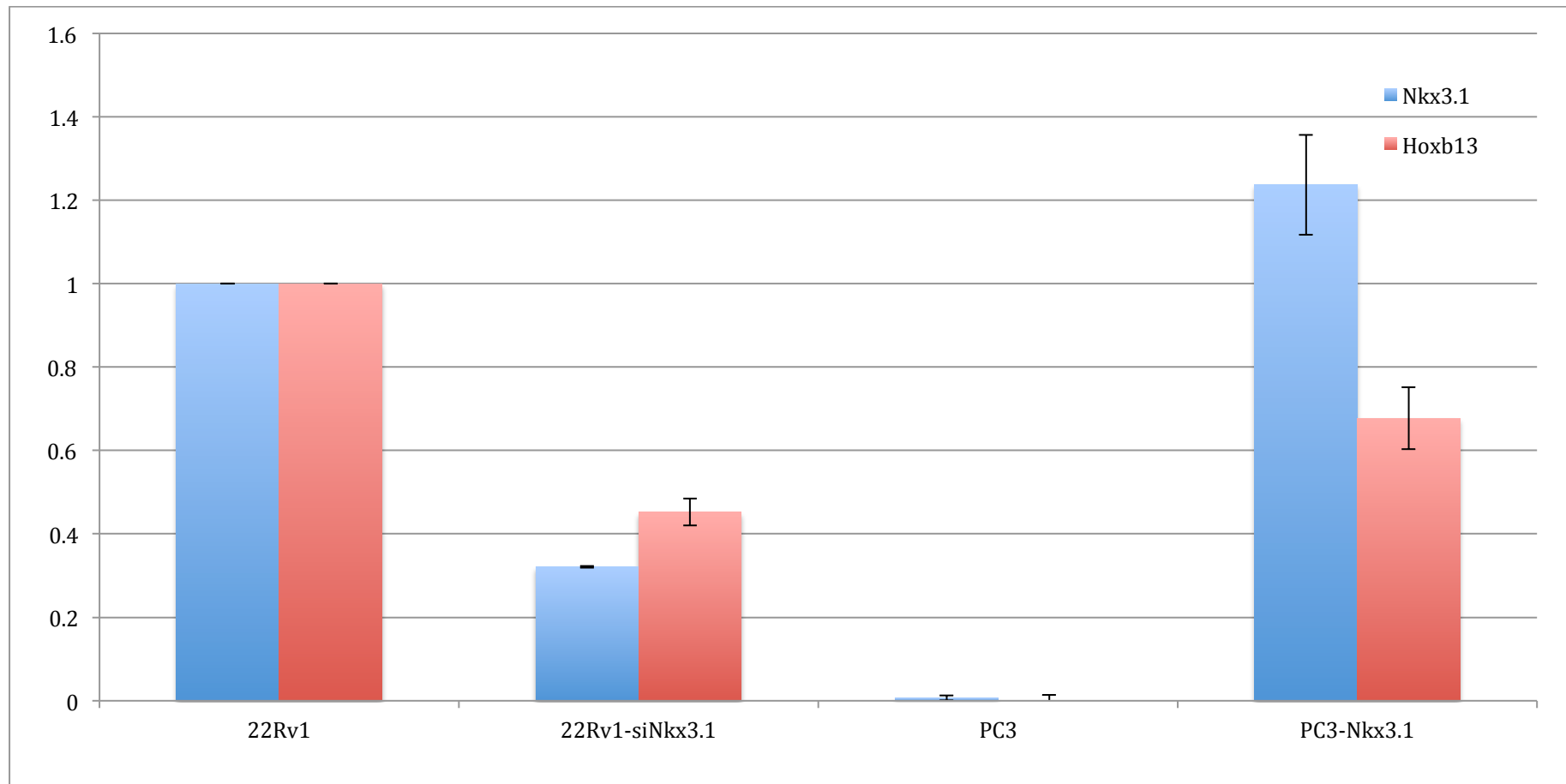


Figure 5.2
mRNA expression levels of *HOXB13* and *FOXA1* derived from microarray data of the prostate cell lines.

5.2.2 Induction of change in expression of *NKX3.1* alters *HOXB13* expression levels in PC3 and 22Rv1

To determine whether or not the expression of *HOXB13* is regulated by *NKX3.1*, the mRNA expression levels of *HOXB13* were determined by qRT-PCR after siRNA mediated knock down of *NKX3.1* in 22Rv1 cells and transfection of *NKX3.1* in PC3 cells. Interestingly, an increase in expression was observed in PC-Nkx3.1 cell line where the expression of *NKX3.1* was induced by stable transfection (**Figure 5.3**). PC-Nkx3.1 is AR negative which re-emphasizes the fact that the expression of *HOXB13* is not regulated by AR and is androgen independent.

**Figure 5.3**

mRNA expression levels of *NKX3.1* and *HOXB13* in control 22RV1, *NKX3.1* knockdown 22Rv1 cells control PC3 cells and PC3 cells transfected with *NKX3.1*. *HOXB13* expression was reduced with *NKX3.1* knockdown in 22Rv1 cells and increased with *NKX3.1* transfection in PC3 cells.

The expression of HOXB13 at protein level in 22Rv1, PC3 and PC3-Nkx3.1 cells was determined by immunofluorescent staining with mouse monoclonal HOXB13 antibody. PC3 cells, as expected based on the mRNA levels, were HOXB13 negative (**Figure 5.4A**). The expression of HOXB13 in PC3-Nkx3.1 and 22Rv1 was mainly membrane-related staining (**Figure 5.4A**). In 22Rv1 cells, HOXB13 staining was very similar to that of F-actin, highlighting the lumens in 2-dimensional culture.

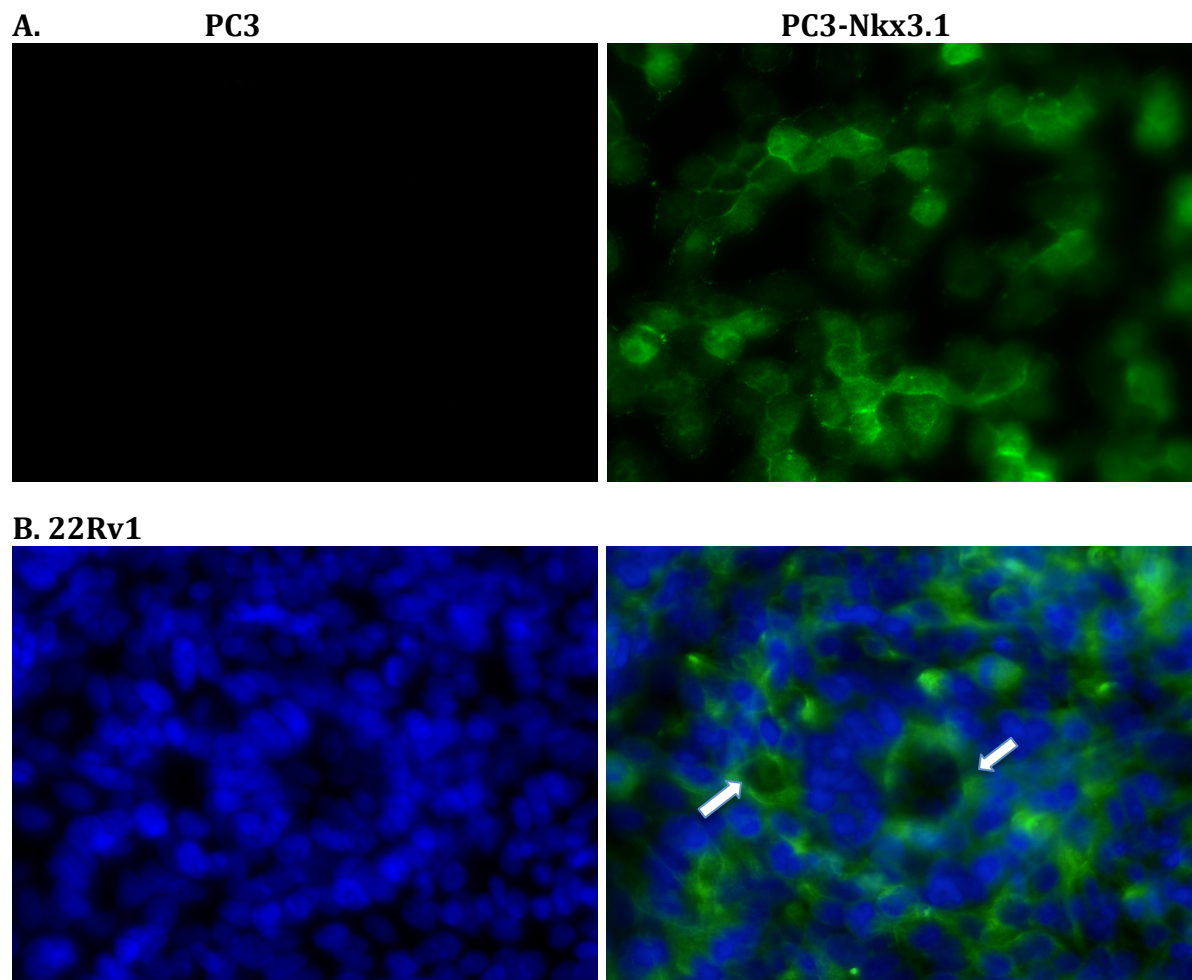
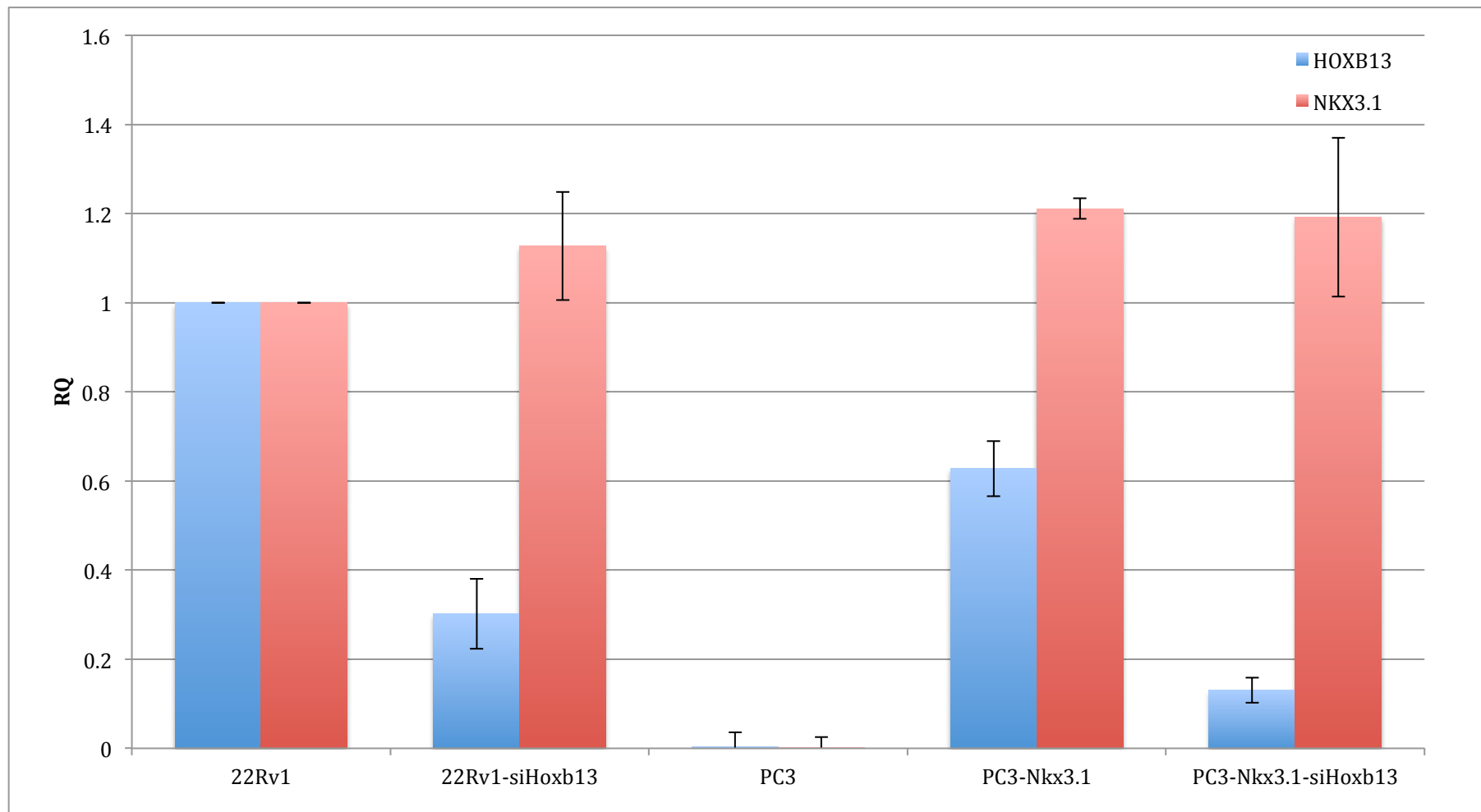


Figure 5.4 HOXB13 protein expression in PC3, PC3-Nkx3.1 and 22Rv1 cells.
A. Protein expression of HOXB13 (green) in PC3 and PC3-Nkx3.1 (20X magnification).
B. DAPI (blue) and HOXB13 (green) in 22Rv1 cells (40X magnification). HOXB13 marks the lumens formed by 22Rv1 cells in 2-dimensional culture.

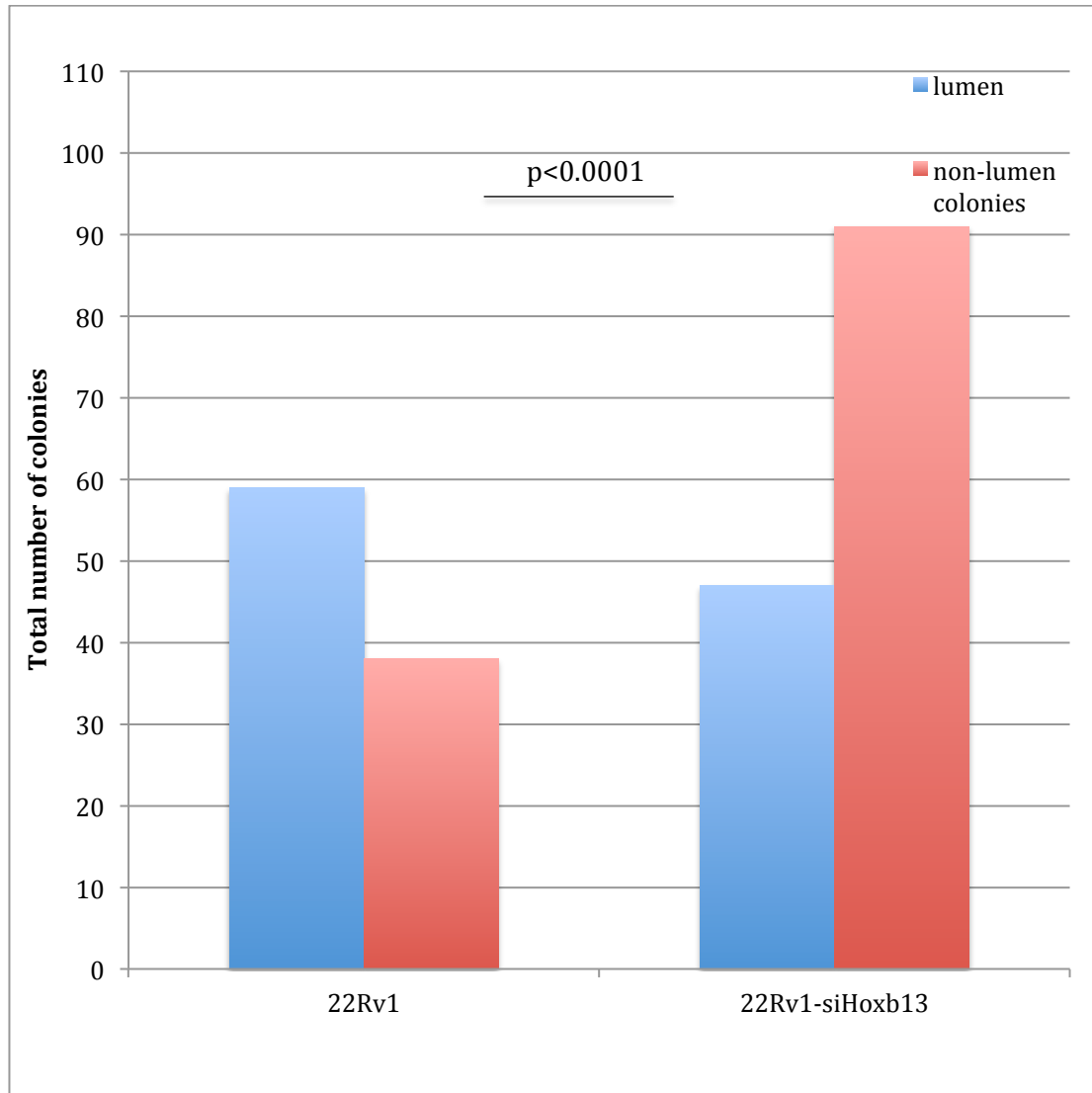
5.2.3 HOXB13 knockdown results in increase in clonogenicity and decrease in differential potential of the cells

To testify the functional effect of *HOXB13*, it was knock down in 22Rv1 and PC3-Nkx3.1 cells by transfecting them with siRNA against *HOXB13* mRNA. The mRNA expression levels were quantified by qRT-PCR. A maximum knockdown of 70% of *HOXB13* was achieved in 22Rv1 cells and a knockdown of 80% was achieved in PC3-Nkx3.1 cells. The expression level of *NKX3.1* was the same in 22RV1 and PC3-Nkx3.1 control cells and in *HOXB13* knockdown cells. *HOXB13* knockdown did not have any effect on the expression of *NKX3.1*.

**Figure 5.5**

mRNA expression levels of *HOXB13* and *NKX3.1* determined by qRT-PCR before and after siRNA mediated knockdown in 22Rv1 and PC3-Nkx3.1 cells.

To study the functional effect of *HOXB13* knock down, single cell suspensions were grown in 3-dimensional culture in 96 well plates as described earlier in Chapter 4. 22Rv1 cells when cultured in Matrigel™:collagen mix for 14 days formed lumen forming colonies. The proportion of lumen forming colonies in 22Rv1 control cells was about 60% (**Figure 5.6**). *HOXB13* knockdown 22Rv1 cells showed increased clonogenicity and the total number of colonies increased by about 42%. Also, the proportion of lumens was significantly reduced ($p<0.0001$) from 60% to 34%. These findings suggest that the knockdown of *HOXB13* is similar to that of *NKX3.1* knockdown in 22Rv1 cells.

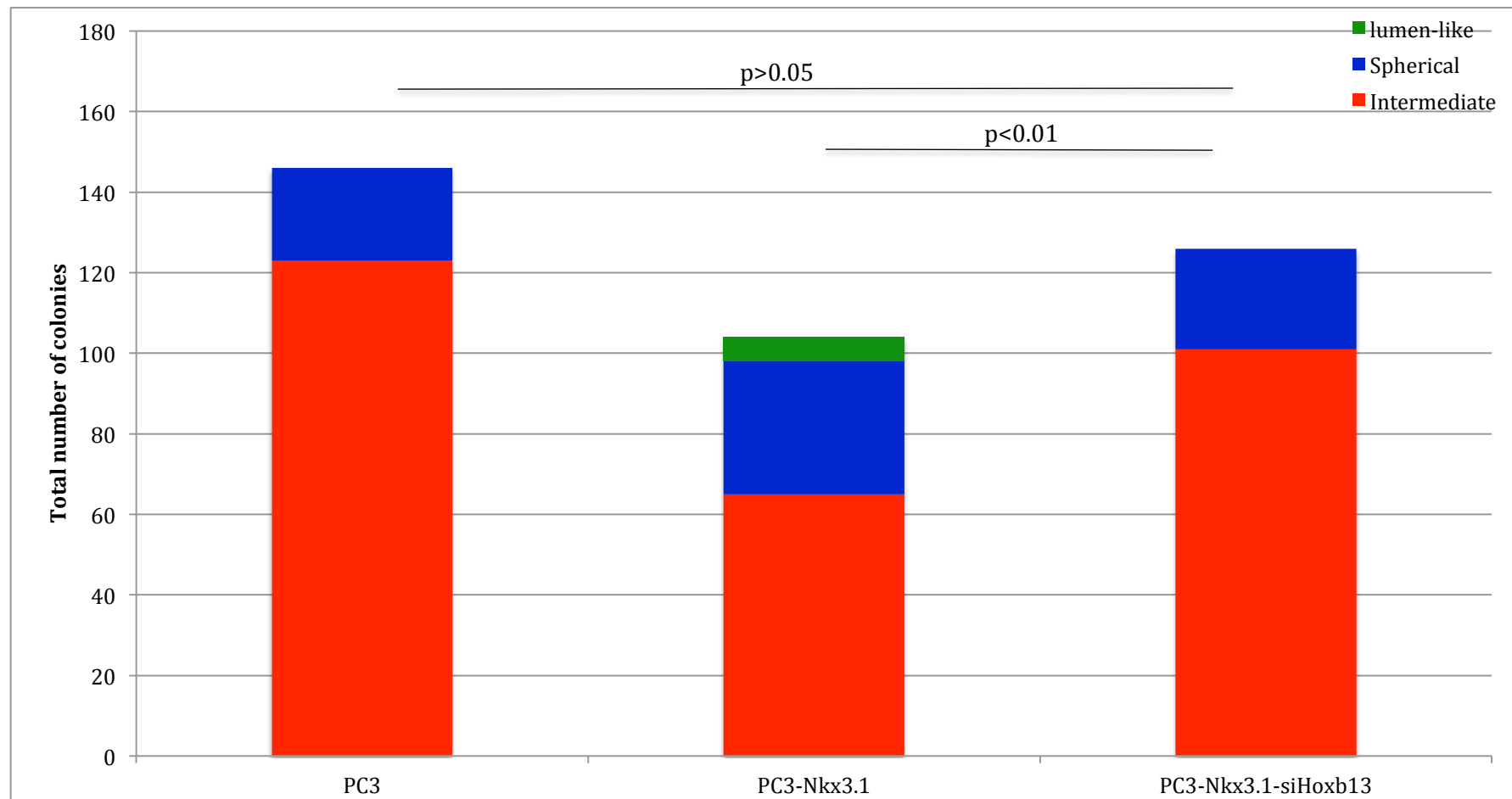


	Proportion of lumens	Total number of colonies	p-value
22Rv1	60.8%	97	<0.0001
22Rv1-siHoxb13	34.0%	138	

Figure 5.6

Total number of colonies and the proportion of lumens in control 22Rv1 cells and *HOXB13* knockdown 22Rv1 cells.

Similarly, *HOXB13* knockdown PC3-Nkx3.1 cells were grown in 3-dimensional culture to determine their clonogenicity and differentiation capacity. PC3 control cells do not form any lumens but they do form spherical colonies that are more structured and differentiated than the intermediate colonies. As described earlier PC3-Nkx3.1 cells however, did form some lumen like structures that were more differentiated than spherical colonies and intermediate colonies. Knocking down *HOXB13* in PC3-Nkx3.1 resulted in increased clonogenicity and decreased proportion of spherical colonies (**Figure 5.7**). No lumen-like structures were formed by PC3-Nkx3.1-siHoxb13 cells. The clonogenicity and the proportion of spherical colonies in PC3-Nkx3.1-siHoxb13 cells were similar to that of PC3 cells with a p-value greater than 0.05.

**Figure 5.7**

Total number of colonies and the proportion of different colonies types in PC3, PC3-Nkx3.1 and *HOXB13* knockdown PC3-Nkx3.1 cells, PC3-Nkx3.1-siHoxb13.

5.3 Discussion

The role of *HOXB13* in differentiation of ventral prostate has been previously studied in mice (Economides, K. *et al.*, 2003). However, since the mouse prostate is considerably different than human prostate, it is important to establish the role of this gene in differentiation in the human prostate. There have been no published studies in this area. Here, we have shown that *HOXB13* knockdown results in reduced differentiation and increased proliferative rates of the cells from prostate cancer or transformed prostate cell lines.

NKX3.1 knockdown resulted in reduced expression of *HOXB13*. However, *HOXB13* knockdown by siRNA mediated silencing had no effect on the expression of *NKX3.1*. This suggests that *NKX3.1* is a regulator of *HOXB13*, but *HOXB13* does not play a role in regulating the expression of *NKX3.1*. In other words, *HOXB13* is a downstream target of transcription factor *NKX3.1*. To further support this hypothesis, it is important to transiently or stably transfect *HOXB13* in prostate cell lines and determine whether or not this induces the expression of *NKX3.1*.

The effect of *NKX3.1* and *HOXB13* knockdown on the colony morphology of cells in 3-dimensional culture was very similar. In both cases knockdown of the genes resulted in a decrease in differentiation potential and increase in clonogenicity. The similarity in the functional effects of expression of *HOXB13* and *NKX3.1* found in prostate cell lines suggests their involvement in a common pathway for differentiation.

The dynamics of transcriptional interplay between *NKX3.1*, *HOXB13* and *FOXA1* can be complex and is not yet well understood. It is important to establish the

transcriptional relationship of these genes to clearly understand the role of each in differentiation. Further studies need to address how *HOXB13* interact with *NKX3.1* in the early events of prostate carcinogenesis, which could contribute towards making *HOXB13* a therapeutic target along with *NKX3.1*.

Chapter 6

Discussion and future directions

6.1 Using colony morphology in a three dimensional culture as a measure for differentiation

Cancer cell lines are generally grown *in vitro* as adherent cultures in plastic flasks, but such culture conditions are quite foreign to the *in vivo* conditions experienced by tumour cells, where they are in contact with each other in three dimensions and are surrounded by an extracellular matrix (ECM) or other protein scaffold. Growth factors diffuse through this matrix to reach cell surface receptors and cells organize themselves into larger structures.

The idea that cells grown in long-term *in vitro* culture still possess the ability to form differential colony morphologies in three-dimensional has been widely accepted. Lang *et al* (2001) grew the prostate cell lines PNT2-C2 and PC3 in Matrigel™ and found that both were able to give rise to colonies, with the PNT2-C2 cells able to differentiate more than the PC3 cells. Bodmer's lab found the SW1222 colon cancer cell line could similarly differentiate in collagen I (Richaman, P *et al.*, 1988). Kenny and colleagues (2007) took this approach further and assayed a 24-cell line breast cancer panel to identify genes whose expression could be associated with each of four colony morphologies listed.

Experiments presented in this thesis show that cell lines grown in ECM-like substrate, Matrigel™, possess the capacity to give rise to three-dimensional colonies with distinctive morphologies. Although each cell line grows differently and the resulting colonies are organized differently (likely a result of the unique characteristics of each primary tumour from which the cell lines were derived), relatively homogenous nature of morphology within individual lines and striking

similarities in the cell lines' colony morphologies were observed. This allowed for the classification of colonies, and cell lines, into five groupings: lumens, dense, spheroid, stellate and intermediate. Each group was characterized by the organization and polarity of cells within a colony.

The most interesting colony morphology was the luminal, where the cell lines formed a central open space around which cells were organized to form acinus-like structures that exist in the prostate. These cell lines possibly possess an ability to give rise to lumens because the cells spontaneously differentiate into the basal, luminal, intermediate and neuroendocrine cells.

The dense cell lines appear to be made up of cells that primarily consist of stem cell populations that do not differentiate, while the lumen cell lines appear to be made up of cells that can differentiate. To understand the control of prostate cancer stemness and differentiation, the results of the cell line panel indicate that the characteristics of dense lines can be studied as surrogates for characteristics for cancer stem cells of cancer stem cells (CSCs), while those of lumen lines can be studied as models of cancer differentiated cells.

The network or the stellate colony classification was the most radically different compared to the other four classes of colony morphologies. The remaining classes formed spherical or semi-spherical colonies, while the network morphology did not. The fibrous spindle shaped cells that grew out of M12 and M2182 are reminiscent of the morphological appearance of epithelial cells undergoing an epithelial-mesenchymal transition (EMT), in which epithelial cells undergo a transformative process that liberates them from well-organized epithelial layers (Agiostatidou, G *et al.*, 2007) to form invasive, migratory cells

similar in behavior to mesenchymal cells. Identification of factors influencing the formation of the network morphology, such as presence or absence of EMT markers and other cadherin proteins would be interesting.

This assay was further used as described below, in studying the role of *NKX3.1* and *HOXB13* in differentiation.

6.2 Effect of *NKX3.1* and *HOXB13* expression on differentiation potential of prostate cells

The *NKX3-1* gene has been described as a tumour suppressor but how it functions is still unclear (Shen, MM *et al.*, 2003). Using chromatin immunoprecipitation coupled with sequencing (ChIP-seq) it has been shown that *NKX3.1* co-localizes with the androgen receptor (AR) across the prostate genome to mediate expression of genes associated with advanced and recurrent prostate cancer. (Tan, PY. *et al.*, 2012). Recently Shancheng Ren's lab, using small activating RNA (saRNA) as a molecular tool, showed that targeted induction of endogenous *NKX3.1* by small activating RNA inhibits prostate tumour growth in xenograft models (Ren, S. *et al.*, 2013). In this thesis, two *NKX3.1* negative human prostate cell lines, PC3 and PNT1a were transiently transfected with *NKX3.1*. mRNA levels and immunofluorescence imaging confirmed that the cDNA was expressed and that this expression could persist for up to seven days. Although the cells were cultured for more than seven days (ten days), the initiation and initial differentiation (if any) of a colony is likely to occur only hours after the start of the culture. Even if the expression of *NKX3.1* drops in the period of 10 days the initiation of generation of clones would have already been in motion. PNT1a-*Nkx3.1* cells showed about a 40% decrease in clonogenicity when grown in three-dimensional culture, however, no apparent difference in the colony morphology or size was observed. *NKX3.1* expressing PC3 when cultured in Matrigel™:collagen mix showed a decrease in clonogenicity and about a 40% increase in the proportion of spherical colonies, which are more differentiated than the intermediate colonies. On the contrary, siRNA directed knockdown of

NKX3.1 in 22Rv1 cells, drastically increased the clonogenicity of the cells and almost a 70% decrease in the proportion of lumens when cultured in Matrigel™:collagen mix.

These results match what has been observed with *CDX1* and its impact on differentiation potential on colorectal cell lines. Bodmer lab has shown that *CDX1* induction promoted the expansion of more differentiated structures (mainly colonies containing lumens), both in lumen forming cell lines and undifferentiated non-lumen forming dense cell lines (Yeung, T *et al.*, 2010). Like *CDX1*, *NKX3.1* transfection also appeared to have dampened cell proliferation and sensitized cells to environment differentiation cues, implying a causal link between *NKX3.1* and differentiation.

The results of microarray analysis are often used as training sets in order to make predictions of biological or clinical significance based upon sample gene expression data. In this thesis, a list of differentially expressed genes in *NKX3.1* positive and negative cell lines was generated in order to identify the genes associated with *NKX3.1*. Microarray analysis on a panel of 14 prostate cell lines yielded 624 genes that were upregulated in *NKX3.1* positive cell lines. Out of the 624 genes, *AR*, *KLK3*, *FOXA1* and *HOXB13* stand out because of their role in carcinogenesis and tissue development. Since, all the *NKX3.1* positive cell lines are also *AR* positive, it could be argued that this list of genes could also be differential to *AR* and not just *NKX3.1*. However, given the mutual regulation of *NKX3.1* and *AR* in a feed-forward loop manner, the microarray analysis would enrich for genes that are associated with both *NKX3.1* and *AR*.

HOXB13 was further explored in this thesis because of its role in prostate development, normal differentiation and secretory function of the prostate (Economides, KD *et al.*, 2003). It has been shown that *HOXB13* suppresses growth of prostate cancer cells by acting as an AR repressor to modulate the complex AR signaling pathway (Jung, C *et al.*, 2004). In this thesis, for the first time, we show that *NKX3.1* regulates the expression of *HOXB13*. siRNA mediated knockdown of *NKX3.1* in 22Rv1 cells resulted in reduced expression of *HOXB13*, while stable transfection of *NKX3.1* in PC3 cells induced the expression of *HOXB13* in PC3-*Nkx3.1* cells. However, no change in expression of *NKX3.1* was observed on knocking down *HOXB13* in 22Rv1 and PC3-*Nkx3.1* cells. This suggests that *HOXB13* is possibly a downstream target of *NKX3.1*.

HOXB13 knockdown in 22Rv1 cells resulted in increased clonogenicity and a decrease in differentiation potential of the cells. The colony morphology of the *HOXB13* knockdown 22Rv1 cells is also similar to that of *NKX3.1* knockdown 22Rv1 cells. Knocking down *HOXB13* in PC3-*Nkx3.1* cell line yielded clonogenicity and colony morphologies that were the same as PC3 cells. This means that *HOXB13* knockdown in PC3-*Nkx3.1* cells reversed the effect of *NKX3.1* on differentiation and clonogenicity. Hence, it could be said that the effect of *NKX3.1* expression on stemness and differentiation potential of the cells is primarily regulated by *HOXB13*. Given its role in differentiation in prostate cancer cells, it should be further studied as a potential therapeutic target in addition to it being a molecular marker.

6.3 NKX3.1 and its association with hypoxia

Hypoxia is associated with poorer prognosis and resistance to chemotherapy. Hypoxia has also been shown to correlate with increased tumour invasiveness, and metastasis (Vaupel, P *et al.*, 2001). Investigation of the effects of hypoxia on cancer differentiation in colorectal cancer cell lines has shown that it increases clonogenicity and prevents their differentiation when grown in Matrigel™ (Yeung, T *et al.*, 2010).

The Bodmer lab has shown that hypoxic colonies express reduced levels of CDX1. There have been no published studies on the direct effect of hypoxia on the expression of NKX3.1. In this thesis, it has been shown that growing 22Rv1 cells in a hypoxic environment increases the expression of NKX3.1. No such increase in expression of NKX3.1 was observed in NKX3.1 stably transfected PC3 cells (PC3-NKX3.1). It has been shown by Park *et al* that hypoxia enhances the transcriptional activity of AR in an androgen dependent manner (Park SY *et al.*, 2006, Mitani, T *et al.*, 2011). The increase in expression of NKX3.1 in 22Rv1 cells is likely to have been mediated by the increased transcriptional activity of AR. Since, PC3-Nkx3.1 cells do not express AR and are androgen insensitive, no apparent increase was observed in the expression of NKX3.1. In fact, like in colorectal cell lines, a small decrease was observed in the expression of NKX3.1 in PC3-Nkx3.1 cells grown in hypoxia. It has been shown that Hif1 α may have a direct inhibitory effect on CDX1 transcription as two putative hypoxia response element (HRE)-like sequences have been identified in the 5' flanking promoter region of human CDX1 (Choi, YJ *et al.*, 2007). It is possible that the decrease in

expression of NKX3.1 is mediated by the activation of Hif1 α under hypoxic conditions in a similar fashion.

Investigation of the effects of hypoxia on colorectal cancer in three-dimensional culture has shown that hypoxia increases clonogenicity and prevents differentiation. However, the prostate cancer and hypoxia scenario is more complicated than colorectal cancer due to the role of androgens in the former. Work in this thesis demonstrates that the effect of hypoxia on stemness is primarily governed by the expression of AR and NKX3.1. AR negative cell lines, PC3 and PC3-*Nkx3.1* when grown in hypoxia showed increased stemness like the colorectal cell lines. However, when AR and NKX3.1 positive 22Rv1 cell line is grown in hypoxia, increase in NKX3.1 expression by transactivation of AR probably conflicts with hypoxia-induced stemness and therefore there is no significant change in clonogenicity or the proportion of lumen forming colonies.

Work in this thesis begins to identify how hypoxia regulates stemness and differentiation in an androgen dependent manner. Since hypoxia reduces the differentiation potential of cells and increases stemness in absence of androgen regulation, this could explain why androgen independent hypoxic tumours behave more aggressively clinically, and are associated with a poorer prognosis. In case of androgen dependent tumours, it is essential to further understand the interplay of AR, NKX3.1, Hif1 α and hypoxia in prostate cancer.

6.4 Mismatch repair and prostate cancer

RER and the resulting MSI have been studied in depth in colorectal cancer where epigenetic silencing or mutation of *MLH1* and less frequently, *MSH2* or *MSH6* has been identified as the cause of the defective mismatch repair. However, microsatellite analysis in prostate cancer primary tissues has revealed that MSI frequency in prostate tissues is considerably lower than in colorectal cancer. Using a combination of four different microsatellite markers the MSI status of the cell lines has been determined. 8 out of the panel of 11 cell lines are MSI^{+ve} suggesting that microsatellite instability is a frequent event in cell lines derived from prostate cancer and those derived from normal prostate. This is in contradiction to widely published data that show that clinically localised prostate cancer is microsatellite stable (Azzouzi, AR *et al.*, 2007).

Although M12 and M2182 are derived by transforming normal prostate cells with SV40T, they are RER⁺. BPH-1 and BPH-1^{caf} are derived from benign SV40T transformed cells and yet they are RER⁺. These cell lines cannot be considered normal based on their RER status. The MMR deficiency in these cell lines is unlikely to be due to SV40T transformation, unless MMR deficiency gave an advantage to the outgrowth of the transformed cells. The high frequency of MMR deficiency in cell lines could also be because RER⁺ tumours more easily give rise to cell lines for some unknown reason. This also would explain why it has, generally, been difficult to grow out cell lines from localized prostate cancer tissues that are microsatellite stable.

Summary

To summarize, the experiments reported herein have identified a link between cell line morphology and differentiation status, and that such status can be utilized to identify regulators of cancer differentiation and markers of cancer stemness. The data in this thesis suggest that *NKX3.1* plays a key role in differentiation in prostate cancer and therefore the prognosis of prostate cancer relies on the expression of this gene. The novel 3-D assay developed in the Bodmer lab and now widely used by various labs for all cancer models, is an efficient technique to determine differentiation potential of the cells. This assay can also be used to understand the behavior of cancer stem cells, high throughput drug testing and identification of potential new chemotherapeutic molecules. We have shown through microarray data that *NKX3.1* is down regulated in a number of prostate cell lines. For normal prostate development, a balance of undifferentiated stem cells, proliferative cells and differentiated cells is necessary. The results in this thesis show the effect of *NKX3.1* expression on differentiation in prostate cancer cells. Therefore, selection for reduced expression of *NKX3.1* in prostate cancers is likely to be associated with a reduced capacity of the cancer stem cells to differentiated cells and so to contribute to an increases rate of growth. We have also shown that hypoxia regulates the expression of *NKX3.1* and differentiation potential of the cells in and AR dependent manner. Clearly, studying the regulation of *NKX3.1* and *HOXB13* within the context of cancer differentiation and stemness proved and shall continue to prove useful in understanding the mechanisms of prostate carcinogenesis and tumour proliferation.

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Appendix

Results of microarray comparisons

Table 1a

List of genes up regulated in *NKX3.1^{+ve}* cell lines, sorted by fold change. All genes are statistically significant at a significance level of 0.02 and are up regulated at least 2 folds as compared to *Nkx3.1^{-ve}* cell lines.

Gene Symbol	Corrected p-value (+ vs. -)	Fold-change (+ vs. -)
AR	1.33E-08	104.234
KLK3	2.95E-05	65.2163
HOXB13	0.000471999	49.5253
TMEFF2	0.000328146	34.7965
TMPRSS2	2.56E-05	34.5037
SLC44A4	8.50E-07	33.1693
PRUNE2	2.08E-07	31.7303
KLK2	5.37E-05	31.2718
KLK4	9.81E-09	25.8116
NAALADL2	1.38E-05	22.5567
PRUNE2	3.19E-07	21.6811
SYT4	5.73E-05	21.6521
SESN3	0.00565851	20.4449
APOD	0.00278875	19.6369
GLYATL1	2.30E-10	18.8658
PLA2G2A	0.00464259	18.0056
LOC100128252	0.0111349	17.376
C11orf92	0.000234438	17.0465
COLEC12	2.66E-07	16.9511
ACPP	1.75E-05	16.9057
FOLH1	0.000332003	16.5333
SEPP1	1.07E-05	14.7784
NKX3-1	4.73E-07	13.7009
ACSM3	0.000170274	13.4286
SELENBP1	0.00108009	13.4201
LUZP2	3.26E-05	13.3489
SPDEF	0.00102226	13.1368
METTL7A	1.02E-05	12.9421
PLA2G7	0.0167389	11.6611
ZBTB16	3.75E-07	11.5815
TC2N	0.00265345	11.4568
KIAA1324	0.00691988	10.9993
GPR158	0.000283617	10.922
STEAP2	1.26E-05	10.6522
NCAM2	0.017647	10.5625
SPON2	0.0116701	10.3973
ZG16B	0.000180198	10.2349
PRKD1	0.00613773	9.91542
FAM110B	0.00401468	9.83119
KIAA1244	4.27E-06	9.51779
SEMA6A	0.000944698	9.39488
PRAC	0.00103678	9.33174
MAOA	0.00135398	9.13948

Appendix: Results of microarray comparisons

SLC45A3	0.000221956	8.84285
PPFIA2	0.00412547	8.78988
UGT2B15	0.00107191	8.66213
LIN7A	0.00143859	8.54574
TSPAN8	0.0101987	8.36512
MAML3	0.000228896	8.23646
STEAP1	0.000119742	7.9978
CAB39L	5.94E-05	7.95767
FOXA1	0.000237714	7.82051
NETO1	0.00100644	7.7846
HMGCS2	0.0040079	7.73541
DNASE2B	0.00533731	7.65113
AMACR	0.000837128	7.63814
EPHA6	0.00301324	7.52116
MOSC1	6.31E-05	7.46142
PPAP2A	4.08E-07	7.31841
PART1	0.000396408	7.20574
CHRNA2	0.000330832	7.20109
ARHGAP6	2.39E-06	7.19692
RAB6C	0.000138065	7.1857
CPNE4	0.00647314	7.06851
GABRB3	0.000195767	7.06291
DSEL	0.000573636	7.03795
ZNF682	0.000317392	6.9716
ANK3	0.00279728	6.9446
FAM174B	0.000924419	6.78933
TP53INP1	0.00172124	6.71275
NLGN1	4.02E-05	6.58652
PBLD	0.000661513	6.5601
TBX3	6.71E-05	6.54556
PRR15L	1.14E-07	6.37134
SLCO5A1	0.000228881	6.24689
CDH26	0.00487426	6.19555
ZNF429	0.00806245	6.12042
ENPP5	0.0105696	6.10037
RASSF3	2.76E-06	6.02081
POTEKP	0.0108299	5.95683
NRN1	0.00317847	5.78129
AZGP1	0.00294644	5.74613
KCNN2	0.00336801	5.71346
CKB	0.00625957	5.69269
SLC25A33	1.19E-05	5.65508
BICD1	8.42E-05	5.64846
ATP8A1	0.0112592	5.56912
RAMP1	0.000273399	5.52372
HPN	5.99E-07	5.47778
ZBTB20	0.00257177	5.47218
RHOA	0.000660349	5.44095
BMPR1B	0.0160895	5.43806

Appendix: Results of microarray comparisons

RAB3A	6.91E-05	5.40134
MB	0.000912808	5.36801
UGT2B17	0.00449352	5.36145
KCNJ3	0.00490431	5.34113
SYTL2	0.0179088	5.3338
USP54	0.00018893	5.26371
MYO6	9.40E-05	5.24582
CDYL2	0.00343971	5.24141
GATA2	1.92E-05	5.2402
PPP3CA	4.50E-05	5.22822
SMPDL3A	0.000365909	5.1564
SI	0.00493187	5.1409
C19orf46	1.17E-08	5.10453
ZNF385B	0.012807	5.10267
ALDH3B2	0.00123896	5.10043
OR51E1	0.00448164	5.00559
ABHD2	0.0025961	4.99343
ARHGEF26	0.00703914	4.91542
ALOX15	0.000214634	4.88837
LRIG1	0.00603502	4.83626
PPFIBP2	2.82E-05	4.81453
CBR4	0.00862317	4.80005
CACNA1D	0.00142751	4.79956
ZNF610	0.00209352	4.7962
LOC286161	0.00349122	4.7825
CDK19	0.000129899	4.70931
PDE3B	6.08E-05	4.69004
AMD1	2.49E-07	4.68016
SLC7A2	0.00793518	4.63383
KIF5C	0.00384499	4.62513
EPHX2	0.000343065	4.58287
NAP1L2	3.77E-05	4.57749
FAM134B	0.00745284	4.54262
ZNF542	0.0149797	4.53922
PLCB4	0.013906	4.49733
GRIN3A	0.00097413	4.45767
B3GNT9	0.000192002	4.45759
LEF1	0.0110646	4.45431
TM7SF2	0.00440915	4.44544
CAMK2B	0.000330867	4.42462
NME5	7.08E-06	4.33664
GADD45G	3.50E-05	4.32099
PODXL2	0.00110953	4.31433
FAAH	3.64E-05	4.31206
FAM36A	0.00105893	4.28352
TLCD1	0.000402901	4.24353
THNSL1	7.06E-05	4.22913
PDZRN3	0.001705	4.22519
DEPDC6	0.0116696	4.21958

Appendix: Results of microarray comparisons

STX19	0.00724601	4.20696
CLDN3	0.00341562	4.19976
GHR	0.00311525	4.19795
CREB3L4	1.53E-06	4.18027
LPPR1	0.003966	4.15145
CAMKK2	0.000651026	4.14207
ALDOC	0.00175528	4.14101
KIF1A	0.0016963	4.13252
SNAP91	0.000356088	4.11837
NFIX	0.00633905	4.11017
FAM155B	8.44E-06	4.10486
FASN	0.00182655	4.10117
ZNF493	0.00210898	4.07293
ACSL1	0.00227062	4.06234
RPL17	0.000232864	4.05676
BLNK	0.000528698	4.03979
SEMA4A	0.000230134	4.03146
ZBTB10	0.00210442	4.01979
CYP2R1	0.0061399	4.01471
GREB1	0.000158802	3.99947
SLC9A2	0.0118733	3.96366
EIF4B	0.00210679	3.9445
ICA1	0.000896951	3.93802
HOOK1	0.0104597	3.93586
INSIG1	0.00442965	3.89525
TRIM36	0.014855	3.85763
ALDH6A1	0.00368396	3.84778
ANKS1B	0.000982396	3.84643
A1CF	0.0191214	3.84366
SLIT1	0.00561838	3.84166
ACACA	1.14E-05	3.84074
C19orf48	3.26E-05	3.8309
PCDHB11	0.00261876	3.82131
DDC	6.37E-05	3.81409
FAM118A	0.000389734	3.81406
MGST2	0.000114388	3.81354
MAP2K6	0.000996371	3.80428
SSBP3	0.000159985	3.79709
MAP9	0.00261779	3.76617
ZNF649	0.0131945	3.76396
SNRPN	0.00112179	3.75451
PCDHB18	0.00139797	3.74027
RPL14	0.000344418	3.73952
LUM	0.0155663	3.71969
SLC19A2	0.000130983	3.69511
UNC80	0.00803086	3.6858
CENPN	0.00901298	3.67424
PRKACB	0.00393394	3.67118
ZNF300P1	0.0162516	3.66195

Appendix: Results of microarray comparisons

RWDD2A	0.00675807	3.6565
HSD17B7P2	0.000117055	3.65328
TPD52	0.00343712	3.64492
RRAGD	0.00564196	3.64156
IGFBP2	0.00568579	3.63863
ALDH1A1	0.0041118	3.6258
ELF5	6.19E-05	3.62385
CHDH	3.81E-06	3.58807
SPATA13	0.00010834	3.57922
FRY	0.00320757	3.56378
RTN1	0.00100541	3.56133
NOVA1	0.0111256	3.56116
ZNF667	0.00193094	3.5407
SLC16A10	0.00465814	3.51362
GPR160	0.0107581	3.48855
TTR	0.00588824	3.48312
TP53TG1	3.68E-05	3.45426
C17orf28	0.0195044	3.44165
DACH1	0.0024287	3.44108
TTC18	0.000261478	3.41973
PTPRN2	0.0120048	3.39702
MAFB	0.00180174	3.3875
PPAPDC2	7.26E-06	3.3836
C11orf75	0.00485354	3.38237
NDN	0.00555816	3.38056
KLF15	2.93E-06	3.37908
ANKH	0.0104358	3.37084
SUOX	6.12E-05	3.36782
THTPA	0.000154514	3.3607
MANSC1	0.00143298	3.35996
ADRA2A	4.89E-05	3.35857
MCCC1	7.74E-05	3.35738
SLC39A6	0.0017869	3.35257
GDAP1	0.00695429	3.33895
BRP44	0.000318795	3.33279
CHPT1	0.000519536	3.31907
FAM117B	0.00608718	3.30861
C1orf21	0.000885424	3.28832
UNC13B	0.00448135	3.28737
LOC147727	0.00124887	3.2713
TMEM144	0.00467096	3.26594
ONECUT2	3.33E-06	3.25176
RPL17	0.000416099	3.24392
NCRNA00287	0.000166211	3.23523
AKAP9	0.000928302	3.228
C7orf63	0.00407501	3.2188
PDCD4	0.00662314	3.20908
POTEM	6.17E-05	3.2062
C12orf26	0.000126296	3.19067

Appendix: Results of microarray comparisons

IL1R1	0.00318851	3.18181
RASEF	0.00220713	3.17813
COBL	0.0180415	3.17014
FAM13A	0.0020196	3.15851
LYRM5	0.00699629	3.14798
LONRF2	0.0133864	3.14162
SFXN2	0.00159705	3.13044
HOMER2	0.00341128	3.12821
RPS4Y1	0.0179079	3.1181
FAM173B	0.00547297	3.11199
FAM13A	3.60E-07	3.08961
TMX4	0.0119786	3.08894
TAS2R19	0.00060076	3.08751
FAM151B	1.91E-05	3.07705
CLCN3	0.00408753	3.06237
FAM188B	0.00128649	3.05728
TMEM168	0.000371306	3.0473
IRX3	0.00773063	3.0468
JPH1	0.0145116	3.04205
PHC1	0.00103546	3.04085
RAB39B	2.54E-06	3.0363
LOC338799	0.00466971	3.0305
TESK2	0.00804734	3.02996
BAGE2	0.0169472	3.0265
CPE	0.00344064	3.02429
RAB17	0.00440895	3.02363
SYT7	0.0139028	2.99617
CMBL	0.00786857	2.98234
PLA2G6	1.70E-05	2.96672
RANBP3L	0.0183747	2.95872
SLC25A45	0.00315737	2.94982
RIC3	0.00518914	2.94068
SERPINI1	0.00315214	2.93973
FLJ44790	0.000953207	2.93655
C11orf71	0.00206137	2.93541
ZNF471	0.0035326	2.93528
GARNL3	0.00788664	2.92374
GPR98	0.00278331	2.91004
FBXO25	0.00014754	2.90958
DPYSL5	0.00411655	2.90793
LASS4	0.00344404	2.87868
PLA2G4F	0.000208024	2.87705
LIG3	0.000177986	2.87658
EPB41	0.000455531	2.87384
MYRIP	0.000259851	2.86786
PAN2	0.00111306	2.86593
ARSG	0.0132518	2.85221
BAIAP2L2	0.00901545	2.84347
TMTC2	0.0109646	2.8404

Appendix: Results of microarray comparisons

PCDHB8	0.0178598	2.83704
CDK5R1	0.0139846	2.83279
BTG1	0.0005141	2.83216
SGK2	0.00172107	2.82615
TBPL1	0.00393787	2.82513
EPB41L4B	0.00295867	2.81611
ZNF487P	0.00157107	2.80741
PRKCD	0.00386614	2.80475
LRBA	0.014648	2.80238
RAB4A	0.000731172	2.79589
SARM1	0.000637432	2.79339
COBLL1	0.00187792	2.79195
RCAN2	0.00743587	2.78832
SEC11C	0.00127302	2.78624
TMTC4	0.000148767	2.77219
DIP2C	0.000461537	2.7591
CCDC110	0.000427029	2.73691
ABHD2	0.0133983	2.7356
KCND3	0.00140277	2.73333
MAPT	0.00962652	2.73205
CBS	4.17E-05	2.73113
BCOR	0.000417236	2.73008
FADS2	0.0118642	2.728
PPAPDC1B	0.000129843	2.70842
EIF3E	0.00511527	2.70762
ZNF680	0.00503363	2.70498
MIPOL1	2.52E-05	2.70389
KCNH6	0.00794909	2.69868
KRTCAP3	0.0129331	2.69617
DEGS1	0.00675819	2.68507
NIPAL2	0.00810288	2.68167
JTB	0.00346446	2.67416
TAS2R31	0.0173861	2.67195
PFKM	4.08E-05	2.66848
MYT1	0.00954805	2.6588
VWA2	0.0106316	2.65834
ENOX1	0.00827313	2.64851
C18orf32	0.000666734	2.64325
CASZ1	4.80E-06	2.63691
DDT	0.00166947	2.63654
RFX3	0.000206991	2.63645
FANCF	0.000456496	2.62841
ICK	0.0158828	2.61863
MLEC	0.000473692	2.61818
SETMAR	0.000109406	2.61788
CLDN8	0.00360626	2.61369
DHRS7	0.00091076	2.61189
MRPS33	0.00726921	2.60588
NACA	0.00119428	2.60502

Appendix: Results of microarray comparisons

TMEM141	0.000397828	2.58738
TRPV6	0.00392988	2.58617
EPHA6	0.012336	2.58269
FKBP4	0.00011031	2.57984
KIAA1683	0.00663439	2.57892
MFSD4	0.0094336	2.57605
HNF1A	0.0107862	2.57457
RBM47	0.011142	2.5727
WASF3	0.000125702	2.56745
GTF3C6	0.00103865	2.56427
PAPSS1	0.000837291	2.56371
RPSA	0.00369122	2.56087
CYB5A	0.00565792	2.54932
ARL17A	0.0141653	2.5462
GOLPH3L	0.00954095	2.54481
SERGEF	0.00651438	2.54459
PNPLA7	4.56E-08	2.54443
GEMIN8	2.78E-05	2.5418
C1QTNF3	0.0106434	2.54168
WNK3	0.00627994	2.5345
C7orf55	1.41E-06	2.53069
SETBP1	0.00375851	2.52094
MAZ	4.44E-05	2.51983
CIRBP	0.00378341	2.51795
SIGIRR	0.0105733	2.51471
TLE3	3.53E-05	2.5104
HSD11B2	5.58E-06	2.50626
MAGI1	0.000743915	2.50101
ZIC2	0.00605332	2.49713
VIPR1	6.27E-05	2.49164
PEG3	0.0042198	2.48981
OGDHL	0.000379386	2.48941
KLHL35	0.0112555	2.48767
ELOVL6	0.00722913	2.48559
C21orf33	6.89E-05	2.48215
SNORD49A	0.00171993	2.47668
RPL14	0.000928597	2.47349
POLI	0.00116121	2.47127
RPL15	0.00263809	2.45896
XYLB	0.00175994	2.4554
ZNF84	0.0147196	2.4532
EEF1A2	0.0130377	2.45062
DGAT2	0.00721803	2.44452
GAS2	0.00519019	2.44264
C9orf43	0.00167568	2.43935
ZNF552	0.00434199	2.43643
TAS2R4	0.0192668	2.43074
CRIPAK	0.0142342	2.42965
RNF43	0.00407165	2.42009

Appendix: Results of microarray comparisons

ZDHHC21	0.000443524	2.41428
DIO1	0.0149691	2.41198
ZNF568	0.0163237	2.40635
RPL37A	0.01904	2.40467
SCOC	2.66E-05	2.40393
ZNF239	0.00281494	2.40094
MDH1B	0.000270428	2.39832
CLYBL	0.00302114	2.39389
TAS2R20	0.001061	2.39289
C10orf118	0.00604964	2.39242
PIGP	0.00277704	2.38872
TCEANC	0.000702433	2.38289
ELMO1	0.019929	2.3817
PEBP4	0.0063963	2.37773
ATP6V0E2	0.000492212	2.37769
COX18	0.00416926	2.37735
CCDC125	0.00469228	2.37688
SYNGR2	0.00347331	2.37138
MAP7	0.010343	2.35831
RORC	0.00163873	2.35775
C22orf32	7.70E-05	2.35651
NFASC	0.0178347	2.35296
MID1IP1	0.00361408	2.34679
RDH11	0.00584819	2.33954
ATP5I	0.0175028	2.33491
LPHN1	0.0145279	2.33389
CPLX2	0.00920365	2.33251
BBS10	7.19E-05	2.33026
TMEM125	0.00304741	2.32905
PARVB	0.00154099	2.32541
LRRC37A3	0.0014052	2.32517
ZNF652	0.00708109	2.32099
SIDT2	0.0198327	2.32082
CSAD	0.00095066	2.31792
STYK1	0.00469142	2.31733
COQ7	0.000127571	2.31652
USE1	0.0186407	2.31634
AACS	1.04E-05	2.3162
NDUFA5	0.000645997	2.31601
EDA	2.81E-06	2.31585
C5orf33	0.00439896	2.31477
FAM89B	0.00654482	2.314
KLHDC9	2.25E-05	2.31266
MRPS35	0.00392294	2.31201
PTMS	0.00182207	2.3073
AFF1	0.00914429	2.30646
RAB11FIP4	0.0113832	2.29879
BEND4	0.000387337	2.29868
ZBTB7B	0.000509848	2.29651

Appendix: Results of microarray comparisons

MMAA	0.00343651	2.29451
MAPK8IP1	8.24E-06	2.29264
IMPDH2	0.00467582	2.29209
COMT	0.000399984	2.28993
HEBP2	0.0023963	2.28786
EPN3	0.00226317	2.28753
CATSPER2	5.73E-05	2.28623
LOC100128508	0.007694	2.28514
LSS	0.0158261	2.28436
C1orf168	0.00348181	2.28396
ACTR3B	0.00541082	2.28263
MYBPC1	0.0048782	2.28131
NME3	4.37E-05	2.27862
BCL9	0.0165813	2.27825
TUB	0.00728015	2.27451
C12orf62	0.00557654	2.27256
SPATA20	0.0145053	2.27246
MCF2L	0.00297411	2.27202
ATP5G2	0.000286252	2.27019
COX6C	0.00994555	2.2691
RNF41	0.000170343	2.26581
EPM2AIP1	0.00729842	2.26374
MRPL24	0.0030102	2.2608
HOXC6	0.00104977	2.25888
KCNH2	0.010756	2.25776
MMAB	0.0060598	2.25773
FAM188A	0.00142538	2.2575
NAPB	0.0124209	2.25557
TTC21B	0.00824996	2.25489
DMXL1	0.00557197	2.25238
ZMYND12	0.00569614	2.25022
ACN9	0.0113073	2.24951
PEBP1	0.000264865	2.24669
NAAA	0.0152287	2.24012
ERGIC1	0.00032095	2.23883
DPH5	0.00875465	2.23877
RPL11	0.0187908	2.23663
RAB5B	0.011524	2.23589
ZKSCAN1	0.00218382	2.23504
PFDN5	0.000491625	2.23336
ZBTB37	0.00669906	2.22776
LYPLAL1	0.00897938	2.22534
KDM6B	0.00243354	2.22434
DSC2	0.0133145	2.22139
C17orf75	4.54E-05	2.22088
KIAA1430	3.65E-05	2.22006
RGL2	0.00912732	2.21944
ABCA2	0.00571353	2.2186
SFXN4	0.00399561	2.21842

Appendix: Results of microarray comparisons

MUC13	1.21E-05	2.21742
NAPEPLD	0.00132453	2.21671
DEFB132	0.00484652	2.21481
HSD17B8	0.00211728	2.21442
C12orf51	0.00604716	2.21256
C5orf30	0.00986346	2.20776
PEX5	2.29E-05	2.20613
ATXN7L3B	4.75E-05	2.20511
RG9MTD2	5.43E-05	2.19977
FSCN2	9.85E-05	2.19872
PACSLN1	8.65E-06	2.1978
PCBD1	0.00445401	2.19747
MRPS28	0.0020814	2.19685
CYHR1	0.000525668	2.19611
ANKRD16	0.000812854	2.19503
SLC1A1	0.0101357	2.19479
CALML4	0.000719729	2.19458
RAB26	0.00147112	2.18802
TCTN1	1.22E-05	2.18612
C12orf57	0.016452	2.17963
ATP5D	0.0161348	2.17918
DYNC1I2	0.00380958	2.17808
CDK14	0.00266252	2.17705
KCNH8	0.00904597	2.17402
NDUFA3	0.0103533	2.16839
RALGDS	0.00854214	2.16743
INPP5J	0.00988049	2.16633
SREBF1	0.00546892	2.16462
NPDC1	1.30E-05	2.16429
C11orf1	0.00851524	2.16233
C10orf32	0.00296979	2.16075
PIGM	0.0163603	2.15357
SLC22A23	0.001963	2.15144
DTWD2	0.00244827	2.14958
C4orf39	0.00348825	2.1487
NME2	0.0185645	2.14829
PMVK	0.00059826	2.14803
GABPB2	0.000589035	2.1474
GNG13	0.000638754	2.14372
SLC25A10	0.000587547	2.14313
FAAH2	0.0187773	2.14221
ACAT1	0.00322499	2.14006
TBL1Y	0.00742693	2.14001
STARD3NL	0.00764326	2.13972
ALKBH7	0.0135582	2.13121
TRPM8	0.0110279	2.13087
ZNF32	0.0145336	2.12967
ALKBH2	0.0120646	2.12959
KLK1	0.00687829	2.12772

Appendix: Results of microarray comparisons

REPIN1	0.000875793	2.12594
C15orf28	0.00392178	2.1234
ZNF233	0.0198072	2.12339
RIMS1	0.00945331	2.12315
ATP5A1	0.0013253	2.12212
GNL3	0.00852925	2.11918
IL17RB	0.00719315	2.11909
GNMT	0.00270906	2.11798
DIRAS1	0.000690955	2.11677
ADCK3	0.0160741	2.11676
SMPD3	0.00724705	2.11508
CYP51A1	0.00424491	2.11445
TMEM134	9.43E-05	2.11312
PDHX	0.00203422	2.1124
TMEM80	0.00107164	2.11093
TTC12	0.000969357	2.11081
CHD3	0.000366811	2.10854
CCDC112	0.00104602	2.10845
PPM1H	8.24E-05	2.1045
ASPHD1	0.000103963	2.1035
TMEM192	0.00283739	2.10187
PPP1R2	0.00285996	2.10035
KIAA0368	0.0194544	2.10002
NVL	0.00744584	2.09774
RNF130	0.00374261	2.09649
ARHGDIG	0.000351405	2.09448
CSMD1	0.00582316	2.09222
DEGS2	0.00320455	2.09151
ZNF880	0.00980996	2.09009
CCDC78	0.000682646	2.08971
TMEM97	0.00560124	2.08882
IDH2	0.00198491	2.08856
HSPE1	0.00730674	2.08747
PCP4	0.000208692	2.08617
FIS1	0.00366356	2.08193
VPS13A	0.00739727	2.08113
ZNF321	0.00729816	2.08058
GAMT	3.69E-06	2.07824
KIF12	0.00977207	2.0776
TMEM25	0.00414212	2.07744
CYP39A1	0.000428276	2.07596
FZD5	0.00155961	2.07232
TARDBP	0.0059452	2.07147
TM2D3	0.00022498	2.07143
BAMBI	0.000998557	2.06989
IMMP2L	0.000145232	2.06934
RPS11	0.00335773	2.06911
TIMM13	0.000566669	2.06781
PLA2G5	0.0111906	2.0652

Appendix: Results of microarray comparisons

NEK8	0.00838033	2.06305
TMEM179	0.000298887	2.06276
NECAP1	0.00043497	2.0623
GALNT11	6.39E-05	2.0586
EEF2	0.00319062	2.0584
CCDC56	0.00903088	2.05736
NOSTRIN	0.000117197	2.05608
PEX12	0.00378916	2.05592
NDUFAF4	0.00237267	2.0538
RAP1GAP2	0.00473934	2.05198
CBX4	8.80E-05	2.04918
FGF13	0.000831214	2.04917
RAB36	0.0190914	2.048
RPL41	0.0156639	2.04589
JTB	0.0086463	2.04493
DCXR	0.00954975	2.04488
TMEM5	0.00014163	2.0438
AGBL3	0.00341133	2.04343
BMX	0.00542594	2.04134
ZNF605	0.0117885	2.04004
NUDT4	0.000411369	2.03783
ZDHHC11	0.0131873	2.03237
TFF3	0.000811599	2.03097
CCNG2	0.00463092	2.03065
PRKAG1	0.00112683	2.02964
TMEM66	0.00190452	2.02562
ARL1	0.0031618	2.02474
C16orf53	0.000405267	2.01732
CPLX1	0.00163252	2.01596
TOMM20	0.000162301	2.01508
CNTD1	4.83E-05	2.01118
ACADM	0.000381443	2.01078
CDC42SE1	0.000359909	2.00994
GDPD1	0.00655431	2.00747
SLC11A2	0.0142291	2.00725
NR3C2	0.00681394	2.00661
POLR3H	2.62E-06	2.00598
LRTOMT	0.00449136	2.00545
CAMK1	0.0158617	2.00521
NUDT9	0.00010476	2.00517
DPYSL4	0.00568069	2.00166
RPS20	0.00184938	2.00143

Table 1b

List of genes down regulated in *NKX3.1^{+ve}* cell lines, sorted by fold change. All genes are statistically significant at a significance level of 0.02 and are down regulated at least 2 folds as compared to *Nkx3.1^{-ve}* cell lines.

Gene Symbol	Corrected p-value (+vs. -)	Fold-change (+ vs. -)
ANXA1	5.16768E-08	-70.6331
GSTP1	4.04212E-09	-50.4909
GPRC5A	3.28972E-09	-49.9108
CAV1	4.3131E-07	-44.0176
DKK1	0.000054469	-40.8665
MYOF	1.37823E-06	-40.5866
MSN	5.85682E-06	-36.8968
IFIT3	0.000255549	-33.4652
IER3	2.88516E-08	-33.4573
S100A16	1.14546E-06	-31.1962
EFEMP1	2.09241E-07	-30.5633
ANXA2P2	5.23898E-09	-28.7686
SNAI2	0.000038134	-27.4173
RND3	1.55309E-07	-27.1417
CLDN1	6.74285E-05	-24.0118
CTGF	0.000107857	-23.7453
OAS2	0.00296965	-22.2337
IFIT1	6.85711E-05	-22.1929
SAMD9	0.000489966	-22.1711
TGFBI	0.00068293	-20.946
MET	6.82029E-05	-20.8816
AXL	9.22023E-10	-20.8384
OSMR	1.98162E-06	-20.6127
ANXA3	2.9317E-06	-20.404
CAV2	3.89935E-06	-20.1746
C3	0.00103884	-19.9608
CYR61	9.2241E-07	-19.854
IFI16	3.22535E-05	-19.6224
SLPI	0.00337092	-19.1324
PLAU	1.00274E-05	-19.1169
IFI6	0.000119716	-18.8202
GLIPR1	0.00205774	-18.6057
CYBRD1	0.000236654	-18.2736
IFI44L	0.013059	-17.8005
LHFP	1.41119E-05	-17.3407
TIMP1	0.00300431	-17.3351
ITGA3	2.16585E-08	-16.9918
SPARC	0.00284035	-16.577
PLP2	9.72309E-05	-16.5606
ANXA2	3.99006E-09	-16.1863
AHR	3.52554E-05	-15.975
SCRN1	2.39752E-06	-15.9657
WWTR1	5.97954E-06	-15.9403

Appendix: Results of microarray comparisons

IFITM1	0.000128657	-15.5625
EMP1	0.000659766	-14.416
LPAR1	6.6321E-06	-14.0495
DCBLD2	1.89032E-05	-14.0004
MX1	0.00199668	-13.8723
IL1B	0.00798583	-13.7313
OAS1	0.000937126	-13.709
SLC43A3	0.00212171	-13.6361
APOBEC3C	6.18344E-05	-13.4858
DAB2	5.88161E-05	-13.2962
NT5E	7.48894E-07	-13.2629
C3orf59	2.66009E-06	-13.1359
SAMD9L	0.00469176	-13.1299
ITGB4	0.00048472	-13.067
TGFB2	0.000153684	-13.0666
FSCN1	2.30821E-05	-13.0451
AREG	0.000455287	-12.9323
KRT7	0.0174223	-12.6261
PLK2	0.000135866	-12.5776
LCN2	0.0180927	-12.4439
IFIT2	0.00319046	-12.4204
RAB34	8.23631E-11	-12.3019
TGFBR2	4.57441E-05	-12.1197
IGFBP4	1.64136E-06	-12.0869
CDH3	0.0067469	-12.0427
HLA-B	0.00508621	-11.9998
IL8	0.000690138	-11.8514
CD109	4.9705E-06	-11.7584
PARP6	0.0055777	-11.6854
ID1	0.00561372	-11.5212
IL18	0.000271218	-11.4122
LGALS3BP	2.50124E-05	-11.3251
SEMA3A	0.000474605	-11.221
PYGL	1.51425E-05	-11.1874
FGF2	0.000123028	-10.8207
IGF2BP2	0.000010237	-10.7982
ICAM1	5.83078E-05	-10.6949
CDA	0.000812047	-10.657
TFPI2	7.60444E-05	-10.5972
LAMP3	0.00193702	-10.5652
IFI27	0.00967459	-10.5106
SLC7A5	0.00427423	-10.4414
SP100	2.23757E-05	-10.3013
PLAC8	0.0110598	-10.2377
PLSCR1	0.000210127	-10.2065
EPAS1	1.05297E-06	-10.1905
FRMD6	0.000031619	-10.0925
KIRREL	4.64404E-09	-9.97754
TACSTD2	0.000092872	-9.94179

Appendix: Results of microarray comparisons

STOM	0.00329347	-9.79599
IFI44	0.00301592	-9.72898
CDH2	0.00711093	-9.65257
GBP1	0.0111508	-9.58318
LAMB3	0.00684951	-9.31125
CTSC	0.000112938	-9.16809
PSMD5	0.00224848	-9.04491
TNFRSF12A	3.80223E-05	-9.04253
VIM	0.0107266	-9.03404
IGFBP7	0.0181775	-8.91606
IL6	0.000463858	-8.91012
BNC1	2.43506E-05	-8.85772
ETS1	7.91407E-07	-8.7161
TNFAIP3	0.00668593	-8.68538
SULF2	0.0100784	-8.66554
OAS3	0.00029932	-8.65705
PLCE1	3.10527E-06	-8.62612
FST	0.0188093	-8.47364
PSMB9	0.00298145	-8.46483
ST6GALNAC2	0.000345032	-8.40577
CPA4	0.00825876	-8.38642
CD44	0.0162994	-8.28548
FOSL1	0.000116492	-8.26836
ZNF655	0.00103507	-8.24796
PAM	0.000143333	-8.20889
SLFN11	0.0107481	-8.15647
PLAUR	4.5709E-07	-8.12493
LGALS1	0.00496802	-8.02715
ACOT9	0.00031475	-8.01358
MYL9	4.29404E-06	-8.00802
RAB31	0.00236505	-7.98628
AHNAK2	0.000531759	-7.98077
FHL1	0.000408973	-7.95562
EMP3	0.000451604	-7.88877
	0.0161298	-7.81047
MMP14	5.69181E-05	-7.75153
MIR31	0.0015359	-7.72488
FXD5	5.54954E-07	-7.7113
C6orf145	2.17813E-05	-7.64802
PLOD2	0.000470573	-7.60594
CNN3	0.00444461	-7.59551
GJC1	2.43296E-05	-7.58275
ETV5	0.00280101	-7.57626
RUNX1	3.74874E-08	-7.35204
C6orf150	3.06037E-07	-7.32741
ITGA2	0.00161816	-7.31426
TSPAN6	0.000885992	-7.28461
PMAIP1	0.000734243	-7.27012
SERPINH1	0.00116133	-7.26164

Appendix: Results of microarray comparisons

RAB38	0.000299177	-7.16652
JUB	9.86331E-05	-7.14485
ABCC3	0.00255337	-7.13022
EPHA2	2.68298E-05	-7.03986
CRIM1	0.000111452	-7.00395
PDLIM1	0.000337075	-6.94102
ETS2	1.33631E-05	-6.88113
HSPA1A	7.81068E-05	-6.87421
NR3C1	8.00491E-05	-6.85557
DFNA5	9.24241E-06	-6.84273
LEPREL1	0.00381346	-6.81456
HES1	0.00369914	-6.80188
SERPINB8	0.000072631	-6.77652
FBN2	0.00525982	-6.64598
TIMP2	0.000802771	-6.63327
RBMS1	0.00517056	-6.62188
AKR1B1	0.00106589	-6.6176
LOXL2	0.00138424	-6.57999
MFGE8	0.000725804	-6.56288
SFN	0.0149737	-6.5134
PARP14	0.00377863	-6.49779
APOL6	0.00168146	-6.46896
CASP4	0.000450889	-6.46399
NID1	0.000837165	-6.45934
EHD2	0.00206327	-6.44549
DUSP5	9.38115E-05	-6.4355
IFI35	0.00994435	-6.42467
SLC12A4	0.000117075	-6.41278
SERPINE1	0.00584303	-6.40238
OAT	0.00749349	-6.36277
PLEK2	0.0038628	-6.3589
KLHL5	2.72337E-05	-6.35794
GPR39	0.000390673	-6.27332
TINAGL1	0.0015486	-6.25971
SH3RF2	0.000458511	-6.22063
MCOLN3	0.000363114	-6.21166
JAK1	0.00027478	-6.19817
DDX60	0.0108783	-6.19362
TGFB1	1.30114E-05	-6.18866
EXT1	1.11964E-06	-6.15107
UBASH3B	0.000868379	-6.13129
HCP5	0.00606405	-6.11819
AMOTL2	4.80253E-06	-6.1149
MAML2	0.00289997	-6.08155
DDX58	0.00278395	-6.07168
PHLDB2	0.00162838	-6.04213
SLC16A3	1.92691E-05	-6.03604
UBE2L6	0.000025547	-6.02761
LRRC8C	0.000220016	-6.01811

Appendix: Results of microarray comparisons

PRSS23	2.73824E-05	-5.99628
GPX8	0.000520479	-5.97627
NTN4	0.0044376	-5.9664
RNF217	7.54146E-05	-5.96621
SERPINE2	6.81478E-05	-5.96094
ERAP2	0.00096284	-5.96083
DSE	9.07876E-06	-5.86154
PAPSS2	0.00409383	-5.84439
HEG1	2.75732E-05	-5.79592
G0S2	0.00803557	-5.78386
GLT8D2	0.00465811	-5.77552
AVPI1	0.000286759	-5.76793
GNA15	0.00539216	-5.7572
PHLDA1	1.98477E-05	-5.73787
NEDD4	0.00193029	-5.72255
RBMS2	3.48761E-05	-5.72135
POPDC3	0.000191658	-5.71502
TUBB6	0.00331311	-5.7068
FLNA	8.39538E-05	-5.67359
KRTAP2-4	0.00350568	-5.66655
LPCAT2	0.00275327	-5.63885
HLA-E	3.45859E-06	-5.59166
DUSP6	0.0073171	-5.57597
MICB	3.72138E-05	-5.57287
ELF4	0.000549189	-5.53112
SMAD3	2.89847E-06	-5.51661
SLCO3A1	0.018327	-5.50716
HLA-F	0.00543064	-5.48607
SGK1	0.0139067	-5.47337
PTRF	0.00246821	-5.42149
IL4R	2.15374E-06	-5.38976
PTPN14	8.60882E-07	-5.38645
ARHGAP42	5.47681E-05	-5.36671
CERCAM	8.85211E-05	-5.35943
ARNTL2	0.000296323	-5.34627
KLF5	0.00150294	-5.34469
SMARCA1	0.000738421	-5.33788
BMP4	3.71531E-05	-5.30155
EPB41L2	0.00222643	-5.29464
GNE	0.000304577	-5.2759
TPBG	3.73177E-07	-5.27125
NCRNA00152	0.00972505	-5.25427
BHLHE40	0.00147059	-5.21708
MEIS2	0.000139278	-5.21155
SERPINB1	0.00744809	-5.20457
B3GNT5	0.000394115	-5.14867
BASP1	7.62554E-05	-5.14656
TGFB1I1	0.000646055	-5.14459
DNM1	2.31725E-05	-5.13769

Appendix: Results of microarray comparisons

RUNX2	0.000719674	-5.13639
KDELR3	0.000510991	-5.12767
CACHD1	1.67527E-05	-5.09986
PRICKLE1	0.0187297	-5.04453
CHST11	0.000172945	-5.04164
FHL2	3.39634E-08	-5.01397
NNT	0.00641112	-4.99533
NMI	0.00128654	-4.9857
CDCA7L	0.0057849	-4.98146
CSF1	0.000172003	-4.93736
SPG20	7.9618E-06	-4.93166
MATN2	0.000352553	-4.92251
ACSL4	0.000107356	-4.91433
TRIP10	0.000021893	-4.91201
ZNF185	2.83744E-05	-4.82287
NAV2	0.000503461	-4.79501
FBLN1	0.000715895	-4.78744
ATP10D	0.000898603	-4.76948
PSMB8	6.73701E-05	-4.76458
GFPT2	0.00122102	-4.75114
ACSF2	0.000468904	-4.72139
C15orf41	4.18617E-05	-4.70904
IRF1	0.00186172	-4.70501
IRF9	0.000991627	-4.66975
AFAP1	0.00133114	-4.66151
PMP22	0.0163226	-4.66041
SDC4	4.37298E-05	-4.66029
PDGFC	0.00124823	-4.65577
FBXO27	2.49849E-05	-4.63434
EREG	0.00423198	-4.61209
TBC1D2	0.0023898	-4.60901
DGKA	0.00103532	-4.56426
P4HA2	7.47402E-05	-4.55203
CYP2S1	3.93886E-05	-4.54149
CD55	0.000932605	-4.53194
OASL	0.00808677	-4.52317
IGFBP6	4.03197E-05	-4.51504
RBMS3	0.00144932	-4.47508
ASAP1	5.99841E-06	-4.46706
TAP1	0.00307706	-4.46584
SAMHD1	0.00318738	-4.45417
IGFBP3	0.00690509	-4.45358
LGALS3	2.71227E-05	-4.45051
STAT1	0.000971949	-4.43597
TIPARP	0.000186296	-4.42966
KRT80	7.45289E-07	-4.42959
CCBE1	0.00969284	-4.42251
SCHIP1	3.04959E-06	-4.40917
GALNT12	0.00167722	-4.39234

Appendix: Results of microarray comparisons

HTATIP2	8.61174E-05	-4.37896
PI3	0.0183282	-4.37883
KDELC2	0.00897955	-4.37778
LIF	0.000400599	-4.37483
DKK3	0.00791292	-4.37081
FGD6	0.0121657	-4.37061
MFI2	5.07912E-05	-4.34591
AMIGO2	0.0111389	-4.33482
RP9P	0.000387531	-4.32471
SIRPA	0.0019722	-4.29989
EDN1	0.00150674	-4.27634
MYO1B	0.00999434	-4.27008
BIRC3	0.0116003	-4.26684
SP110	0.00368091	-4.25829
CXCL2	0.00103764	-4.25815
TNS3	0.00296049	-4.25805
OBFC2A	0.000236759	-4.25516
ZNFX1	0.000222283	-4.25258
CAPN2	0.00143281	-4.23004
CCDC88A	0.00111163	-4.22754
HSPA2	0.00722202	-4.21764
FBN1	0.018795	-4.20836
ITGA5	0.000453505	-4.20826
SPRY2	0.0152283	-4.18125
MCAM	0.00404461	-4.17653
FLNC	0.00918426	-4.13301
RRAS	0.000137652	-4.11338
PTPRK	0.0027898	-4.11155
CLIP4	4.36767E-05	-4.09099
CDKN2A	0.000254247	-4.07647
STK17A	0.00138504	-4.07174
BICC1	0.00721331	-4.07121
P2RY2	0.017965	-4.06036
WWC2	4.44487E-05	-4.05584
MRC2	0.00147234	-4.05192
YAP1	0.0112131	-4.04364
CNN2	0.00023208	-4.0314
DPYD	0.00610759	-4.02668
CTSB	0.0162006	-4.02111
SWAP70	0.00533442	-4.01658
C1R	0.0191819	-3.99821
GPRC5B	0.00169907	-3.99648
SLFN13	0.00711799	-3.97464
RIN3	7.89575E-05	-3.96637
NFE2L2	0.00392796	-3.9564
F2R	0.0143239	-3.91925
CMTM3	0.00144303	-3.90886
ARSJ	7.87538E-07	-3.90312
LY6K	0.0052448	-3.90252

Appendix: Results of microarray comparisons

CD68	0.000708252	-3.87222
SH3KBP1	0.00107491	-3.86493
QKI	0.00739872	-3.82561
NUAK1	0.00197859	-3.7968
FMNL2	0.00263554	-3.78923
PLCD3	1.94844E-05	-3.77625
MBNL2	1.60738E-05	-3.77517
DLC1	9.68736E-05	-3.77188
ETNK2	9.73443E-05	-3.76996
GBP3	0.00204395	-3.76807
ALDH3B1	0.00512099	-3.73627
IFI30	0.0115	-3.71907
SLC46A3	0.0132481	-3.71502
EGFR	0.00651366	-3.68763
DMKN	0.000202324	-3.68026
KLF12	0.0150024	-3.67942
CHRNA1	0.0180727	-3.65463
DBNDD2	6.52017E-05	-3.65242
C19orf33	0.000254486	-3.63345
OSBPL3	0.000501535	-3.63213
PHF11	8.89275E-05	-3.62708
FYN	0.00403403	-3.62237
TMEM173	0.000072566	-3.61753
SP140L	0.0127948	-3.60897
SERTAD1	0.000151448	-3.60627
HTR1D	0.000944569	-3.60489
CORO1C	0.000138563	-3.59391
FAM40B	0.00709172	-3.57897
ADAMTSL5	8.85724E-05	-3.57175
SERPINA1	0.0183034	-3.53154
TRIP6	0.000020611	-3.52008
LY6E	0.000752511	-3.51972
TAP2	0.000199305	-3.49403
CERK	0.000473186	-3.48296
KCTD14	0.000449054	-3.48242
ERO1L	0.000167391	-3.48226
NXN	0.00599637	-3.48004
TRIM38	0.0059393	-3.47271
COL4A2	8.21848E-05	-3.46627
DST	0.00191561	-3.46041
CDC42EP3	4.94305E-05	-3.45706
PDCD1LG2	0.0188449	-3.45501
LHFPL2	0.000031103	-3.44852
TNFAIP2	0.00355173	-3.43986
PLEKHH2	0.0171887	-3.42829
PHF19	6.72837E-05	-3.42461
OPN3	0.00362928	-3.41594
GAS6	0.0010925	-3.4104
CCDC82	0.00486016	-3.39138

Appendix: Results of microarray comparisons

GSN	6.06685E-05	-3.38646
TCF7L2	0.000167555	-3.38214
SPRED1	0.0151636	-3.37712
PHLDB2	0.00535228	-3.37559
MR1	0.00398758	-3.36982
PYCARD	0.00148601	-3.36205
VEGFC	0.00102392	-3.35851
ARPC1B	0.00744241	-3.3575
SRGAP1	0.0165639	-3.34836
CDKN2B	0.00501016	-3.33292
LCAT	0.000643132	-3.32667
TRAM2	0.000729826	-3.30978
BCAR3	0.0108391	-3.30624
CPVL	0.00629335	-3.29425
CTSA	0.000538384	-3.29424
CAPG	1.72027E-05	-3.29045
RARRES3	0.00616892	-3.28613
TSPAN5	0.000506771	-3.28448
NCEH1	0.00184348	-3.27916
CNTNAP1	0.00293832	-3.27559
FOSL2	4.32398E-05	-3.27066
IFIT5	0.0155928	-3.26158
TCF7L1	0.000386691	-3.25634
QSOX1	0.00677695	-3.24946
EHD4	0.000321846	-3.24707
OPTN	0.00497186	-3.23443
BMP1	0.000751619	-3.22228
CHST3	2.75586E-05	-3.20791
WFDC2	0.0136399	-3.20058
RGNEF	0.0112131	-3.19632
SERTAD4	0.0179364	-3.19529
CCNJL	0.000331849	-3.19273
LMNA	0.00535256	-3.19051
GLIS3	0.0168871	-3.17912
KCTD12	0.00218214	-3.1646
SYDE1	0.00441943	-3.15305
CA2	0.00910664	-3.14172
MCC	0.0127797	-3.13828
CDC42EP1	0.00196693	-3.13602
TCIRG1	4.97185E-06	-3.11335
HOXB9	0.00883356	-3.1074
GPC1	0.000242612	-3.10429
B2M	0.00212458	-3.09993
FERMT2	0.00171672	-3.09935
MDFIC	0.0127176	-3.09367
RFWD3	0.0148966	-3.07704
C14orf169	0.000519035	-3.07089
SPATS2L	0.000315017	-3.06779
CLIC4	0.0101526	-3.06594

Appendix: Results of microarray comparisons

GLCE	2.78336E-05	-3.0587
ARHGAP29	0.00276495	-3.05789
ITPRIP	0.00151201	-3.05731
SLC25A24	0.00038662	-3.05527
PPL	0.00285255	-3.05488
OLFML2A	0.00170088	-3.05002
GALNT6	0.000630979	-3.04553
CD59	0.00287743	-3.039
LARP6	0.00437967	-3.03513
PPP1R15A	0.00426881	-3.03104
PLA2R1	0.000502539	-3.03029
ELK3	0.00554329	-3.02898
NEXN	0.00795114	-3.02642
FCHSD2	0.000110486	-3.02564
SLC25A43	0.00152065	-3.02301
FNDC3B	3.12014E-05	-3.00513
NHS	4.56228E-05	-3.00463
ZAK	0.000487885	-3.00327
SYNPO	0.000957347	-2.99327
FAM92A1	0.00538032	-2.99204
SRPX2	0.0110941	-2.98645
IKBIP	0.000637889	-2.96734
RAB32	0.00181187	-2.96254
TSPAN2	0.00100449	-2.92152
HPSE	0.00571712	-2.91759
ADAM19	0.00649031	-2.91414
WIPF1	0.0195622	-2.91153
TMEM63A	0.0129047	-2.90662
PTPRE	0.0180511	-2.90614
ARL4D	0.000668322	-2.90496
ITM2C	0.0158906	-2.90312
NALCN	0.00807821	-2.89465
FSTL3	0.0180061	-2.88833
PLEKHG2	0.00454579	-2.88668
COTL1	0.0185972	-2.8851
FAM129A	0.003693	-2.88415
DENND3	0.000309059	-2.88197
IL1RAP	0.00778028	-2.87754
KIAA1949	8.69343E-07	-2.87421
ZFP36L1	0.000441021	-2.87212
MLKL	0.00120265	-2.86793
LPCAT4	0.00129927	-2.85668
RARG	0.014756	-2.84893
INF2	6.06556E-05	-2.84561
C19orf66	0.00427581	-2.84305
FRMD5	0.00203196	-2.83686
FUT8	0.0119251	-2.83401
IL13RA1	0.00413332	-2.82708
CEP55	0.0152214	-2.81412

Appendix: Results of microarray comparisons

THSD4	0.00185106	-2.80714
AGPAT9	0.0134836	-2.79707
FAM46A	0.0122073	-2.7916
TLR6	0.00631173	-2.78086
ACTN1	0.000500857	-2.77651
STK38	0.00185769	-2.76999
UHRF1	0.0112788	-2.7678
COL4A1	3.42849E-05	-2.76655
40422	6.16562E-05	-2.76535
C9orf30	0.00131238	-2.76525
CEBPB	0.000735269	-2.76436
C19orf21	0.00279991	-2.76203
UGCG	0.00330229	-2.75819
C3orf52	0.00245943	-2.75713
ADORA2B	0.000344455	-2.75165
EIF2AK2	0.00743996	-2.75164
UPP1	0.0149309	-2.74129
FGD1	5.51095E-05	-2.7376
PLAGL1	0.00941376	-2.71264
ANTXR1	0.00431226	-2.71154
GRN	0.00224519	-2.71034
IQGAP1	0.000112849	-2.70928
ABCA1	0.00833285	-2.70923
MYO1E	0.00351238	-2.70617
NAB1	0.00150347	-2.68232
ADAM17	0.00117794	-2.67848
DNAJB6	0.00233971	-2.67675
DOCK5	0.00826903	-2.6745
TLE1	0.00707571	-2.67034
NFKBIZ	0.0123457	-2.66343
DONSON	0.00690817	-2.66207
C9orf125	0.00645313	-2.65872
HBEGF	0.00701702	-2.65642
SNX24	0.00882094	-2.65323
ANKS6	0.000148803	-2.65012
FAM126A	0.00458266	-2.64417
ARHGEF2	0.000641844	-2.64233
BAG2	0.00877702	-2.63484
PPPDE1	0.000248375	-2.63297
HIST1H3G	0.0114912	-2.62396
AGPS	7.70203E-05	-2.62284
B3GNT3	0.0134445	-2.61227
WARS	0.00576342	-2.59939
FZD6	0.0082699	-2.59686
FHL3	0.000612765	-2.59235
PLBD1	0.002504	-2.58192
CEACAM1	0.000005412	-2.58183
PRDM1	0.00262905	-2.58148
TAX1BP3	0.00109161	-2.57897

Appendix: Results of microarray comparisons

B3GNT9	0.00014877	-2.5775
PML	0.00127849	-2.57194
PTPLA	0.0169543	-2.56875
KIAA0802	1.00902E-05	-2.56472
IL32	0.00167237	-2.55877
APOBEC3G	0.00784562	-2.55775
TJP2	0.0032469	-2.5513
CBL	0.000422814	-2.54911
CYP27A1	0.00209349	-2.53952
INPP1	0.0117295	-2.53684
FLT3LG	0.0053888	-2.53618
SMO	7.03577E-05	-2.53545
ARL4C	0.000435855	-2.5321
HMG20A	4.57568E-05	-2.52593
BCL3	0.000304669	-2.5227
LRP1	0.00876388	-2.52072
PLCG2	0.00914964	-2.51731
PRKD3	0.00411772	-2.51556
NCOA3	0.00111445	-2.51488
PXN	0.0185467	-2.50812
UST	0.0136053	-2.49985
NSUN7	0.00863788	-2.49497
GPR176	0.000852769	-2.49415
SLC20A1	0.00364846	-2.49297
BRWD3	0.000171206	-2.48089
ERAP1	0.00987217	-2.47877
AURKA	0.015733	-2.47756
PRELID2	0.000233223	-2.47326
SNAPC1	0.00439699	-2.47031
OSBPL6	0.0137516	-2.46678
SLC1A5	0.0177585	-2.46281
SLC12A6	0.00138209	-2.46106
EXOC2	0.00249656	-2.45385
TRIM6-TRIM34	0.000354329	-2.4503
MSLN	0.000563558	-2.44965
PDPR	0.000394285	-2.4468
IL6ST	0.00196496	-2.44503
TGFBR1	0.000111998	-2.44126
PANX1	0.000123475	-2.43364
C1orf38	0.00204653	-2.43342
ERLIN1	0.00135759	-2.43269
JDP2	0.0167538	-2.43117
PPP4R1	0.00196197	-2.42897
C13orf31	0.00081224	-2.42717
SOCS3	0.00123559	-2.42448
SERTAD2	0.0178537	-2.42176
ADCY3	0.00780957	-2.41593
QSER1	0.0102279	-2.41258
ZYX	0.00835412	-2.41014

Appendix: Results of microarray comparisons

STK10	0.000753706	-2.40982
FAM70B	0.00583374	-2.40873
C7orf23	0.00728557	-2.40699
PHTF1	0.000881417	-2.40591
EHD1	0.00439023	-2.40079
AKAP12	0.00955217	-2.39209
CCDC68	0.0198443	-2.38912
RAI14	0.00205823	-2.38839
SHB	0.000393748	-2.38387
PALLD	0.00239932	-2.38143
LRRK1	0.00139235	-2.37843
CDC14B	0.000244555	-2.37736
EFHD2	0.0147068	-2.37679
KPNA3	0.000709863	-2.37672
LRRK1	0.00282222	-2.37627
IFITM4P	0.0097814	-2.36344
PITPNM1	0.00109724	-2.35977
SPHK1	0.00224237	-2.35064
PATL1	0.000187504	-2.35037
FZD2	0.000142542	-2.34843
KIFC3	9.49691E-08	-2.33831
ARAP2	0.0014128	-2.32753
PI4K2A	0.000416398	-2.32345
ITPR3	0.0117436	-2.3159
FADS3	0.00124603	-2.31567
TFAP2A	0.00288416	-2.30776
PHLDA3	0.00422684	-2.29998
ADCY7	5.07922E-05	-2.29695
METTL4	0.0180498	-2.29335
C11orf63	0.00287636	-2.29156
LAMP1	0.000126355	-2.29106
AIFM2	1.86124E-06	-2.29101
CDCP1	0.0166911	-2.29035
TUFT1	0.00670961	-2.28766
RHOD	0.00366146	-2.27417
POLD3	0.0135681	-2.26894
NDE1	0.00701393	-2.26376
ASPHD2	0.00687124	-2.25763
HOXA1	0.000633176	-2.25546
PRDM5	0.00976598	-2.25004
RRN3P3	0.00371524	-2.24842
ANKMY2	0.00104908	-2.24179
KIF13A	0.00178145	-2.24131
C18orf19	0.000237095	-2.24109
PRSS12	0.00999511	-2.24055
IRS1	0.00363825	-2.23409
TP53I3	0.000286225	-2.23088
FOXE1	0.00564283	-2.22749
SKIL	0.0110897	-2.22294

Appendix: Results of microarray comparisons

RASA3	2.56543E-05	-2.22128
MOBK1B	0.00598773	-2.21119
STK17B	0.00208684	-2.20789
SPSB1	7.37313E-05	-2.20747
COL5A1	0.0145023	-2.20498
DIS3	0.0144974	-2.20302
OSBPL9	0.000107213	-2.19623
IL27RA	0.0169643	-2.19507
HDGFRP3	0.003575	-2.1893
ANXA2P1	0.00125299	-2.1882
TNFRSF10B	0.0107433	-2.18535
RBMS1	0.0119101	-2.18534
MICALL1	0.00651847	-2.18438
RAB23	0.00180208	-2.18159
PRKD2	0.000767538	-2.18133
CELSR1	0.00553905	-2.18019
MSRB3	0.00481723	-2.17206
NIPAL1	0.0106445	-2.16731
LAMA5	0.000801292	-2.16585
KLK7	0.0043878	-2.16059
PDE8A	8.46695E-06	-2.15763
HRH1	0.00195341	-2.15459
GGH	0.00711286	-2.15407
DNMBP	0.0100468	-2.15386
PTPN12	0.000530333	-2.15231
TMEM188	0.000016127	-2.15071
SYNJ2	0.00558197	-2.14739
SLC9A6	0.000148714	-2.14699
RB1	0.00521074	-2.14673
DCBLD1	0.0111368	-2.14443
TMEFF1	0.00745407	-2.14119
PAQR7	0.00808154	-2.13468
SLC2A1	1.42765E-05	-2.12869
SVIL	0.00928382	-2.12733
RGS19	0.000296728	-2.12137
DPP9	0.003149	-2.10783
BCL2L1	0.0011135	-2.1072
SNX6	0.00240248	-2.10626
NFKBIA	0.000155081	-2.09698
TMOD3	0.0142028	-2.09132
C16orf57	0.013861	-2.08895
TSPAN4	0.0143737	-2.0813
PCGF5	0.00748014	-2.08029
STARD13	0.0158959	-2.0774
TFDP1	0.0118179	-2.0753
PLA2G15	0.0121235	-2.07193
GBP7	0.00695921	-2.06956
AP3B1	0.00376697	-2.06932
DRAM1	0.00353264	-2.06696

Appendix: Results of microarray comparisons

LOC100131541	0.0017188	-2.06308
SPECC1	0.00758082	-2.05849
PMS2CL	0.0134691	-2.0576
SLC39A14	0.00345628	-2.05299
SLC16A5	0.00187633	-2.04871
PARP12	0.0158484	-2.04859
REC8	0.00609705	-2.04656
AGPAT4	0.00109437	-2.04653
ETHE1	3.77763E-05	-2.04554
HTRA1	0.000820379	-2.04329
PLSCR3	0.000176161	-2.04307
NTN1	0.0139239	-2.03977
	0.000119829	-2.02415
OSBPL11	0.0122347	-2.02122
CD58	0.00165668	-2.02027
TEAD3	0.00170693	-2.01592
CWF19L1	0.000878611	-2.01474
B4GALT5	0.00749645	-2.01401
YWHAH	0.0032156	-2.01299
FZD7	0.000738928	-2.01268
CDC25B	0.00776135	-2.00972
EZR	0.00354844	-2.00948
OBSL1	0.0153688	-2.0077
RIN1	0.00115229	-2.00677
ACOT4	0.00163674	-2.00381
GLIS2	0.00104233	-2.00092
ITGB1	0.00502797	-2.00046
CALU	0.0138539	-2.00037

Table 1c

List of genes up regulated in PC3-Nkx3.1 cell line as compared to PC3 cells, sorted by fold change. All genes are statistically significant at a significance level of 0.02 and are up regulated at least 2 folds as compared to *Nkx3.1^{-ve}* cell lines.

Gene Symbol	p-value	Fold-Change
C11orf92	0.000134064	205.878
KLK4	5.16E-06	201.526
LUZP2	6.02E-05	190.148
KLK2	0.000639323	161.028
SLC44A4	0.003892	134.921
PRUNE2	8.59E-05	133.299
KIAA1324	0.00443998	127.428
USP9Y	0.0195614	124.54
SLC44A4	0.00311076	103.339
GLYATL1	2.56E-05	100.33
KLK2	0.00340032	97.8169
TMEFF2	0.00191158	95.0582
PART1	0.000332855	90.3468
ACPP	0.000275051	87.4079
LUZP2	0.000938171	85.6227
PART1	0.000349848	85.2851
TXLNG2P	0.0121123	83.3722
USP9Y	0.0161678	81.7292
TMEFF2	0.000884111	74.5925
HIST1H3I	0.00323669	69.9164
ZNF542	0.0145325	67.6872
USP9Y	0.00733524	67.2826
UGT2B15	0.000912258	65.3851
USP9Y	0.014282	64.1003
ACPP	0.0016746	63.5895
ACPP	0.00117458	59.8836
TMEFF2	0.00465766	59.8601
RIC3	0.00435873	56.5069
HIST1H3I	0.00775976	52.8736
SLC44A4	0.0024468	50.3862
KIAA1324	0.00947606	45.9616
SLC44A4	0.00405128	44.8624
NKX3-1	5.87E-05	43.3751
USP9Y	0.0156628	42.776
KLK2	0.0095004	42.4746
TMEFF2	0.001479	42.1537
ACPP	0.00903616	41.1312
USP9Y	0.00825998	40.7535
PRUNE2	0.00080969	40.3309
KIAA1324	0.00850209	40.2835
FOLH1	0.000796717	40.1156
SLC44A4	0.00617921	39.9171
SYT4	0.00218232	38.8385

Appendix: Results of microarray comparisons

PRUNE2	0.000240155	38.3801
ACPP	0.00377368	38.3674
ZNF682	0.00220637	37.9459
SLC44A4	0.00117883	37.3892
DSEL	0.0175191	37.2827
TXLNG2P	0.0105149	37.0732
KLK2	0.00379096	36.2729
KIAA1324	0.0138067	36.0838
NME5	0.00108005	36.0806
UGT2B15	0.018352	35.797
CHRNA2	0.00265531	35.2834
nkx3-1	3.14E-05	35.0369
TMEFF2	0.00441453	34.5968
SLC44A4	0.000811795	34.5379
KIAA1324	0.0123794	34.4836
ACPP	0.00552459	34.435
SLC44A4	0.00422317	34.3477
TMEFF2	0.0146514	33.8006
USP9Y	0.0108302	33.1423
KIAA1324	0.0175209	32.2544
ZNF542	0.0174745	32.2095
SLC44A4	0.00317662	32.1971
ZNF610	0.00868887	32.1607
ZNF542	0.0130831	31.6908
SLC44A4	0.0019343	31.5973
USP9Y	0.0179075	31.1911
TMEFF2	0.00521163	30.0606
NKX3-1	2.93E-05	28.3653
NETO1	0.00577463	28.2785
SI	0.007213	28.1692
SLC44A4	0.00745738	28.0879
NKX3-1	2.90E-05	28.0343
MAOA	0.00593193	27.8869
SYT4	0.00171814	27.6817
LUZP2	0.00170887	27.5785
PRUNE2	0.00436461	26.4535
USP9Y	0.0141838	26.3625
FAM111B	0.0181191	25.9235
PRUNE2	0.000643057	25.7442
ACPP	0.0045089	25.4827
HPN	0.000249425	25.4023
SI	0.00834174	25.3985
MYBPC1	0.00704042	25.3044
SLC44A4	0.00270343	24.5612
NETO1	0.0137313	24.4761
GLYATL1	0.000184272	24.4747
SLC44A4	0.0002491	24.4288
ACPP	0.0110893	23.8028
SI	0.0168398	23.7515

Appendix: Results of microarray comparisons

KIAA1324	0.0158498	23.6303
NETO1	0.0115932	23.3947
SLC44A4	0.000985717	23.3189
CHRNA2	0.00242189	23.2629
RIC3	0.0050313	23.0475

Table 1d

List of genes down regulated in PC3-Nkx3.1 cell line as compared to PC3 cells, sorted by fold change. All genes are statistically significant at a significance level of 0.02 and are up regulated at least 2 folds as compared to *Nkx3.1^{-ve}* cell lines.

Gene Symbol	p-value	Fold-Change
ZNF655	0.00769441	-112.111
SPON1	2.88E-05	-104.141
CAV1	0.0110286	-89.8046
ZNF655	0.00354584	-88.499
COL4A1	0.000104111	-75.7586
ANXA3	0.0144359	-71.9997
ANXA3	0.0139191	-71.9337
	0.00050079	-71.5856
ZNF655	0.0106965	-68.764
AXL	0.00570065	-68.1277
PLAU	0.0197113	-66.6734
ANXA3	0.0179851	-66.1254
COL4A1	0.000205715	-66.0387
PLAC8	0.0122877	-65.4932
MCOLN3	0.00261173	-64.1731
C3	0.0146578	-60.3212
PLAU	0.0169961	-59.7421
CALB2	0.000410335	-55.6821
	0.00426049	-55.1458
FLT1	0.000524613	-53.8169
IFITM1	0.0111962	-53.7809
	0.000400095	-53.4298
IGF2BP1	0.00293824	-53.0476
ANXA1	0.0170331	-51.7178
ELSPBP1	9.76E-10	-50.6997
S100A16	0.0107769	-50.4462
FAM92A1	0.00162963	-49.9177
SERPINF1	0.000680264	-49.583
SERPINF1	0.000830207	-49.4655
CALB2	5.62E-08	-46.8097
ANXA1	0.0193363	-46.7409
CAV2	0.0063939	-46.5688
IFI16	0.00587386	-46.4848
IFI16	0.00360267	-45.9688
CALB2	0.000637121	-45.8149
ANXA3	0.0128904	-45.2236
AHR	0.0174931	-43.1437
FLT1	9.12E-07	-42.5309
ANXA1	0.0112555	-41.9128
IGF2BP1	0.00218191	-41.8094
	0.00262056	-41.7275
SEMA3A	0.0138788	-40.8006
ANXA3	0.0187398	-40.6405

Appendix: Results of microarray comparisons

ANXA1	0.0184631	-40.0437
CAV2	0.0060842	-39.7114
TSPAN2	0.000543297	-39.5012
AFF2	2.60E-05	-39.4835
IRS4	1.55E-07	-39.3583
SORCS1	7.20E-08	-38.8083
KLK6	0.0029559	-38.8045
	0.00197685	-38.6436
IGF2BP1	0.00117548	-38.2463
IFI16	0.000806392	-38.0729
FOXC1	0.0102975	-38.0193
SNAI2	0.00188321	-37.2823
ANXA2	0.00688656	-37.2755
	0.0012027	-36.6601
SLCO3A1	0.0165549	-36.6547
SNAI2	0.00157785	-36.4956
MCOLN3	0.00312546	-36.3496
SCRN1	0.0178007	-36.0855
	0.00129687	-36.0487
TSPAN2	0.000698544	-35.7681
SEMA3A	0.0152219	-35.7482
CAV1	0.0082828	-35.5064
FLNC	0.00973329	-35.3671
MYOF	0.0178632	-35.3315
NID1	0.00421071	-35.1272
IGF2BP1	9.72E-05	-35.1106
IFI16	0.00215648	-35.0476
	0.000561	-35.0239
ZNF655	0.0136229	-34.8457
BCAT1	0.0170118	-33.7653
SPON1	2.88E-05	-33.7432
IFITM1	0.0138693	-33.704
FOXG1	6.90E-05	-33.6428
MYOF	0.0147785	-33.4741
COL4A1	0.000187456	-33.3748
ZSCAN12	0.00696273	-33.1903
S100A16	0.0105229	-33.1018
	0.00247914	-32.7394
POU4F1	0.000217472	-32.4061
SCRN1	0.0133424	-32.2166
CAV1	0.00845316	-32.1518
IFI16	0.00321097	-31.998
MYOF	0.0134579	-31.7711
S100A16	0.0124632	-31.6559
FHL1	0.0102661	-31.6534
PLCE1	0.00258727	-31.336
C3	0.0143791	-31.2509
IFI16	0.00165123	-31.1894
IFI16	0.00392133	-31.0036

Appendix: Results of microarray comparisons

MYOF	0.0151048	-30.9591
AFF2	5.50E-05	-30.5873
	0.0050035	-30.3909
CA2	0.00459086	-30.2363
PLCE1	0.00132622	-30.1327
ANXA2	0.0119742	-29.9229
SCRN1	0.0117272	-29.8405
AHR	0.0174061	-29.8213
ANXA1	0.0184862	-29.6686
IFI6	0.0069872	-29.536
CAV2	0.00235619	-29.3383
GPR50	2.57E-06	-29.2794
UBASH3B	0.012798	-29.2713
UBASH3B	0.0169773	-29.2659
FLT1	1.24E-05	-29.1147
PLCE1	0.00348605	-29.0472
MCOLN3	0.00850663	-28.8632
NCAM1	0.000272376	-28.7946
MET	0.018675	-28.4337
TSPAN2	0.00234357	-28.2531
NCAM1	0.000817776	-28.2172
	0.00115934	-28.0407
GLT8D2	0.0185774	-27.9508
	0.00167598	-27.938
LRCH2	8.92E-09	-27.9284
	0.000909753	-27.8872
MCOLN3	0.00532375	-27.8862
ST6GALNAC3	0.00074435	-27.8853
	0.00137734	-27.8745
APOBEC3G	0.00322716	-27.7909
PYGL	0.00613428	-27.784
SULT1C4	2.14E-05	-27.7149
SNAI2	0.00209654	-27.6615
ANXA3	0.0158847	-27.4987
CPVL	0.0110779	-27.4488
MYOF	0.0158755	-27.4338
RAB34	0.00327164	-27.4138
CAV2	0.0085685	-27.3775
ANXA3	0.0175233	-27.2943
FGF2	0.0158106	-27.2301
SCRN1	0.0119182	-26.9658
GPRC5A	0.0199248	-26.6734
CALB2	0.000805603	-26.673
SORCS1	2.62E-06	-26.6
PCDH10	0.000732933	-26.5728
PLCE1	0.00814884	-26.5155
MAGEB2	7.49E-07	-26.4503
MET	0.0193396	-26.2504
SEMA3A	0.0127814	-26.1874

Appendix: Results of microarray comparisons

HEY1	0.00219889	-26.0266
MCOLN3	0.0014472	-25.9481
EPB41L3	0.00226391	-25.7914
MYOF	0.0169677	-25.7913
	0.0016677	-25.63
	0.000507405	-25.566
C3	0.0120971	-25.4691
MCOLN3	0.00485091	-25.2396
SNAI2	0.00299352	-25.1554
MSN	0.0171617	-25.1519
MUC15	0.000243765	-25.1219
MEIS2	0.00812822	-25.0583
PYGL	0.00331021	-25.0166
DDX60	0.0129131	-24.7527
GPRC5A	0.0167187	-24.6714
MYOF	0.015796	-24.6268
IFI16	0.00181769	-24.4839
KLK6	0.000990129	-24.4002
ST6GALNAC3	0.000124495	-24.0109
