NOTTINGHAM TRENT UNIVERSITY

Molecular and Functional Analysis of the Tumour Antigen T21

Bader M. Alshehri

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Copyright statement

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Abbreviations

^{3}H-thymidine	Tritriated radioactive thymidine
Ab	Antibody
ACT	Adoptive T Cell Therapy
ADCC	Antibody Dependent Cellular Cytotoxicity
AFP	Alpha-feto protein
Ag	Antigen
AIRE	Autoimmune Regulator protein
ALL	Acute lymphoblastic leukaemia
AMACR	alpha-methyl-CoA-Racemase
AML	Acute myeloid leukaemia
APC	Antigen-Presenting Cell
APML	Acute promyelocytic leukaemia
APS	Ammonium persulphate
ATP	Adenosine tri-phosphate
BC	Blast crisis
BLAST	Basic Local Alignment Search Tool
BM	Basement membrane
BM-DC	Bone Marrow Derived Dendritic Cell
BPH	Benign Prostatic Hyperplasia
BSA	Bovine serum albumin
CAGE	Cancer-associated antigen
CARs	Chimeric Antigen Receptors
CD	Cluster of Differentiation molecules
CDC	Complement Dependent Cytotoxicity
CDK	Cyclin-dependent kinase
cDNA	Complementary Deoxyribonucleic Acid
CEA	Carcioembryonic antigen
CEP290	Centrosomal Protein 290 kDa
CMC	Complement-mediated cytotoxicity
CML	Chronic Myeloid Leukaemia
CMML	Chronic myelomonocytic leukaemia
cpm	Counts per minute
CTAs	Cancer/Testis antigens
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen-4
DC	Dendritic Cell
ddH_2O	Double-distilled H_2O
DISC	Death Inducing Signalling Complex
DLBCL	Diffuse large B cell lymphoma
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic Acid
DPBS	Dulbecco's phosphate buffered saline
DTT	Dithiothreitol
EBV	Epstein Barr virus
ECM	Extracellular Matrix
EGF	Epidermal growth factor
ER	Endoplasmic Reticulum

FCS	Foetal calf serum
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
GAP	GTPase activating protein
GEF	Guanosine exchange factor
GFP	Green Fluorescence Protein
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GPCR	G-protein coupled receptor
GSK-3	Glycogen synthase kinase-3
GTP	Guanosine tri-phosphate
HCC	Hepatocellular carcinoma
HEF	Hepatocyte Growth Factor
HIF	Hypoxia inducible factor
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HPV	Human Papilloma Virus
HR	Homologous recombination
HRP	Horseradish Peroxidise
HTLV-1	Human T-lymphotrophic virus type 1
IAP	Inhibitors of apoptosis
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin growth factor
IGRT	Image guided radiation therapy
IHC	Immunohistochemistry
IL	Interleukin
mAb	Monoclonal Antibody
MAGE	Melanoma-associated antigen
MAPK	Mitogen activated protein kinase
MDS	Myelodysplastic syndromes
MDSC	Myeloid Derived Suppressor Cell
MHC	Major histocompatibility complex
MM	Multiple myeloma
MMP	Matrix Metalloproteases
mRNA	Messenger Ribonucleic Acid
NFκB	Nuclear Factor kappa beta
NGS	Next generation sequencing
NK	Natural Killer Cell
NKT	Natural Killer T Cell
NTC	Non-template control
PCR	Polymerase chain reaction
PDGF	Platelet Derived Growth Factor
PH	Pleckstrin homology
PI3K	Phosphotidylinositol-3-kinase
PIN	Prostatic Intraepithelial Neoplasia
PIP2	Phosphotidylinositol-bis-phosphate
PIP3	Phosphotidylinositol-tris-phosphate
PKB	Protein kinase B
PSA	Prostate Specific Antigen
PTEN	Phosphatase and tensin homologue

qPCR	Quantitative Polymerase Chain Reaction
RACE	Rapid Amplification of cDNA Ends
Rb	Retinoblastoma
RNA	Ribonucleic Acid
RP	Recombinant Protein
RT	Room temperature
RTK	Receptor tyrosine kinase
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
SDS	Sodium dodecyl sulphate
SEREX	Serological analysis of recombinant cDNA expression
shRNA	short hairpin RNA
siRNA	small interfering RNA
T21	Testis clone 21
TAA	Tumour associated antigen
TAP	Transporter Associated with Antigen Processing
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper Lymphocyte
TIL	Tumour Infiltrating Leukocyte
TIMP	Tissue inhibitor of metalloproteinase
TKI	Tyrosine kinase inhibitor
TNFα	Tumour Necrosis Factor alpha
TSG	Tumour suppressor gene
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
VitE	Vitamin E

Abstract

Immunotherapy is a valuable approach to target tumour cells by stimulating the body's adaptive immune system. To achieve this objective, it is important to identify and study antigens that are distinctively expressed in cancer and can be used effectively to initiate or enhance immune responses.

T21 (Testis clone 21) was identified as a member of cancer/testis antigen (CTAs) family of proteins using a modified SEREX technique and can be considered a promising prostate-associated tumour antigen, shown to elicit a humoral immune response in prostate cancer patients. T21 has also been shown to be over-expressed in malignant glands of the prostate compared to benign glands and stroma at the mRNA level. Since T21 shares significant similarity with the Centrosomal Protein, CEP290, which has been implicated in several cilia associated syndromic disorders such as Joubert syndrome, it was necessary to determine similarities and differences in the expression and functionality of these two molecules to facilitate further studies on the role of T21 in prostate cancer tumourigenesis.

As with the majority of identified cancer/testis antigens, the role of T21 in cancer remains undetermined. Therefore, investigations were initiated to understand the potential function of T21 in cancer cells. Next Generation Sequencing (NGS) data have been previously obtained following T21 knockdown/silencing in PC3 cells and the expression profiling of this data indicated that T21 function was related to several pathways involved in tumourigenesis. Genes that were either up or down regulated in the presence of T21 were validated by qRT-PCR and the results provided evidence that T21 may share functional activity these various genes and be implicated as a key driver of important signaling pathways.

The up or down regulation of molecules associated with key signaling pathways was established from the NGS data and validated by qRT-PCR. The effect of T21 silencing on the associated MAPK pathways was further investigated by using proteome profiler arrays. The results indicated the potential role that T21 may play in cancer cell growth regulation and suggested that T21 is potentially a central player in the cancer process. Finally, the data presented here strongly supports the hypothesis of T21 having a significant role in the biology of malignant cells. This, together with its potential use in immunotherapy, that warrants further investigation.

CHAPTER I

INTRODUCTION

1 CHAPTER ONE: Introduction

1.1 Cancer

1.1.1 Cancer overview

Cancer has remained as one of the biggest challenges in the biological and medical fields which have continuously attracting people's attention, especially scientists and medical professionals. The recent global data provided by World Health Organisation (WHO) (on line at http://www.who.int/cancer/en/) shows that the cancer is considered to be the leading cause of morbidity and mortality worldwide and as the second most lethal diseases following the cardiovascular diseases. The cancer incidence relates mainly to lifestyle behaviours and increases in aging populations significantly in most developed countries such as UK and the USA. Globally, there were 12.7 million new cancer cases diagnosed in 2008 and approximately 7.6 million cancer-related deaths. In 2011, there were 331,487 people in the UK diagnosed with breast, lung, prostate and bowel cancers accounting around 54% of the new cases. In the UK population, there is a one in three lifetime risk of cancer with lung, prostate, breast and colorectal cancers as the leading causes. Furthermore, in 2012, a total of 161,823 deaths of cancer were reported. (Cancer Research U.K).

Cancer, in medical diagnosis, is categorised as benign or malignant, based on the cancer behaviour in terms of spreading and metastasising. Benign cancers (non-malignant) are considered non-invading tumours to other parts except the origin tissue. However, they might exist as a complex, or continuously growing large mass that can cause damage to surrounding tissues or organs. In contrast, malignant tumours are represented as metastatic cancers that can spread to other parts of the body. The majority of cancers (around 85%) occurs within the epithelial cells and is classified as Carcinomas (Cancer Research U.K). Adenocarcinomas, on the other hand, are originated from glandular tissues (e.g. in breast) and Sarcomas are derived from mesoderm cells that exist in organs such as bones and muscles.

1.1.2 Prostate Cancer

Prostate cancer is considered as the most common male non-cutaneous malignancy in the United Kingdom (http://www.cancerresearchuk.org/). The prostate gland anatomically is involved within the males' reproductive and urinary systems as an oval shaped organ with a rounded tip. It is approximately 2-3 cm in size and enlarges gradually with age. Its exact location is at the bottom of the bladder, surrounding the upper part of the urethra and the seminal vesicles where it encourages seminal fluid liquefaction. The prostate gland is composed of various types of cells covered in a layer of connective tissue (the prostatic capsule). These cells types are: (1) gland cells type that have a role in producing the fluid portion of semen; (2) muscle cells that control urine flow and ejaculation; and (3) fibrous cells to provide the prostate gland's supportive structure. The gland structure is surrounded by seminal vesicles on both sides to produce semen for the sperm that is carried to them from the testicles by the vas deferens. Moreover, there are nerve bundles that have the function of controlling the bladder and erection process beside muscles that control urination.

The function of a prostate is mainly governed by endocrine signalling which has been widely noticed in having an important role in prostate cancer progression. The prostate gland, structurally, is divided into peripheral, transition and central zones. The peripheral zone is the largest part of prostate gland which locates closer to the rectum and, subsequently, it is the easiest zone to be examined by physicians during a Digital Rectal Examination (DRE). The transition zone location is at the middle area of prostate gland between the peripheral and central zones surrounding the urethra. This zone size forms up to 20% of the prostate gland and is gradually enlarged in men over 40 years of age. As a result, the transition zone is the largest zone in prostate gland where benign prostatic hyperplasia (BPH) is extensively observed.

It has been reported that the majority of prostate cancers originate in the peripheral zone of the prostate gland (70-75%), while 15% arises from the central zone and 10-15% occur in transitional zone (Bracarda et al., 2005) (Figure 1.1.1). In addition, the significant majority (95%) of the prostate cancers are diagnosed as adenocarcinoma that has the ability to spread to the surrounding tissues or invade seminal vesicles and the bladder. Therefore, there is a considerable potential of metastasis to distant organs such as lymph nodes and bones.

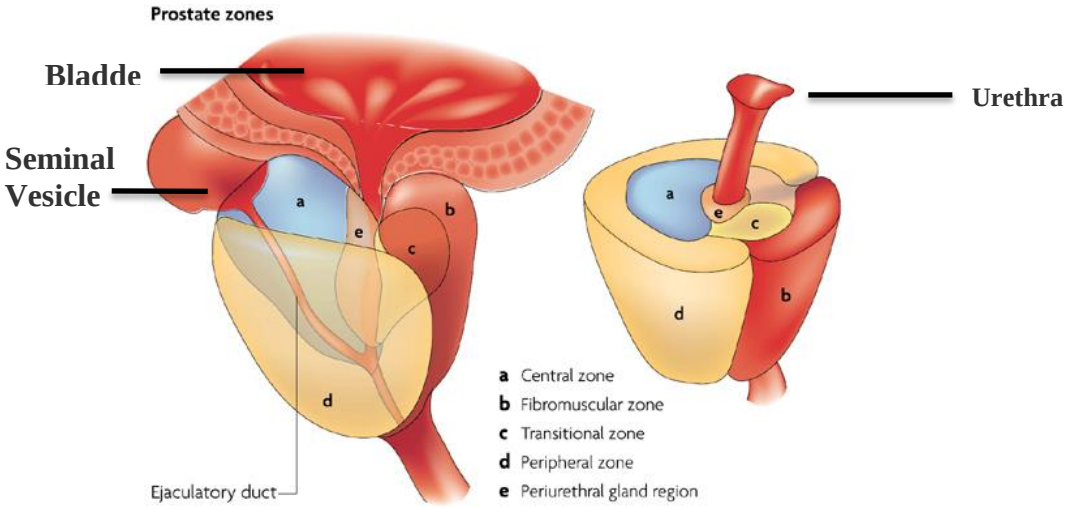


Figure 1.1. 1 Prostate zones.

(adapted from Nature Reviews Cancer 2007)

1.2 Current methods of diagnosis

1.2.1 Diagnosis and screening for prostate cancer

Prostate cancer can often be asymptomatic, however patients may present with lower urinary tract symptoms which are also associated with other conditions such as prostatitis and benign prostatic hyperplasia (BHP). In addition to this, patients with prostate cancer may also present with lower back or pelvic pain as a major indication of metastatic disease. Currently there are three diagnostic tools used for detecting prostate cancer when patients present with such symptoms. An initial measurement of serum PSA level, Digital Rectal Examination (DRE), transrectal ultrasound and biopsy sampling, the results of which are taken together to form a diagnosis of disease status and the course of action that should be taken.

Prostate-specific antigen (PSA) is a 34kDa glycoprotein, a serine protease with chymotrypsin-like activity which is produced only in prostatic epithelial cells. From here it is secreted into the prostatic ductal system where it proteolyses gel proteins (SEMG1 and SEMG2) in order to liquefy seminal fluid. The production of PSA is restricted to the prostate epithelium and in healthy males, is largely limited to this area with little outflow into the circulatory system. PSA levels found in blood range from <0.1 to 10^4 ng/ml with levels the lower range found in < 50 year old males, a figure that increases slightly with age. Men with advanced prostate cancer, whatever their age, have a marked increase in PSA levels found in the blood (10^2 ng/ml). High levels of PSA are also found in normal healthy males and in those with other prostate related conditions (prostatitis and BHP) and is influenced by age, body mass and racial differences, therefore can not be exclusively associated with prostate cancer. PSA levels in the blood are therefore a reflection of its release into the blood and not the elevated expression of PSA in the prostate due to presence of cancer. The numerous factors that contribute to elevated levels of blood PSA have made it difficult to pin down an exact threshold for evidence of prostate cancer and for this reason PSA screening would be unsuitable for population screening due to its lack of specificity and sensitivity (Lilja et al., 2008).

PSA testing may be advantageous for earlier diagnosis of disease, however the primary drawback of PSA testing is the lack of distinction between high risk and low risk prostate cancers that would be clinically insignificant and would not result in patient death. Importantly, the increasing incidence of newly diagnosed cases of prostate cancer in the UK

has not resulted in a reduced mortality rate bringing into question firstly the true benefit of PSA guided diagnosis and secondly its lack of improvement on treatment and disease management over the past few decades.

With this said, assessing for increased levels of PSA in blood can not be used in isolation to detect and diagnose prostate cancer but instead used as a preliminary assessment for more invasive procedures, namely DRE and biopsy examination which gives more definitive evidence of disease. Biopsy cores are used to determine the TNM staging along with a Gleason score based on cell morphology and cellular arrangement. Further analysis using tissue from core biopsy is performed by assessing specific markers by immunohistochemistry staining. A cocktail of markers are used containing basal markers such as high molecular weight cytokerins (34βE12, cytokerin 5/6) and nuclear protein p63 (absent in prostate adenocarcinoma) and a prostate cancer specific marker such as alpha-methyl-CoA-Racemase (AMACR), which is present in 80-100% of prostate cancers. These markers are used to determine disease status and in addition distinguish between malignant glands and benign lesions. Additional diagnostic procedures may be undertaken if metastatic disease is suspected using CT, MRI and bone scans to determine locally advanced or metastatic spread to distant organs (Yu et al, 2007; Lin et al, 2012).

1.3 Current Treatment

Treatment strategies are devised on criteria derived from stage and spread of disease and Gleason grading of disease as well as the age and general health of the patient. As described earlier, prostate cancers are slow growing and are often asymptomatic and are not always the cause of mortality in many elderly patients who have the disease. The option of unnecessary and potentially risky invasive treatment may be avoided by clinicians who opt for watchful waiting or active surveillance policy, whereby patients are monitored via PSA levels and core biopsies in order to delay invasive treatments and potentially avoid overtreatment of non-aggressive forms of prostate cancer.

Currently, radical prostatectomy in which the prostate gland and areas of surrounding tissue are surgically removed is the mainstay of treating localised disease and can be curative. Following surgery or as an alternative treatment, external beam radiotherapy (EBRT) and brachytherapy (internal radiotherapy) can be used to target and destroy rapidly proliferating cancer cells in order to limit spread of disease. In more recent years, further technological advancements in the field of radiotherapy, including 3D-conformal radiotherapy and intensity modulated radiotherapy, have increased targeting precision.

Progression beyond the capsule of the gland to the surrounding tissue where there is local or further advanced spread requires further treatment using radiotherapy and hormone therapy with the addition of chemotherapy as treatment for androgen refractory prostate cancer and metastasis of which a significant proportion of patients gradually progress to and currently remains incurable. Hormone therapy is then used to reduce androgen levels in order to stem androgen-dependant tumour growth and can be approached surgically (bilateral orchiectomy) or using combinations of chemical castrators namely competitive binding of the androgen receptor using anti-androgens such as Flutamide and Bicalutamide, the use of luteinising hormone blockers (LH-RH) and gonadotrophin releasing hormone analogues (Gn-RH analogues) used to down regulate the production of androgens in the pituitary. After cancer fails to respond to previous hormone therapy intervention, a second line hormone therapy may be used such as steroids Prednisolone and Dexamethasone or diethylstilbestrol (Stilboestrol), which mimics the female hormone oestrogen which can in turn reduce testosterone levels (Malkowicz 2001).

Chemotherapy combinations of taxanes (paclitaxel and docetaxel) and prednisone, have been shown to increase overall patient survival and in addition have a positive impact on quality of life after hormone-refractory prostate cancer (Tannock, 2004 and Haas et al., 2001). Existing hormone therapies are partially effective in limiting the progression of disease, however the majority of patients relapse to an androgen resistant form of disease and have limited in further treatment options, thus increasing evidence suggests that better tumour targeting strategies are required.

In recent years, there has been an emergence of a number of therapeutic approaches to hormone refractory prostate cancer, all with varying degrees of success. Treatments such as FDA approved taxane cabazitaxel, a semi-synthetic taxane which has proven to reduce susceptibility to taxane resistant prostate cancer, in addition to increasing the effective impact on metastatic lesions in the brain (de Bono et al., 2010). Another anti-androgen antagonist drug, MDV-3100 which is effective in Bicalutamide resistant prostate cancer, blocks testosterone binding to the androgen receptor, obstructs movement of androgen receptor to the cell nucleus and inhibits DNA binding (Swayers et al., 2009). Targeting androgen synthesis using small-molecule inhibitors to enzymes such as CYP17 (such as Abiraterone) has been utilised for its anti-tumour activity (de Bono et al., 2008). On the other hand, the long established radiation-based therapy has recently been further advancement using alpha radiation instead of gamma and beta radiation. Alpharadin (radium-223 chloride) targets and destroys tumour cells and has been shown to more effectively target bone metastases than other radiation treatments (Wissing, 2013).

Research into effective treatment of cancer has turned to the body's own immune system to identify and destroy tumour cells, in particular, circulating tumour cells. This concept of instructing the immune system to specifically target tumour cells to avoid more invasive treatments which carry a high mortality risk and unwelcome side effects poses an attractive approach with recent evidence showing strong potential for successful outcomes. One such treatment, called Sipuleucel-T, is an autologous cellular immunotherapy consisting of PBMC's cultured with a recombinant fusion protein PA2024 Prostate Associated Protein (PAP) linked to granulocyte-macrophage colony-stimulating factor (GM-CSF) to initiate an immune response to tumour cells exhibiting PAP antigens with the overall survival of patients on Sipuleucel-T being 4.1 months longer compared to the control patient cohort who received conventional treatments (Fishman et al., 2011). Recently, three epitopes of PAP

(PAP-114-128) were addressed as a highly relevant peptides that can be used for developing vaccine and to be applied in prostate cancer immunotherapy (Saif et al, 2014).

1.4 Tumourigensis of cancer

Cancer, as described previously, is a group of diseases with unregulated cell growth and division due to acquired accumulations of genetic mutations. These mutations lead to loss of the regular control system of the cellular homeostasis (the systems of controlling and maintaining the environmental balance in cells) causing dysregulation and imbalanc in the cellular differentiation and proliferation (Bertram, 2001). Normal cells are composed of complex molecular networks which respond to any instructional signaling from other cells in order to control their proliferation, differentiation and programmed cell death (apoptosis). These signalling commands are to ascertain the controlled preservation of organ size, function and tissue architecture constantly with the body's requirements. Any disruption of these signaling pathways due to genetic mutations may lead to an increase in cell proliferation, overtaking inhibitory growth signals and unregulated apoptosis. Furthermore, these transformed cells gain the ability to divide and grow indefinitely without restricted growth control. These unregulated cells accumulate further genetic mutations which give them cell selective growth feature over normal cells and invade (metastasise) to nearby tissues, blood and even to the lymph nodes and subsequently form further masses at distant organs. Any disruption that might occur to the cellular homeostasis process will noticeably destroy the systematic balance of the cell. Meanwhile, the tumour formation will begin to use the cellular defining features to be self-sufficiency in terms of growth and inhibitory signal insensitivity, an infinite capacity for self-renewal, a resistance to apoptosis, sustained angiogenesis and invasion of tissue and metastasis. Hanahan & Weinberg (2011) illustrated the hallmarks characteristic of cancer and demonstrating the capability of tumours to reprogramme their cellular activities. Cancer cells have the ability to acquire the functionality of cell metabolic pathways, promote genomic stress and diversity, escape immune destruction and promote inflammation.

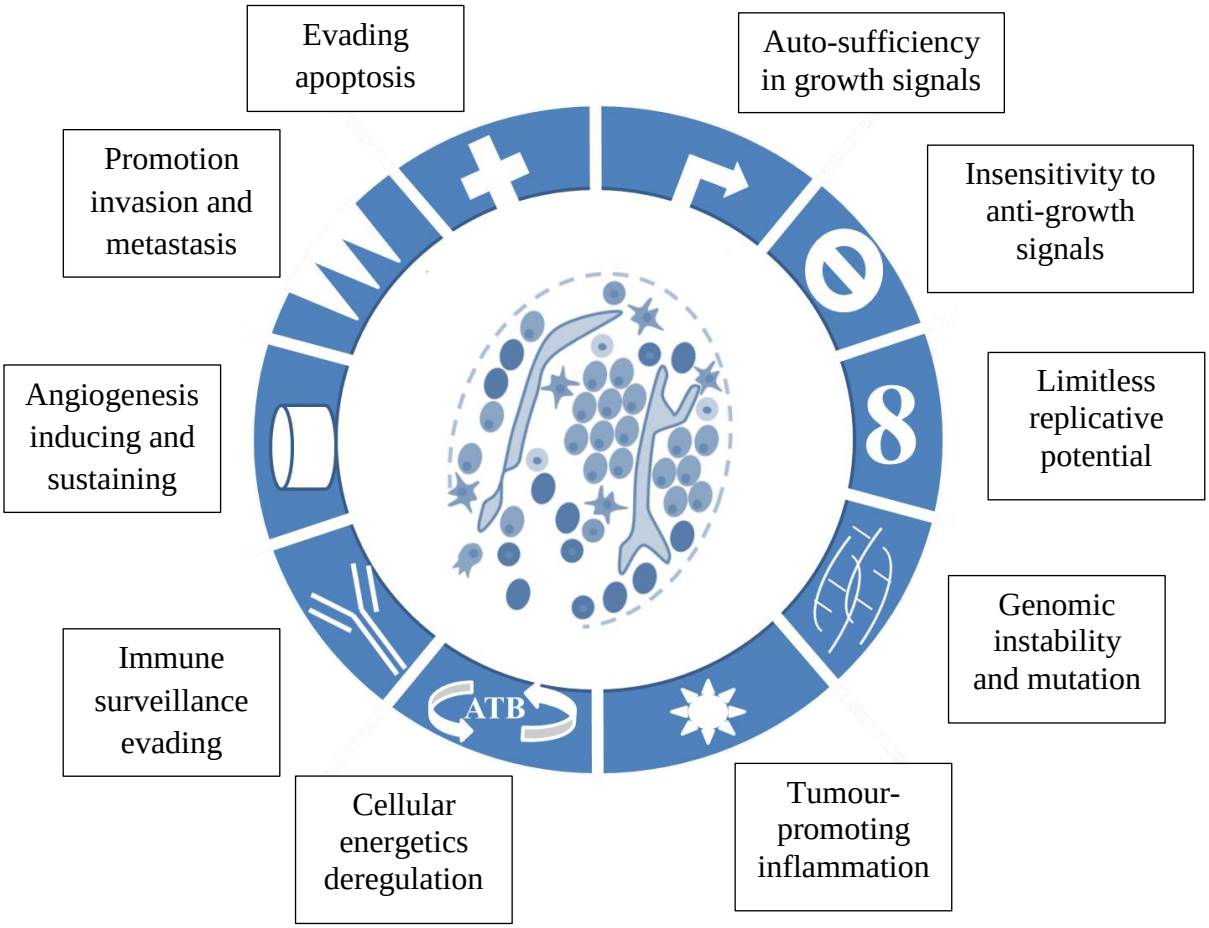


Figure 1.4. 1 The hallmarks of cancer.

(Adapted from Hanahan & Weinberg, 2011).

DNA damaging due to carcinogetic materials or exposure to any external environmental is a frequent occurrence (Fortini et al., 2003; Lodish et al., 2004), leading to accumulated genetic mutations overtime and an increase in the possibility of cancer development. Advancing age has also been reported to increase the incidence of various forms of cancer. The influence of genetic and epigenetic factors can be classified as following:

Environmental carcinogenic factors:

Both chemical and physical causative agents lead to genes mutations which may initiate cell transformation. Chemical carcinogens, for instance, are mostly related to industrial sources linked with occupational cancers such as lung cancer and skin malignancies. Smoking cigarettes is a cause of lung cancer due to the carcinogenic elements such as benzo-pyrene and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Shopland, 1995; Hecht, 1999). DNA structure can be affected, on the other hand, as a result of radiation through prolonged exposure to ionising radiation such as ultra-violet radiation (UV) (Pfeifer et al., 2005; Belpomme et al., 2007).

Infectious viral pathogenic factors:

Many viruses are known to cause cancer. During the infection cycle, the viral genome is integrated into the host cell genome transferring malignant characteristics to the human genome (Butel, 2000; Carillo-Infante et al., 2007). The viral oncogenes are effective agents inducing neoplasia (Parkin, 2006). For example, cervical cancer is caused as result of Human Papilloma Virus (HPV) infection and strongly associated with the development of head and neck carcinoma (Schiffman et al., 2007; Lajer and von Buchwald, 2010). HPV carries E6 and E7 genes that bind to host cell p53 and retinoblastoma (Rb) proteins, increasing proliferation signals that preventing apoptotic cell death. This effect is similar to Hepatitis C Virus (HCV) core protein, which causes hepatocellular carcinoma (HCC) (Hassan et al., 2009; Ghittoni et al., 2010; Tsai and Chung, 2010). It has also been shown that bacteria (such as helicobacter pylori) also have a strong association with cancer due to their role in causing chronic inflammation (Eslick et al., 1999).

Epigenetic factors:

Carcinogens and viruses directly impact on cell transformation, however, there are also abnormal processes which have a modifying role on cell behaviour leading to cancer development (Cui et al., 2002). The DNA demethylation/methylation status influences gene expression. Numerous human gene promoter regions contain CpG islands which may lead to demethylation/methylation spontaneously. This can lead to the expression of genes that promote malignant behaviour or inhibit the action of those that act to inhibit transformation respectively (Esteller et al., 2001; Ehrlich, 2002; Eden et al., 2006).

Epigenetically altered gene expression can be restored, unlike genetic changes, and in some instances can be used for diagnostic and prognostic applications. Reversing inversed methylation patterns in leukaemias and lymphoma using 5-azacytidine and 5-aza-2'-deoxycytidine (Kantarjian et al., 2006) has been shown, and similarly in Food and Drug Administration (FDA) approved histone deacetylase inhibitors, Vorinostat and Romidepsin in cutaneous T cell lymphoma (CTCL) and acute myloid leukemia (AML) (Marks & Breslow, 2007).

The failure of DNA repair pathways to repair genomic damage is important and will lead to the development of cancer over time through a multistage transformation process (Duesberg and Li, 2003). Primary mutants, such as dominant oncogenes, are passed onto the daughter cells (Bertram, 2001) and all tumour masses develop as a multistep process following unrepaired primary cell mutations (Greenman et al., 2007; Stratton et al., 2009). Moreover, carriers of germline mutations are at a greater risk of developing cancer, for example, the irregular BRCA gene is the most known germline event related to various types of cancer including breast, ovarian, prostate, stomach, pancreas, and colon cancers (Hall et al., 1990; Friedenson, 2005).

The genetic abnormalities which are involved in the process of carcinogenesis occur due to the accumulation of mutations in particular two classes of genes that play significant roles in cellular pathways that control cellular proliferation and differentiation: proto-oncogenes and tumour suppressor genes. Proto-oncogenes are normal genes essential to the control of growth and are linked to programmed cell death (apoptosis) which, if mutated, produces excessive cellular proliferation. Tumour suppressor genes are key "protector genes" which operate to inhibit mitosis and promote cellular destruction in case of DNA damage and therefore provide crucial stop signals to ensure correct regulation of cell function and growth.

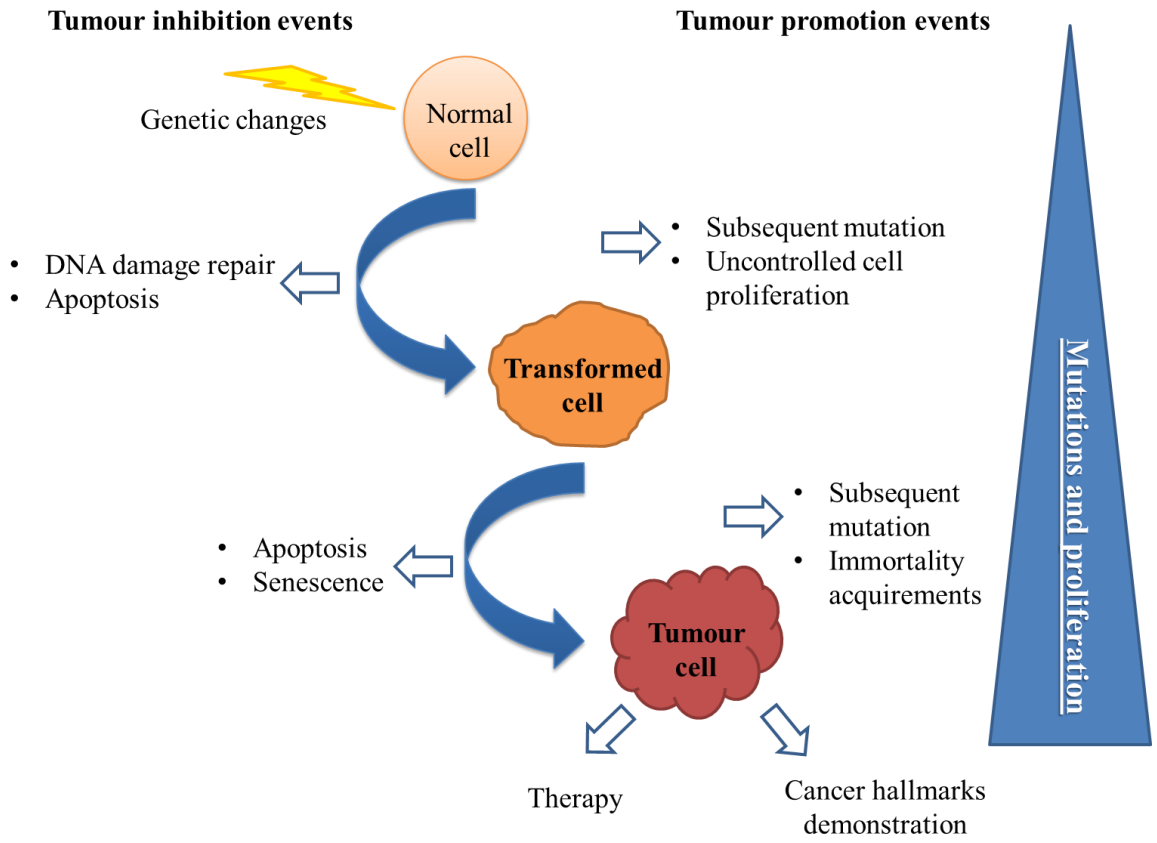


Figure 1.4. 2 The cancer pathways.
An overview showed the progression from normal cells to tumour state.
(Adapted from Bertram, 2000)

The cell reaches to the stage of demonstrating the considered hallmarks of cancer when there are mutations in at least five genes. Each gene mutation must enhance the cells capability to be adapted and can grow in the host organism; as has been shown in modelled colorectal carcinoma development (Fearon & Vogelstein, 1990). Subsequently, the solid tumours have been extensively investigated in order to determine the early events that are involved that can lead to cancer progression. In prostate cancer, for instance, the Nkx3.1 homeobox gene down regulation, appears as a result of promoter methylation (Asatiani et al., 2005), is an early initiation event which represents roles in responses to oxidative stress and DNA damage (Ouyang et al., 2005; Bowen & Gelmann, 2010).

The MYC oncogene somatic amplification is also associated and contributed in the early onset of the prostate cancer beside TMPRSS2-ERG fusions causing chromosomal

rearrangement of 21q resulting in a truncated ERG protein driven by androgen-responsive promotor elements of TMPRSS2 (Tomlins et al., 2005). The cell cycle reduction associated PTEN tumour suppression in prostate cancer has been shown to precede the gradual progression to an androgen independent phenotype (Mulholland et al., 2006). Finally, the histone-lysine N-methyltransferase is up regulated and encoded by EZH2 gene as a result of of miR-101 frequently associated to the late stage of metastasis and promoted cellular pathways such as RAS and NF-kappaB which are deregulated in various forms of cancer (Chiaradonna et al., 2008; Sun & Xiao, 2003) (Figure 1.4.3). The prostate cancer development is predominantly slow and shown as asymptomatic disease. When the symptoms of the disease are observed, this means the cancer usually has been at advanced stage and can metastasis to distant organs such as bones, colon, lymph nodes and brain.

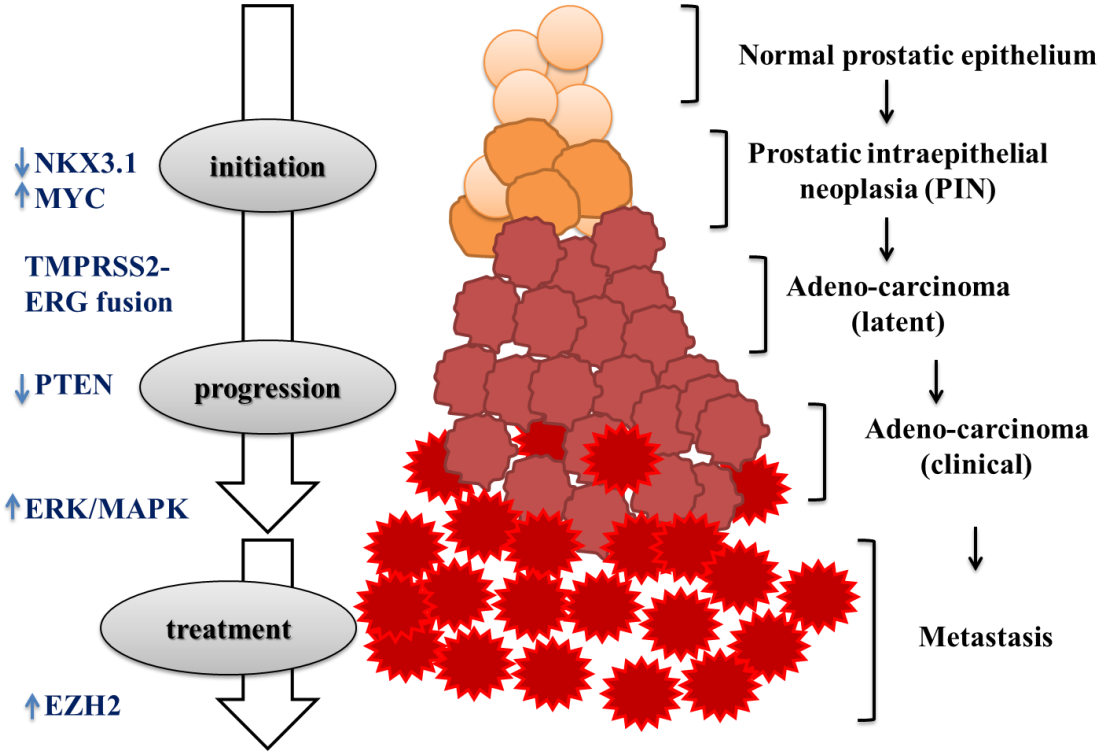


Figure 1.4. 3 Prostate cancer multi-step progression.

(Adapted from Abate-Shen & Shen, 2000).

1.4.1 Oncogenes

Oncogenes are a class of genes that play a role in stimulating the cell growth and originate from modified normal genes called proto-oncogenes and if mutated, can lead to controlled cell proliferation. However, a single oncogene requires another mutated gene (viral or cellular) through several mechanisms including point mutations, gene amplifications and chromosomal translocations to be dominant for causing malignant tumour. The introduction of a viral oncogene can be dominant and altered self-genes (proto-oncogenes). An example of a viral oncogenes that can lead to and cause cancer is the Sarcoma or Src oncogene induced by Rous retrovirus (Martin, 1970; Martin, 2004). There are several additional genetic alterations that are important in cancer such as fusion gene production arising by translocation (e.g. bcr-abl in CML) and gene amplification.

Oncogenes can be divided into four subgroups dependent on the where their protein product acts. The first two are growth factors and growth factor receptors, for example platelet derived growth factor, PDGF, which has a role in initiation of wound healing, but in cancer the PDGF BB isoform over-expression results in enhanced signalling for cell division (Barnhill et al., 1996). The amplification of growth factor receptor genes such as ERB-B2 has a similar outcome where the ERB/Her pathways are continually stimulated in some breast cancers. Oncogenic activation of signal transduction pathways such as genes encoding for the Ras family genes produce proteins and protein regulators for intra-cellular signalling cascades through kinase pathways and also transcriptional factors, which triggers cell proliferation and inhibit cell death. Table 1.4.1.1 lists these oncogenes that have a direct link to prostate cancer.

Name of oncogene	Function	Reference
c-myc (transcription factor)	Transcription factor which activates genes involved in driving cell proliferation and preventing apoptosis	Swayers et al., 2003
Bcl-2 (B-cell lymphoma 2)	Suppresses apoptosis preventing cell death	McDonnell et al., 1992
STAT5 (signal transducer and activator of transcription 5)	Transcription activators with an anti-apoptotic function	Dagvadorj et al., 2008
c-kit/tyrosine kinase receptor	Promotes cell survival, proliferation, division, adhesion and migration	Paronetto et al., 2004

Table 1.4. 1 Oncogenes associated with prostate cancer and their function which is enhanced during tumourigenesis.

1.4.2 Tumour suppressors genes

Tumour suppressors, on the other hand are key gatekeeper genes which operate to restrict mitosis and are inducers of cell suicide when the need arises. They therefore provide crucial stop gaps to ensure correct regulation of cell function and growth. Mutations in tumour suppressors lead to a reduction in their protein activity or efficiency and can leave the cell vulnerable to further mutations. Unlike oncogenes, tumours suppressors require both alleles to be inactivated/altered in order lose normal gene function and therefore are termed as recessive in nature. Gene inactivation may result from a hereditary germline mutation of one allele and with the wildtype acquiring a damaging mutation later in life, making those individuals with germline mutations more susceptible to cancer (Knudson, 1971).

There are over 20 known tumour suppressors that are inactivated in various cancers, p53 and retinoblastoma (Rb) genes are key pathway regulators and are mutated in the majority of cancers due to their essential role in delegation of cellular processes**,** and are therefore the most well characterised and studied tumour suppressors.

The p53 protein is a 53kDa multi-functional transcription factor that binds directly to a DNA response element to initiate the synthesis of a vast array of proteins. Identified mutations in the core binding domain of p53 have been shown to greatly reduce activity in the majority of p53 defective cancers (Cho et al., 1994). p53 operates as a central nexus point for signalling of cell cycle pathways and orchestrates various protein cascades. p53 concentration of p53 may increase and accumulate in the nucleus in response to “danger signals” in the event of DNA

breakage. This allows monitoring of cellular stress and damage to DNA which subsequently leads p53 to override cell proliferation signals, initiat cycle cell arrest and trigger apoptotic pathways, thus having an anti-oncogenic function within the cell. Its own regulation is tightly controlled by MDM2, a ubiquitin proteolysis enzyme that operates on a controlled feedback loop mechanism when the concentration of p53 rises. Often referred to as "the guardian of the genome" p53 has a fundamental role in preserving cell integrity for future division and cell growth, including initiation of DNA repair (Kastan et al., 1992; Meyer et al., 1999; Smith and Seo, 2002).

Like p53, the protein encoded by the Rb gene interacts with a many cell cycle proteins and on integration of these signals, determines whether the cell undergoes division and growth. In cancer, the deactivation of the Rb protein prevents its effective suppression of S phase, promoting transcription factors of the E2F family. As a consequence, the cycle cell can progress from G1 phase to the S phase of cell proliferation (Lees et al, 1993). Interestingly, both tumour suppressors are targeted for degradation by oncogenic viruses, most notably by Human papillomavirus (HPV) for which a national vaccination programmed for all girls aged 9 to 18 was introduced in 2008, in order to prevent cervical cancer in the UK. Here E6 and E7 viral protein bind to the tumour suppressors gene fraction of p53 and Rb protein respectively (Scheffner et al, 1990; Cheng et al, 1995). Table 1.4.2 lists these tumour suppressors genes that have been associated with human prostate cancer.

Name of tumour suppressor	Function	Reference
Rb (Retinoblastoma)	Key regulator for entry into cell division	Kubota et al., 1995
p53 (Tumour suppressor protein p53)	Key regulator of cell cycle, division and apoptosis	Gumerlock et al., 1994
PTEN (Phosphatase and tensin homolog)	Involved in regulating AKT/PKB pathway	Li et al., 1997
GSTP1 (Glutathione S-transferase P1)	Detoxify environmental carcinogens and oxidants preventing DNA damage	Nelson et al., 2004
p27/KIP1 (Cyclin-dependent kinase inhibitor 1B)	P27 controls cell cycle progression at G1	Kyprianou et al., 1997
NKX3.1 (NK3 homeobox 1)	Transcription factor involved in prostate development	Shen et al., 1999
KLF6 (Kruppel-like factor 6)	Transcriptional activator	Dong et al., 2003
BRCA2 (breast cancer 2, early onset)	Genome stability homologous repair pathway for double stranded DNA repair	Mitra et al., 2008

Table 1.4. 2 Tumour suppressors specific to prostate cancer and their normal function which are lost during tumourigenesis.

In most cancers, tumours develop and sustain the ability to continually proliferate in the absence of mitogenic growth signals. Tumour cells have been shown to increase pro-proliferation signalling using a number of mechanisms; briefly, they may have the capacity to produce their own growth ligands resulting in autocrine induced proliferation. In addition to this, tumour cells may disrupt signalling in normal cells within the surrounding stroma to induce the production of growth factors to promote tumour growth, demonstrated by the up regulation of hepatocyte growth factor, by fibroblasts in breast carcinoma (Cheng et al., 2008). Tumour cells may also upregulate the synthesis of growth factor receptors presented on their cell surface, the HER2 receptor being a frequent example and one that has been successfully utilised as a therapeutic target in HER2+ breast cancer. Structural alterations in growth factors and their receptors which enhance the rate of activation of their subsequent downstream pathways also sustains cell proliferation. Not only do tumour cells increase mitogenic signalling through growth factors and their receptors, they also maintain "pro-growth" conditions by becoming increasingly insensitive to anti-growth signals (O'Regan et al., (2012). An important aspect of cell maintenance is programme cell death which serves to eliminate aberrant cells from the population. This in essence is an anti-cancer defence which must be

overcome by tumour cells in order to continually replicate. Evading intrinsic apoptotic pathway activation by bypassing homeostatic sensors (p53, Bcl-2 family proteins) and their subsequent apoptotic effector molecules (death signals and caspases) means an additional bias toward cell growth (El-Deiry and Jin 2005).

In order to survive and sustain continual turnover, tumour cells must achieve replicative immortality unlike cells which normally (not-transformed) undergo a predefined number of division cycles. Telomeres are composed of multiple base-pair sequences located at the chromosomal ends, although they hold no genetic information, they protect the ends of chromosomes and determine the cell's capacity to divide in that after every round of division they shorten and eventually trigger cell senescence. Tumour cells increase the synthesis of a telomere extending polymerase (telomerase) and are therefore able to counter the senescence countdown and become in effect "immortalised cells". Protein components of telomerase, in particular TERT, have been shown to have a much more diverse involvement in support of cell proliferation, anti-apoptosis and DNA damage repair all of which aid tumourigenesis. Recently hTERT has been considered as a target for cancer therapy, especially immunotherapy (Thomas et al., 2002; Yang et al., 2012). Continual proliferation allows tumour cells to monopolise nutrients and oxygen and removal of waste produces by the formation of tumour associated neovasculature. To achieve this, tumour cells upregulate angiogenic inducers such as vascular endothelial growth factor (VEGF) and suppress their counterpart inhibitors which results in the initiation of endothelial cells that form the basis for blood vessel formation. This process is referred to as the "angiogenic switch" and is activated in metastatic disease and has been considered a viable target for therapy. By using inhibitors to VEGF (bevacizumab) and its receptor (sunitinab) to target tumour driven angiogenesis, clinical observed responses have been transient, as increasing evidence suggests tumours inhibited by angiogenesis go on to enhance activation of invasion and metastasis around surrounding normal tissue to access existing vasculature (Bergers and Hanahan 2008; Ellis and Hicklin (2008).

Tumour cells have a final acquired capability which is absent in the majority of normal cells, their ability to invade surrounding tissue, pass through the blood and lymphatic system and colonise distant tissue. This complex multistage process is termed invasion and metastasis, and the cause of cancer lethality. Briefly, tumour cells begin to invade by penetrating and moving through the basement membrane of surrounding local tissue by releasing proteases such as matrix metalloproteinases (MMP's) and alter their expression of cell adhesion (intergrin) molecules to favour invasion. Degradation of the surrounding extracellular matrix (ECM)

allows tumour cells to gain access to surrounding blood and lymphatic vessels (intravasation) and be transported through these networks and disembark at distant organs (extravasation). Tumours adapt to the organs specific microenvironment may proliferate, initiate angiogenesis and metastasise once more (Itoh and Nagase, 2002; Talmadge and Fidler, 2010).

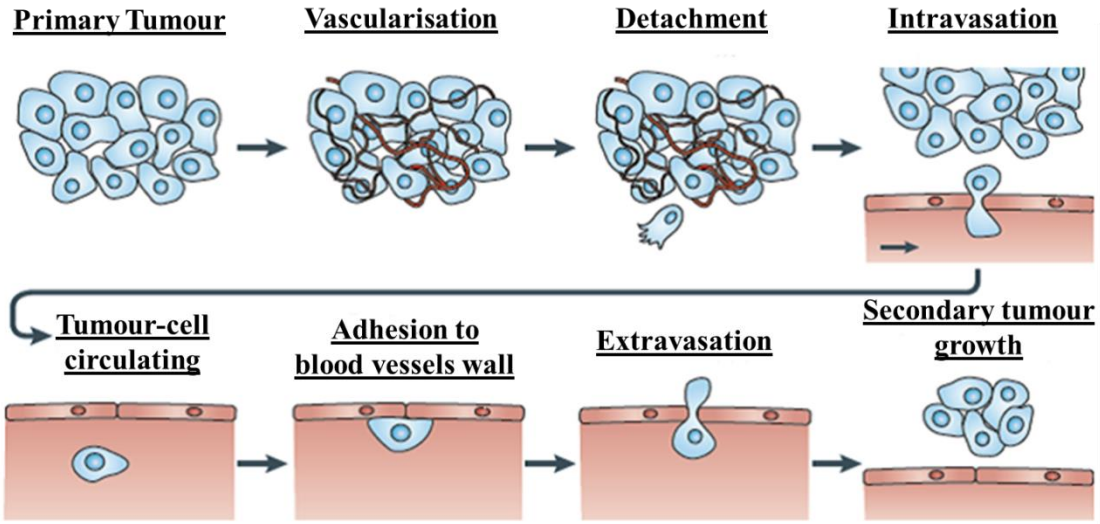


Figure 1.4.2.1 Schematic of metastasis cascade process.

(Adapted from Nature Reviews Cancer; Wirtz et al.,2011)

Intensive research into tumourigenesis over the last decade has begun to reveal further tumour hallmarks that explain tumour enabling characteristics and how they survive or evade elimination by the body's own defence mechanisms. The increasing genomic instability of cancer cells allows rapid cell turnover and gives them a selective advantage over other cell populations. This is achieved by the tumour cells ability to dismantle genomic maintenance machinery and subdue the cells genomic surveillance systems avoiding cell suicide. Another emerging hallmark is the deregulation of cellular metabolism, namely the tumour cells ability to reprogramme and redirect cellular energy towards fuelling cell growth and division. At the forefront of research into tumourigenesis and strategies for therapy, is to better understand the role that the immune system plays in supporting tumour growth. Tumour cells are able to manipulate immune activity in the surrounding vicinity to circumvent immune destruction of the tumour, and to promote tumour growth through inflammation. The ways in which immune evasion and tumour expansion is achieved will be discussed below how by harnessing the

body’s most important defence mechanism; the immune system, opportunities to oppose cancer can be exploited.

1.5 The immune system

The immune system is comprised of cells, originating from precursor haematopoietic stem cells produced in the bone marrow which under influence from specific cytokine stimulations are able to differentiate into a distinct array of myeloid (differentiating into granulocytes and monocytes) and lymphoid progenitor cells (T and B lymphocytes), which differentiate further into specialised subsets. For T lymphocytes repertoire generation and selection take place in the thymus, an essential process that selects against T-cells recognising cognate "self-antigens". The immune system has a principal function to survey and eliminate any potential dangers to the body arising mostly from incoming pathogens. There are two types of the response mechanisms which work together to achieve this: the innate and the adaptive response.

1.5.1 Innate immunity

The innate arm is referred to as the first line of defence as it constantly "seeks and destroys" any posing threat to the body. The innate system operates in a primitive response context which is comprised of a series of transmembrane receptors, called Toll-like receptors (TLR's) located on dendritic cells (DC's) and macrophages. TLR's are a family of 13 pathogen associated molecular pattern recognition receptors which recognise a diverse range of molecules typically essential to the function of invading pathogens but are not present in host cells, examples include bacterial flagella (recognsing TLR 5) and lipopolysaccharides (reconising TLR 4) and ligands for nucleic acids derived from viruses (specific for TLR 3 and TLR 7) (Ouchi and So 2010). Stimulation of macrophages through activation of their TLR leads to the release of inflammatory response elements which recruit phagocytic cells for the elimination of target pathogens and infected host cells. This type of response is dependent on TLR recognition of pathogen associated molecules and provides an immediate, albeit fixed, transitory immune response (Seong & Matzinger., 2004).

1.5.2 Adaptive immunity

In comparison, on encountering pathogens, the adaptive response requires an array of specialised cells (T cells and B cells and their subsets) all of which have specifically engineered receptors to recognise small segments of peptides which in this context are termed antigens (Ag's). Through presentation of Ag's by DC's, T and B cells undergo specific cell

expansion and trigger a diverse immune response towards those pathogens carrying the same Ag resulting in pathogen destruction through the production of antibodies (B cells, humoral response) and the activation of "immune" cells through MHC-peptide and cytokine facilitated stimulation (T cell, cell mediated response). This is a far more flexible response mechanism to the innate system, which also retains memory cells which act immediately in response to any future encounter with the same antigen/pathogen to trigger a much more rapid response (González et al., 2008; Moretta & Moretta, 2004)

Despite their different mechanisms of action, the humoral and cell mediated responses are coordinated through 3 types of professional antigen presenting cells (APC's), these are macrophages, DC's and B cells which scour the body sampling the surrounding environment, internalising and processing pathogen Ag's and finally presenting these Ag's on their cell surface in the context of major histocompatibility complex (MHC) molecules to T cells. In order to mobilise a T cell response, DC's must undergo alteration from an immature to a mature state (Salio et al., 2001; Medzhitov, 2001).

This switch occurs on encounter with a pathogen and the release of interleukin-1 (IL-1) and Tumour Necrosis Factor- α (TNF-α), which causes mature DC's to migrate from the peripheral tissue to the lymphatic system. Full maturation of DC's occurs through interactions with co-stimulatory molecules CD28/CD80-86 and CD40/CD40L present on both APC's and T-cells, following which appropriate presentation of Ag's can take place in the context of MHC presentation (Moretta & Moretta, 2004).

1.5.3 Major histocompatibility complex (MHC)

Human leukocyte antigen (HLA) or MHC molecules are presented on the surface of cells and provide the correct presentation context for T cells to recognise Ag's and initiate a cascade of immune responses. MHC are subdivided into MHC class I and MHC class II antigens according to their structure. MHC class I comprises of a transmembrane polypeptide α-heavy chain component consisting of 3 domains (α1, α2, and α3) and an associated β2-microglobulin (β2m) component. MHC class II consists of two transpolypeptide chains α-chain (comprising of α1, α2) and β-chain (comprising of β1, β2). Highly polymorphic HLA gene combinations, where each individual inherits two sets of each gene, gives rise to a very diverse range of MHC molecules. MHC class I antigens are present, with very few exceptions, on all nucleated cells and are able to bind endogenous peptides 8-11 amino acids

in length and present these to CD8+ T cells. MHC class II are present on all APC's or can be induced on other cells such as epithelial cells by cytokine stimulation, and are able to present exogenous peptides 12-25 amino acids in length to CD4+ T cells. Ag's that are presented by the MHC complex share a unique binding motif with T cell receptor (TCR) molecules found on CD8+ and CD4+ T cell sub populations. Only when correct recognition between MHC, Ag and TCR occurs along with corresponding CD8+ and CD4+ glycoprotein and the correct co-stimulatory signal (CD28 and CD80/CD86) interaction can T cells become activated. A lack of co-stimulation between these signals leads to T cell anergy towards that particular Ag. (Harding et al., 1992; Bennett et al., 1997; Ossendorp et al., 1998; Wang & Livingstone, 2003).

T cell activation on encounter of Ag displayed in the correct MHC complex leads to clonal expansion of T cells possessing the specific receptor for the Ag. Activated T cells proliferate into active effector T cells with specialised functions as either cytotoxic T lymphocytes (CTL's) (CD8+) and/or T helper (Th) cells (CD4+) responding to MHC class I and II antigens respectively. CD8+ cell are described as the fundamental component required for for direct elimination of tumour cells. Under the influence of IL-2 they are able to undergo clonal expansion not only with amplified expression of the TCR matching the Ag of interest but also expression of Fas ligand (FasL). Activated CD8+ T cells are then able to migrate throughout the body and target MHC class I receptors presenting the same antigenic peptide. Once CTL recognise tumour cells expressing the antigen they are able to engage the target and release cytotoxin (perforin) into the tumour to initiate caspase signalling cascades which leads to tumour cell death. CTL cells can also induce apoptosis in tumour cells via the FasL/Fas death receptor pathway. FasL also activates caspases resulting in degradation of DNA structure and deregulation of the cells cytoskeleton framework leading to cell arrest or cell suicide. (Porakishvili et al., 2004; Stalder et al., 1994; Thomas & Hersey, 1998)

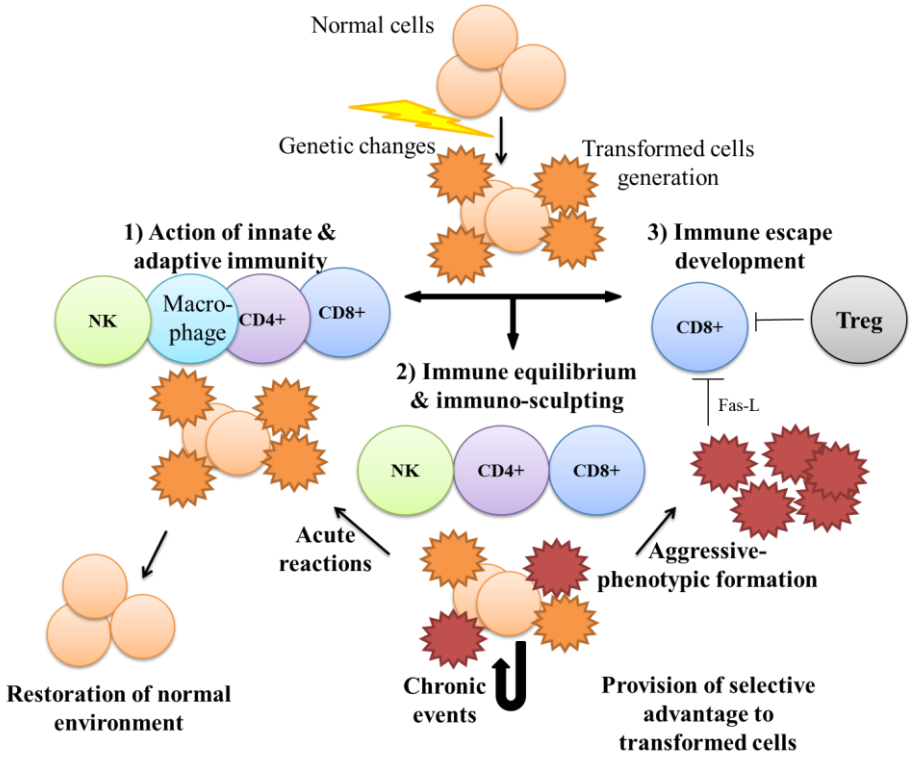


Figure 1.5.3.2 Overview of the three phases of cancer immunoediting.

(Adapted from Dunn et al., 2004).

T helper (Th) cells originating from CD4+ T cells support the cellular response to Ag by orchestrating the attack led by CD8+ T cells. Upon presentation of exogenously processed Ag's through MHC class II, CD4+ T cells secrete various IL's and IFNγ cytokines leading to the production of subpopulations of Th1, Th2 and Th memory cells. These subset are critical in determining the nature of the immune response through cytokine dependent cell stimulation and ultimately dictating whether the response initiated is predominantly humoral (Th2) or cell mediated (Th1). The release of IL-2 and IFNγ cytokines by Th1 cells is favourable to activation and expansion of CD8+ T cells, bolstering the CTL response and promoting them to proliferate to assist in maintaining a longer CTL response and avoiding T cell anergy. Th1 cells release IFNγ which also promotes the production of IL-2 and activation of APC's, which in turn operates in a positive feedback loop to maintain the Th1 response. Conversely, IFNγ works to hinder IL-4 production which subsequently suppresses Th2 cell development. Taken together, immunomodulation of cell mediated immunity through cytokine release mechanisms mean that Th responses are mutually inhibitory and function as a counter balance to initiate T-cell activity. Given that Th1 driven CTL responses can

distinguish between a tumour cell and a normal “self” somatic cell with the added advantage of immunological memory (the humoral response is unable to make this crucial discrimination), it stands to reason that a Th1 response is advantageous in eliminating tumour cells (Sica & Mantovani, 2012; Martinez et al., 2009)

1.6 Tumour immunology

The immune system is able to specifically detect, target and then eliminate newly arising tumour cells in the body, and this process is therefore termed immunosurveillance. This is believed to occur throughout life, however, tumours still arise which persistently and successfully evade these detection methods and continue to proliferate to form clinically detectable cancers. In response to the surveillance mechanisms employed by the immune system, tumours have been shown to use several "escape" mechanisms to subdue or avoid immune attack. Tumours may down regulate the production of MHC class I Ag's presented to T cells with in some instances the complete loss of MHC class I molecule on their cell surface, achieved by mutations or loss of gene components that assemble to produce the complex (β2m, TAP, HLA gene and MHC heavy chain) (Janeway, 2001)

Secondly, tumour cells can reduce surface expression of a particular tumour Ag and lose the immunodominance of that Ag, whereby a subclass of previously immunorecessive tumour Ag's emerge whereby immune T-cells are less able to target tumours cell due to less effective MHC/TCR interactions with the new antigenic epitopes. Tumour cells may also begin to circumvent the activation of death signalling pathways such as FasL and TRAIL found on the tumour cell surface by deactivating them (Thomas and Hersey, 1998). This may result in a state of T-cell anergy through lack of correct receptor co-stimulation of activated T cells. Finally, tumour cells subdue the immune response by releasing immunosuppressive cytokines, (increasing VEGF, IL-10 and TGFβ and reducing the production of IL-2 and IL-12) generating an environment that favours tumour expansion without immune constraints (Khong and Restifo 2002).

Building on this notion of immunosurveillance, Burnett and colleagues (Burnett et al., 1970) proposed the theory of immmunosurveillance and more recently the concept of immunoediting. It was proposed that natural killer (NK) cells scour the body disposing of tumour cells that pose a potential cancer threat. Observations of an increase of cancer in immunocompromised individuals such as those with immune deficiencies and supressed immune systems such as transplant patients, added further weight to the idea of immunosurveillance, which operates through activation of danger signals or tumour specific markers resulting in the destruction of tumour and a predominantly Th1 response. If however the tumour is only partially cleared, the remaining cells are maintained in equilibrium. Immune editing determines the antigenic composition of the new population of tumour cells

through an immune selection process. Here, selection pressures applied by the immune system allow tumour cells most resistant to immunity to emerge and escape through various mechanisms in a “Darwinian-like” evolutionary fashion (Schreiber et al, 2002).

Evidence suggests that the immune response plays a further role in tumour formation in that the response may in fact stimulate the growth of tumour cells by inducing Th2 T-cell responses. This can result in the infiltration of mast cells into some tumours and tumour cells themselves may be incapable of stimulation a strong immune response (Imada et al., 2000). Here, tumour cells use the same pro-growth mechanisms that promote wound healing to maximise their own growth.

1.7 Tumour antigens

1.7.1 Tumour associated antigens

As a direct consequence of genetic mutation described in earlier in section 1.4 various tumour associated protein products consisting of endogenously processed peptides are presented as Ag's on the cell surface of tumour cells have been found to be unique in their expression on tumour cells. More significantly, these tumour associated antigens (TAA's) provide viable targets for their applications as in immune based therapy against tumour cells.

Over the past decade many TAA's have been identified across a whole range of cancers, theses TAA's can be broadly separated into five categories. 1) Normal (unmutated) self Ag's that are re-expressed in large quantities in tumour (MAGE-1, NY-ESO-1) also referred to as cancer/testis Ag's due to their unique expression in specific tumours, and in immunoprivileged sites (testis and placenta) or in embryonic stages (CEA) (Boon et al, 1992; Chen et al., 1997). Genes encoding for these Ag's are usually silent in normal tissue however become activated in tumours. 2) TAA's are differential Ag's that are derived from tissue specific Ag's which are over-expressed in tumours and present in low levels in tissues (gp100, MART/melan) (Schwartzentruber et al., 2011). 3) Over-expressed normal (unmutated) Ag's found in tumours, these can be amplified products of genes (HER2/neu, hTERT) (Tsai et al., 1997). 4) Oncogene and tumour suppressor gene products and genes as a result of translocations that are expressed by the tumour (CDK4, ras, p53, bcr/abl). 5) Virally encoded proteins that can be expressed on the tumour cell surface (HPV derived E6 and E7 Ag's) (Song et al., 1999; Kawashima et al., 1998; Rojas et al., 2005). It is considered important to identify new target antigens for immunotherapy, in order to formulate a vaccination starategy.

In order to combat tumour cells, cancer immunotherapy strategies must counteract various immune evasion manoeuvres described earlier. The crucial aspect of all strategies are to target tumour cells by activation of APC's and T cells by augmenting the existing response which has been suppressed by tumour cells. On this basis, the identification of tumour Ag's are critical that have been shown to already elicit an antibody response in patients; p53, for example, provides evidence that an antibody and cell mediated response can be achieved as anti-tumour targets.

Tumour antigen	Expression in tumour	Normal tissue distribution
Oncofetal antigens:		
AFP	Hepatocellular carcinoma	None
CEA	Colorectal carcinoma	None
Oncoviral antigens:		
EBV	Burkitt's and Hodgkin's lymphoma Cervical	-
HPV(E6/E7)	cancer, head and neck	-
HBV	Hepatocellular carcinoma	-
Tumour-specific unique antigens:		
Bcr/Abl	Chronic myeloid leukaemia	None
Ig idiotype	B cell non-Hodgkin's lymphoma	None
Cancer-Testis antigens:		
MAGE, BAGE,GAGE families	Multiple	Testis
NY-ESO-1	Multiple	Testis
Overexpressed antigens:		
Her-2	Breast, ovarian, lung	Ubiquitous (low)
Muc-1	Breast, ovarian	Breast
p53 (mutated)	Pancreatic, colon, lung	None
p53 (wild type)	Multiple	Ubiquitous (low)
Survivin	Multiple	Ubiquitous (low)
WT-1	Gastric, lung, HCC, leukaemia	None
Differentiation antigens:		
Melan-A/Mart-1	Melanoma	Melanocytes
gp100	Melanoma	Melanocytes
Tyrosinase	Melanoma	Melanocytes
PAP, PSA, PSMA	Prostate	Prostate

Table 1.7.1.1 Tumour associated antigens.

A list of the major classes of TAAs found in cancer; examples showing the types of malignancies in which they can be found and their expression in normal tissue.

1.7.2 SEREX technique and identification of prostate tumour antigens

An attractive category of tumour antigens, first discovered in the analysis of T cell recognised epitopes, has been referred to as cancer testis antigens. CTAs are expressed in variable proportions, approximately 10-40 %, of a wide range of different human tumour types, and their unique expression pattern makes them attractive targets for vaccination purposes. CTAs are derived from non mutated genes that are expressed in various tumours but not in normal tissues except testis, embryonic ovaries and placenta (Osterlund et al., 2000) (Li et al., 2004; Scanlan et al., 2004). These genes are attractive candidates for tumour immunotherapy because of their unique expression pattern and because their expression can be shared by multiple tumour types (De Plaen et al., 1994). Additionally, induction of CTL responses does not cause a risk of inducing autoimmunity because CTAs in testis are expressed by spermatogonia and spermatocytes which do not carry HLA molecules, hence the CTAs cannot be expressed by these cells and recognised by antigen specific CTLs (Coulie., 1997). One of the first CTAs identified via a serological screening approach was NY ESO 1. NY ESO 1 belongs to an expanding family of immunogenic testicular antigens that are aberrantly expressed in human cancers in a lineage non-specific fashion (Gure et al., 1997). With the exceptions of SCP 1, OY TES-1, and CT15/fertilin β, the biological function of most CTAs is not known.

The SEREX technique (Serological analysis of cDNA expression libraries) first devised in 1995 (Sahin et al 1995), combines serological analysis with antigen expression and cloning approches to identify, in this instance, human tumour antigens which elicit IgG antibodies. This method involves extraction of mRNA from tumour derived tissue which is then expressed in a λ-phage display vector to create a cDNA expression library; these libraries are screened with patient sera, the positive colonies are isolated, cloned and sequenced and are finally put forward for DNA databank search. This then allows further sequence analysis to be performed to define tumour antigens, the identification of any gene abnormalities, assessment of mRNA expression patterns in normal, benign and malignant tissues and frequency of antibody responses relative to disease status.

By applying this principle in a modified SEREX approach, cDNA expression libraries were constructed from normal human testicular mRNA and were used to screen pooled allogeneic sera from prostate cancer patients in order to identify novel tumour associated antigens. Following this, positive clones underwent databank searches, analysis of gene anomalies, and assessment of

mRNA expression in normal and malignant tissue. A panel of serologically defined antigens expressed human prostate cancer cells were identified, included T21 and the findings published in 2007 (Miles *et al.*, 2007). Further examination of T21 serological reactivity using recombinantly expressed protein showed T21 reactivity with sera from 5/10 prostate patients whereas no reactivity was observed in 10 healthy individuals with benign prostatic hyperplasia (BPH). RT-PCR using cDNA reverse transcribed from whole tissues also supported the notion of a potentially good immunotherapeutic target with expression restricted to normal testis, kidney, gastric and prostate cancer, mild expression in BPH and little or no mRNA expression observed in normal essential tissues examined. Later, T21 was found to share significant sequence similarity with a centrosomal protein called CEP290 to which the presence of CEP290 antibodies has been previous reported in a number of malignancies (Eichmuller *et al.*, 2001; Chen & Shou, 2001). To date, SEREX technique allowed identification of novel tumour antigens including T21, T128 and Pr genes published in 2007 (Miles et al., 2007).

1.7.3 Previous investigation of T21

In-silico analysis revealed the T21 DNA sequence to be situated on chromosome 12q21.33 and further sequence analysis showed the full nucleotide sequence to be 2797bp in length, encoding a novel 524 amino acid protein. Structurally T21 was found to consist of 19 exons with 12 exons covering the coding region of the gene.

Expression analysis studies, using both conventional and RT-qPCR demonstrated the aberrant expression of T21 mRNA in various human neoplasms including gastric, kidney, and prostate cancer tissues and cell lines.

Mild expression in BPH was observed for T21 but low or no expression was observed in other normal tissues examined (Miles et al., 2007). Although T21 is not an X chromosome linked gene, in view of the restricted expression pattern of T21 with expression in germ cells of the testis and a variety of malignant tissues but generally not in other normal tissues, the presence of a SPAN-X domain and the antigenic property of T21, it has been tentatively assigned to the cancer/testis antigen family.

It has also been previously shown that the expression of prostate cancer-associated antigens can be diluted out in heterogeneous tissues when RNA is extracted from whole tissues and not specifically from prostate cancer glandular epithelium. Using, laser-capture micro-dissected material to obtain pure populations of malignant glands and benign prostatic

epithelium and stroma, a significant up regulation in T21 expression was found in cancerous glands.

Initial immunohistochemistry studies using an antibody directed against T21 were performed and demonstrated its specific expression at the protein level in tumour tissue with no or little expression found in normal and benign tissues. In addition T21 protein expression, as assessed using the same anti-T21 antibody, was found to be associated with tumour grade and significantly with tumour stage. Further studies have also demonstrated that knocking down (silencing) the function of T21 in a prostate cancer cell line reduces the proliferation of these cells significantly, indicating T21's potentially crucial role in cell survival (Miles et al., 2012).

Interestingly, it was subsequently discovered that T21 may be a splice variant of another protein called CEP290 (mutations of which have been associated with Joubert syndrome) and that a portion of a CEP290 intron was now part of the coding region of the T21 variant making this coded sequence of T21 unique (exon 10).

CEP290 is a 93.2kb gene located on chromosome 12q21.32, it spans 54 exons and forms a large protein product of 290kD and was first identified from proteomic screening of a human lymphoblast cell line. Although the exact role of CEP290 protein is still largely unknown, it has been shown to play a role in the centrosomes and basal bodies, cilia function and has been shown to associate with microtubule-based transport of proteins including centrin, gamma-tubulin, KIF3A, ninein and pericentrin (Chang et al., 2006). It has also been shown to interact with PCM1 a trafficker of centrosomal proteins and has been linked with maintaining spindle pole integrity prior to and during cell mitosis (Rhee and Kim 2010).

Further investigation using a custom designed mono-specific polyclonal antibody raised in rabbit designed in coding exon 9 of T21 with the peptide sequence:

(VELERQLRKENEKQKNEL) showed protein expression to be restricted to normal stomach, ovary, breast and prostate with greater expression demonstrated in numerous cancer tissues. Protein expression has been extensively studied in prostate cancer in which this laboratory reported significant over expression of T21 in prostate cancer glands compared with those derived from benign tissue. Additionally, expression was positively associated with pathological stage of tumours and suggested a correlation with increasing Gleason grade

and PSA recurrence (Miles et al., 2012). Moreover, T21 was found to be highly homologous to CEP290 with the exception of a unique T21 region derived from a CEP290 intron.

1.7.4 The strategy of gene knockdown to determine the gene function:

Gene knockdown technology using small interferon RNA (siRNA) was first described in 1999 by Baulcombe's group when they showed three types of transgene-induced post-transcriptional gene silencing and one further example of virus-induced post-transcriptional gene silencing. They analysed and detected antisense RNA complementary to the targeted mRNA in plants. Their findings showed that the RNA molecules have constant size which was estimated at 25 nucleotides (Hamilton and Baulcombe, 1999). Gene knockdown or "mRNA silencing'' is a regulatory cellular mechanism using RNA interference (RNAi) which is recently elucidated effectively. This useful technique is used mainly to disrupt unknown coding sequence and observe which structure and/or function will be resulting from the knockdown (Capecchi, 1989; Napoli 1990). Full knowledge of the genome requires knowledge of the function of each of the gene products of the putative genetic coding sequences. Effective studies using ribozymes directed against the mRNA expression product of a targeted gene have shown the ability to down-regulate or ''knockdown'' the expression of these targeted genes (Sokol & Murray, 1996).

RNA interference (RNAi) is an RNA-dependent gene-silencing phenomenon which is initiated by ~20-bp double-stranded RNA in the cytoplasm (Sontheimer and Carthew, 2004; Liu et al, 2004; Meister and Tuschl, 2004). The activation of RNAi can be exogenous or endogenous. A synthetic small interfering RNA (siRNA), for example, is considered to be exogenous RNAi. In contrast, siRNA that is expressed from its respective gene within the cell is represented as endogenous RNAi as well as pre-micro RNA (miRNA)-like short hairpin RNA (shRNA) (Brummelkamp et al, 2002; Miyagishi and Taira, 2002; Zheng et al, 2004).

Both short hairpin RNA (shRNA) and small interfering (siRNA) are endogenously transcribed in the nucleus and then exported to the cytoplasm, where the Dicer enzyme will split or (cleave) the characteristic stem–loop of shRNA (Hannon and Rossi, 2004; Mello and Conte, 2004). siRNA and its shRNA precursor are involved in pathways which converge at RNA-induced silencing complex (RISC) and, subsequently, the sense strand from the siRNA duplex will be removed and then and then degraded (Figure 1.7.4.1). Therefore, the antisense strand within RISC can recognise sequence-homologous RNAs and mediate target specificity (Sontheimer and Carthew, 2004; Zhou et al, 2006).

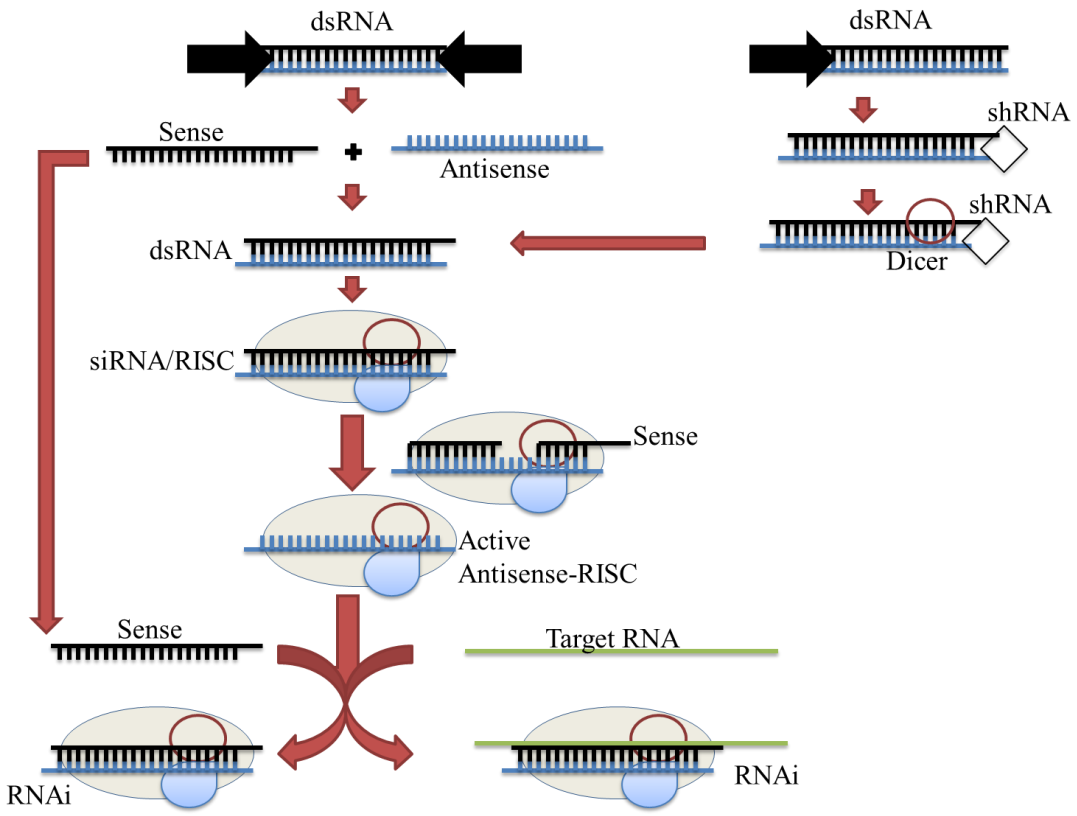


Figure 1.7.4.1: Schematic representation of RNAi pathways and how both siRNA and shRNA are generated within cells.

(*Adapted from Nucleic Acids Research, Jin et al, 2012*)

1.8 Aims of the study

The serological identification of novel tumour antigens by screening with prostate cancer sera is an interesting development requiring further investigation. The aims of this study were therefore to further characterise T21 at its molecular mechanism and to determine its potential role in cancer by:

- Study the expression of the T21 tumour antigen in prostate cancer cell lines, prostate cancer cell by characterising T21 at the molecular level and understand its relationship with the CEP290 gene.
- Determine T21 function and its involvement in tumourigenesis by:

- Investigating the effect of T21 knockdown on key cellular pathways through the analysis of genes expression profiles obtained by Next Generation Sequencing (NGS)
- NGS data pathways modelling using bioinformatics tools
- Pathways analysis using molecular and proteomics approaches

CHAPTER II

MATERIALS AND METHODOLOGY

2 CHAPTER TWO: Materials and Methodology

2.1 Materials:

2.1.1 Laboratory materials and equipment

2.1.1.1 Reagents and a list of suppliers

Culture media and supplements	**Supplier**
DMEM	Lonza
KSFM	Invitrogen
OPTIMEM	Invitrogen
RPMI 1640	Lonza
Blasticidin	Invitrogen
Fetal Calf Serum (FCS)	Perbio Thermo Fisher
HAM-F-12K	Lonza
HEPES buffer	Lonza
L-glutamine	Lonza
ddPBS	Lonza

Cell culture reagents	**Supplier**
Dimethyl Sulphoxide Hybri-Max (DMSO)	Sigma
INTERFERin	Polyplus Transfection
Lipofectamine 2000	Invitrogen
Trypsin/Versene	Lonza
Trypan blue	Sigma

Chemical reagents	Supplier
Acrylamide	Geneflow
Agarose	Bioline
Ammonium persulphate (APS)	National Diagnostics
Bovine serum albumin (BSA)	Calbiochem
Chloroform	Sigma
Decon 90	Decon Laboratory Ltd
Sodium Deoxycholate	Sigma
Dithiothreitol (DTT)	Sigma
dNTP	Bioline
DNA ladders (1kb and 100bp)	Promega
Dried marvel skimmed milk	Premier Brands
Ethanol	BDH
Hydrochloric acid (HCl)	Fisher
Isopropanol	Fisher
Liquid Nitrogen	British Oxygen Company
Methanol	Fisher Scientific
M-MLV RT and 5X Reaction Buffer	Promega
Oligo(DT) 15 primer	Promega
Paraformaldehyde	Sigma
Phosphate buffered saline (PBS) tablets	Oxoid

Presept	Johnson and Johnson
RNasin Ribonuclease Inhibitor	Promega
RNA STAT-60	AMS Biotechnology
Sodium Chloride (NaCl)	Fisher
Sodium dodecyl sulphate (SDS)	Sigma
Sodium fluoride	Sigma
Sodium orthovanadate	Sigma
Stacking gel buffer for SDS gels	Geneflow
Sybr Green SuperMix	BioRad
TEMED	Geneflow
Tris-base	Sigma
Tween20	Sigma
Western-C Precision Plus Mwt marker	BioRad

Consumables	**Supplier**
10ml syringes	Becton Dickenson
Sterile hypodermic needles 25 G	Becton Dickenson
0.5/1ml needles and syringes	Becton Dickenson
15ml Universal tubes	Bibby Sterilin
Microscope glass slides	SLS
24 well plate round cover slips	SLS
0.2 µm filters	Sartorius

Pre-filter separation columns 70 µm	Miltenyi Biotech
LS Columns	Miltenyi Biotech
5ml/10ml/25ml plastic pipettes	Sarstedt
15ml/50ml polypropylene tubes	Sarstedt
24-well/96-well tissue culture plates	Sarstedt
$25cm^2$ /$75cm^2$ / $175cm^2$ tissue culture flasks	Sarstedt
0.5ml/1.5ml eppendorf tubes	Sarstedt
10µl/200µl/1000µl Pipette tips	Sarstedt
Plastic pasteur pipettes	Sarstedt
1.5ml Cryovials	TPP
10µl/20µl/200µl/1000µl Pipette tips	Sarstedt, Starlabs, Deuchter Scientific

2.1.1.2 Equipment

All glassware and plastic boats were washed out using presept then rinsed and soaked in ddH_2O overnight and left to dry then sterilised using an autoclave prior to use.

Equipment	**Supplier**
Autoclave	Rodwell
Bench top vortex mixers	Scientific Industries
Cryostore, Cryo 200	Forma Scientific
Cell Harvester, Filtermate Harvester	Packard
Centrifuges (MSE Mistral 2000R)	Sanyo

Class II safety cabinets, Microflow biological safety cabinet	Walker
ELISPOT Reader, Immunospot Analyzer	Cellular Technology Ltd.
-20 freezer	Lec
-80 freezer, Ultima II	Revco
4°C fridge	Lec
Fluorescence microscope	Olympus BX51
4°C refrigerated centrifuges	Eppendorf/Sanyo
Haemocytometers	Weber
Incubators CO_2 water jacked incubator	Forma Scientific
Light microscopes	Nikon/Olympus
Light microscope camera	Nikon
MACSMix Tube Rotator	Miltenyi Biotech
MACS MultiStand	Miltenyi Biotech
Microcentrifuge, Microcentraur	MSE
MidiMACS Separator	Milteny Biotech
Nanodrop 8000 Spectrophotometer	Thermo Scientific
PALM Laser-capture microdissector (LCM)	Carl Zeiss
PCR Workstation Cabinet	Grant
pH meter	Metler Toledo
Plate rocker	VWR
Pipettes and multichannel pipettes	Gilson, Star labs, Eppendorf

Real Time qPCR Thermal Cycler	Qiagen
Ultra Sonicator	VWR
UNO-Thermoblock	Biometra
Water baths	Grant

Buffers

Buffers used for cell culture

Trypan Blue for cell count

0.1% (v/v) solution Trypan Blue in DPBS.

Immunochemical Reagents

Antibodies

Primary:

Anti T21 polyclonal Ab (Rabbit)	Pacific Immunology
Anti Unique T21 polyclonal Ab (Rabbit)	Pacific Immunology
Anti CEP290 polyclonal Ab (Goat)	Everest Biotech
IgG Rabbit Isotype control	Serotec
IgG Goat Isotype control	R&D Systems
Anti-Beta actin Ab (Rabbit)	Cell Signalling

Secondary:

Alexafluor 488 – goat anti-rabbit IgG	Invitrogen
Alexaflour 568 – donckey anti-goat IgG	Invitrogen
Rabbit anti-goat-HRP secondary Ab	Dako
Swine anti-rabbit-HRP secondary Ab	Dako

Reagents and buffers

VectorShield Mounting Media with DAPI — Vectorlabs

Fixing solution

X1 ddPBS

4% (w/v) Paraformaldehyde

pH 7.2, Storage 4°C

PBST wash buffer

X1 ddPBS

0.1% (v/v) Tween-20

Blocking solution

X1 PBST wash buffer

10% (w/v) BSA

Storage 4°C

Buffers used for western blotting

1X 8% Resolving SDS Gel:		1X 6% Resolving SDS Gel:	
ddH_2O	4.6mL	ddH2O	5.3mL
30% acrylamide	2.7mL	30% acrylamide	2.0mL
1.5Mm Tris (pH 8.8)	2.5mL	1.5Mm Tris pH (8.8)	2.5mL
10% SDS	0.1mL	10% SDS	0.1mL
10% Ammonium persulphate	0.1mL	10% Ammonium persulphate	0.1mL
TEMED	0.006mL	TEMED	0.008mL

1X 5% Stacking SDS Gel:		Reducing sample buffer:
ddH_2O	3.4mL	0.5M Tris-HCl (pH 6.8)
30% acrylamide	0.83mL	2% (w/v) SDS
1.5Mm Tris pH 6.8	0.63mL	10% (v/v) Glycerol
10% SDS	0.05mL	1% DTT
10% Ammonium persulphate	0.5mL	
TEMED	0.005mL	

Tris buffered saline + Tween-20 (TBST)	TBST + Marvel blocking buffer:
1.21g Tris base	100mL TBST
22.33g NaCl	10% (w/v) Marvel milk powder
pH made to 7.5 with HCl	
100mM sodium orthovanadate	
100mM sodium fluoride	
0.05% Tween-20	

5X SDS gel running buffer:	SDS gel transfer buffer:
15.1g Tris-base	5.8g Tris-base
94 Glycine	2.9g Glycine
50mL 10% (w/v) SDS	0.37g SDS
1L ddH2O	200mL Methanol
Made to 1X for each use in ddH2O	800mL ddH_2O

Real time PCR

Primers

All oligo-primers were purchased from Eurofins MWG Operon and diluted to a working concentration of 10pg/µl using ddH_2O and stored at -20°C.

Reagents and buffers

- *RNA extraction:*

RNA STAT-60 Amsbio

- *Reverse transcription:*

M-MLV Reverse Transcriptase and 5X Reaction Buffer	Promega
Oligo(DT) 15 Primer	Promega
RNasin Ribonuclease Inhibitor	Promega

- *PCR setup:*

iQ Sybr Green SuperMix	BioRad
100mM dNTP's	Promega

2.1.1.3 Thymidine incorporation assay materials

[6-^{3}H]-Thymidine (^{3}Thy), 1mCi (37Mbq)	Amersham
96 Uni/Filter scintillin-coated plates	PerkinElmer
Microscint-O Scintillation media	Perkin Elmer
Packard Instrument Co. 96-well plate	Ultima Gold

Wash buffer	Reagent diluent
X1 ddPBS	Storage 4°C, 0.2µm filtered before use
0.05% (v/v) Tween-20	X1 ddPBS
	1% (w/v) BSA

2.1.1.4 Cell line transfection

- *siRNA transfection:*

siRNA negative control	Eurogentec

siRNA CEP290 and T21 Eurogentec

- *shRNA transfection:*

shRNA negative control OriGene Technologies

shRNA T21 OriGene Technologies

2.1.1.5 Cell lines and growth Conditions:

All cells were grown as a monolayer in incubators supplying a 37°C, 5% CO_2 atmosphere

Cell Line / Source	**Tumour origin and cell line features**	**Culture media**
DU145 / ATCC	Prostate carcinoma derived from brain	DMEM + 10% FCS, 1% pyruvate
LNCaP / ATCC	Androgen dependent prostate carcinoma derived from lymph node	RPMI 1640 + 10% FCS + 1% L-glutamine
PC3 / ATCC	Prostate adenocarcinoma derived from bone	HAM-F-12K + 10% FCS
OPCT-1 and clones / Onyvax	Derived from prostate tumour epithelium	KSFM + 2% FCS

Table 2.1. 1 List of cancer cell lines used in this study, describing their origins, features and growth conditions.

2.2 Methods and procedures:

2.2.1 Cell lines and Growth Conditions

2.2.1.1 Cell line freezing

Cell lines were maintained in a humidified incubator at 37^{o}C, 5% CO_2. Confluent monolayer cells were rinsed and washed three times with an appropriate volume of DPBS to remove residual media, and dislodged by using 1X Trypsin/Versene, centrifuged at 400g, 4^{o}C for 3 minutes, counted using trypan blue exclusion method and seeded at the appropriate density into cell culture flasks or plates.

Suspension cell lines were passaged by centrifuging cell suspension and splitting the resuspend pellet into flasks, which were replenished with fresh culture media and antibiotics as required.

All cell lines were frozen to be grown, if required, for further investigations in 1ml cryovials using freezing media comprising of 50% FCS, 40% culture media and 10% DMSO. Cells were frozen at -80 ^{o}C for one day prior to transfer into liquid nitrogen for long term storage.

2.2.2 RNA extraction, transcription and qRT PCR

2.2.2.1 RNA extraction from cell lines

RNA was extracted using RNA STAT-60 according to the manufacture's instructions. Cells were homogenised using 500µl per 2x10^{6} cells of RNA STAT-60 in 1.5ml eppendorf tubes and left at room temperature for 3 minutes. To this, 100µl of chloroform per 500µl of RNA STAT-60 was added and mixed by shaking vigorously for 60 seconds and again tubes were left for 3 minutes at room temperature. Tubes were then centrifuged at 14,000g, 4°C for 15 minutes and the colourless upper aqueous phase containing RNA was carefully removed into a fresh 1.5ml eppendorf and the phenol red phase discarded. To this, 250µl of isopropanol per 500µl of RNA STAT-60 was added and the solution left at room temperature for 8 minutes in order to precipitate out RNA before again centrifuging at 12,000g, 4°C for 10 minutes. The supernatant was removed and discarded leaving a white pellet, and the RNA washed using 1ml of 75% ethanol followed by centrifugation at 7,500g, 4°C for 5 minutes. The supernatant was again removed and discarded and the white pellet left to air dry completely until clear. The pellet was resuspended in an appropriate volume between 10-30µl of ddH_2O and stored at -80°C and used within 6 months. RNA concentration was measured using a Nanodrop spectrophotometer and resuspended to a concentration of 1µg/µl in ddH_2O ready for Reverse Transcription.

2.2.2.2 Reverse Transcription

For first-strand cDNA synthesis from RNA was prepared following manufacture's instructions described below per sample. Into a 0.5ml eppendorf, 2µg of RNA, 0.5µg of the Oligo (DT) 15 primer and ddH_2O were added to give a final volume of 15µl. The samples were then heated using UNO-Thermoblock to 70°C for 5 minutes to melt secondary structures. Samples were then immediately put on ice before the following were added to each sample:

- M-MLV 5X Reaction Buffer 5.0µl
- 40mM dNTP's 1.0µl
- RNasin Ribonuclease Inhibitor 0.7µl
- M-MLV RT 1.0µl
- ddH_2O 2.3µl

Samples were mixed gently and incubated for 80 minutes at 39.2°C in a water bath. After incubation, samples were finally heated using an UNO-Thermoblock to 95°C for 5 minutes and immediately stored at -20°C ready for qRT PCR.

2.2.2.3 Quantitative Real Time PCR (qRT-PCR)

qRT-PCR was set up into PCR tubes using Sybr Green SuperMix following manufacture's instructions described below per sample. In cases of Non-Template Controls (NTC), cDNA template was substituted with ddH_2O.

- Sybr Green 6.25µl
- ddH_2O 4.75µl
- 5' end primer 0.5µl
- 3' end primer 0.5µl
- cDNA template 0.5µl

Samples were run on a qRT-PCR Thermal Cycler. Samples were run in duplicate and repeated at least 3 times to ensure validity of results which were then analysed using ΔCt equation.

Gene	Primer	Sequence (5'-> 3')	Tm [°C]
Centrosomal Protein (CEP290)	CEP290 new F CEP290 new R	5'-CGTCAAGAAGAACTGGCAGA-3' 5'-GCTCATTTACTTCCACCTTGG-3'	61
T21 unique region	T21 qPCR F T21 qPCR R	5'-CAAAGAATGAAATCATAGCACAGG-3' 5'-TTCTGTTCTGCCTGGCTTCT-3'	55
HKG: TBP-1	TBP-1 F TBP-1 R	5'-TGCACAAGGAGCCAAGAGTGAA-3' 5'-CACATCACAGCTCCCCACCA-3'	57
HKG: HPRT-1	HPRT-1 F HPRT-1 R	5'- TGACACTGGCAAAACAATGC-3' 5'-GGTCCTTTTCACCAGCAAGCT-3'	57

Table 2.2. 1 Primer summary table showing primer sequences and optimal annealing temperatures when using Real Time qPCR Thermal Cycler.

(A)

Primer	Sequence (5'-> 3')	Tm [°C]
CHD8	F: CACTGACTGCTTTCGGGTGGAA R: GGTCTCCACATCTCGTTCAGTC	63
TP53INP2	F: TTCGTGTCGGAGGAGGATGAAG R: AACCAGCTCTCGTCCATCAAGG	63
DUSP14	F: GTAGGCTTCTGGAGGCAACTGA R: ATCAGGTGTCGGGACTCCTTCT	63
IGFBP5	F: CGTGCTGTGTACCTGCCCAATT R: ACTTGTCCACGCACCAGCAGAT	63
ZBTB7A	F: GCAACATCTGCAAGGTCCGCTT R: TCTTCAGGTCGTAGTTGTGGGC	63
FRS3	F: CTATGGCTACGACTCCAACCTC R: GCACTGCATCAGATCCTGAAGG	63
RAB6B	F: CAACAAGACGGACCTGGCTGAT R: CGTTGTAGCCAGTCTTCGCACT	63
FGF1	F: ATGGCACAGTGGATGGGACAAG R: TAAAAGCCCGTCGGTGTCCATG	63
PDZD2	F: AGTGACTGTCGCTGGCTTTCAG R: GACAGAGCACTGGCTAGTTCAC	63
BCL2L12	F: AGACCGCAAGTTGAGTGGAGGA R: AGCCTCACCACGCCTAAGGAAG	65

(B)

Primer	Sequence (5'-> 3')	Tm [°C]
MAPK6	F: GACATGACTGAGCCACACAAACC R: GATGGGAGAGTGCTTCTTCTGC	63
CALM3	F: GAGAGGCGTTCCGTGTCTTTGA R: ACCTCCTCATCGGTCAGCTTCT	63
HIPK2	F: AGCGTCATCACCATCAGCAGTG R: AGTCGTGGACTGTGACACAGCT	63
RAP2A	F: TCTACAGCCTCGTCAACCAGCA R: TCTGCCTTCGCTGGACGATACT	63
BCL10	F: CACCCTTGTTGAATCTATTCGGC R: GAGGTTGTTCGTGGCTCCATCT	61
MAP2K	F: GGTGTTCAAGGTCTCCCACAAG R: CCACGATGTACGGAGAGTTGCA	63
TP53BP2	F: AGCACTGGGAATGCTCTGGATC R: GGCATTGGACTGGTCTACTGCA	63
CDK6	F: GGATAAAGTTCCAGAGCCTGGAG R: GCGATGCACTACTCGGTGTGAA	63
NDFIP2	F: GTGATGCAGACCAGCTCAGAGT R: CGCAGATAGCACCATACCTTCC	63
CEP290	F: GCTTCGATTGCCTGCCACTGC R: GCAGTGGCAGGCAATCGAAGC	62

Table 2.2. 2 List of primers used for NGS data validation by qRT-PCR.
(A) Primers were used for up regulated gene validation and (B) Primers for down regulated genes.

2.2.2.4 Statistical Analysis

For various experiments performed in this study, statistical analysis was carried out to highlight their significance. In the first instance, on studies where biological repeats were performed, the difference between repeats was determined using standard deviation, calculated from average values obtained for the control or test measures used in each experiment. In certain cases, the significance between values gained was examined. On these occasions, the p value was calculated using a student's t test, which would allow the recognition of differences that were significant ('*'/p=<0.05), very significant ('**'/p=<0.01), highly significant ('***'/p=<0.001) or very highly significant ('***'/p=<0.0001).

2.2.3 Immunofluorescence staining

2.2.3.1 Cell preparation

A day prior to immunofluorescent staining, cells were dispensed into a 24 well flat bottom plate at a concentration of $1x10^5$cells/ml at 1ml per well each containing one sterile round glass cover slip. The cells were then incubated overnight at 37°C, 5% CO_2 so that cells would be between 85-95% confluent at the time of staining.

2.2.3.2 Immunofluorescence staining

The following day, media was removed from the wells and 200µl of cold 4% paraformaldehyde solution was added to each well for 15 minutes at 4°C in order to fix cells.

Wells were then washed once for 10 minutes using ddPBS whilst on a plate rocker. Wells were then blocked for 60 minutes using blocking buffer. Following blocking, primary antibodies and IgG isotype controls were prepared in blocking buffer at optimal dilutions as suggested by manufacture's instructions and added to appropriate wells and left to incubate at room temperature on a plate rocker. After 60 minutes, the wells were washed using wash buffer x3 times for 10 minutes and the secondary antibodies prepared using blocking buffer and added to appropriate wells. Plates were then wrapped in foil and incubated at room temperature whilst rocking for 60 minutes. Following washing with wash buffer as before

round glass cover slips were removed from the base of the wells and placed cell side down onto mounting fluid containing DAPI on microscope slides. Slides were placed in slide holders and wrapped in foil and kept at 4°C until ready to view using a fluorescence microscope.

2.2.4 Western Blotting

For immunoblotting, DU145, PC3 and LNCaP prostate cancer cell lines were collected, washed with 1X PBS, lysed in 1X solution containing 50mM Tris-HCl (pH 6.8), 100mM dithiothreitol, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, and 10% (v/v) glycerol, and loaded on Tris/glycine SDS-polyacrylamide gels. SDS gels were prepared according to recipes shown in materials description. This was topped with a propanol organic solvent layer to inhibit bubble formation and allowed to set. Once the resolving gel had set the organic solvent layer was removed and replaced with a gel comb. The stacking gel solution is then added around the comb filling the gel mould. When the stacking gel had polymerised the comb was removed to reveal the gel channels (wells). Following denaturisation the gel moulds were placed into the electrophoresis tank and were filled with x1 running buffer, to the far left well 4 μL of protein standard ladder was added (Bio-Rad Western C precision plus™ protein standards). Once the samples were loaded, the gels were run at 100V until the dye front reached the bottom of the stacking gel, approximately 30-40 minutes, the voltage was then increased to 150V until the dye front reached 1cm from the bottom of the gel, approximately 1 hour.

Protein bands were transferred onto Amersham Hybond-P PVDF membranes (GE Healthcare). Membranes were blocked with 10% Marvel milk/Tris buffered saline (TBS) solution with 0.05% Tween-20 (TBST) containing sodium orthovanadate and sodium fluoride at 100V for 1 hour. Following TBST washes 3x 10 minutes each, membranes were incubated with primary antibodies (in blocking solution) at 4ºC for overnight. Then, the membranes were washed with TBST solution before adding the secondary antibodies and incubated for 1 hour at room temperature. Visualisation of bands was performed with Rapid Step ECL reagent (Calbiochem) and viewed using a CCD camera (Fujifilm).

2.2.4.1 Total Protein Assay

To calculate cell lysate protein concentration, total protein assay were performed using BioRad Dc protein assay reagents according to the manufacturer's protocol. Standards were diluted from a stock BSA solution (10mg/ml) to create a series of standard dilutions (2, 1.5, 1.0, 0.8, 0.5, 0.4, 0.2 and 0mg/ml). Standards were tested in duplicate, while protein extracts were tested in triplicate. All assays were carried out in 96-well round bottom plates. 25µl of reagent A was applied to all samples followed by 200µl reagent B. Plates were covered in foil and left to incubate for one hour at RT. Absorbance measurements for plates were read using a plate reader set to a 750nm wavelength. Using these absorbance values, protein fraction concentrations were calculated.

2.2.5 siRNA (Small Interfering RNA) knockdown

Transfection of OPCT-1, PC3 and DU145 using CEP290 siRNAs was done using INTERFERin transfection reagent according to the manufacturer's protocol. Briefly a day prior to transfection, cells were dispensed into a 24 well flat bottom plate at a concentration of $5x10^4$ cells/ml and left to incubated overnight at 37°C, 5% CO_2 so that cells would be between 30-50% confluent at the time of transfection.

The following day, siRNA stocks were prepared at 40µM/well in 100µl/well of serum free OPTIMEM media and mixed. To these mixtures 2µl/well of INTERFERin reagent was added and vortexed for 10 seconds. Further controls of INTERFERin only and OPTIMEM media alone were prepared and all mixtures were incubated at room temperature for 10 minutes to allow complexes to form. These complexes were then added drop wise whilst on a rocking platform at a volume of 100µl/well to appropriate cells containing 100µl/well of complete media. All plates were left to rock for 10 minutes before incubating at 37°C, 5% CO2 for 6 hours after which 500µl of pre-warmed complete media was added to each well and incubated.

2.2.6 Proliferation assay

2.2.6.1 Proliferation assays preparation

Transfected cell lines were harvested and centrifuged at 400g, 4°C for 3 minutes resuspended their growth media and counted using trypan blue. Cell concentration was adjusted to $5x10^4$

cells/100µl per well using appropriate media and dispensed into appropriate wells of a 24 well plate.

2.2.6.2 Post transfection procedure

Cultures were incubated for between 24-48 hours at 37°C, 5% CO_2, and [^{3}H]-Thymidine was added at 37 MBq/well in the last 18 hours of incubation. Following incubation, the media were then removed and the cells were washed with 100µl of ddH_2O and scrubbed to be transferred into a 96-well round bottom plate. An additional 100µl of ddH_2O was added to wash out residual cells in the wells giving a final volume of 200µl/well. Plates were then harvested using a cell harvester onto 96 Uni/Filter scintillation plates and were counted using a Top-Count counter. Results are expressed in counts per minutes (cpm) and as a means of the quadruplicate wells.

2.2.7 shRNA (short hairpin RNA) transfection

2.2.7.1 shRNA (short hairpin RNA) transfection

Stable transfection of cell lines using a pGFP-B-RS vector with blasticidin resistance gene and containing T21 shRNA designed as follows: (sense:5'GCACAGGAATTCTTGATCA3' TCAAGAG - antisense:5'TGATCAAGAATTCCTGTGC3') (purchased from Origene Technologies) was performed using Lipofectamine-2000 transfection reagent according to the manufacturers protocol (Performed by Dr Amanda Miles). A day prior to transfection, cells were cultured into a 24 well flat bottom plate at a concentration of 5x105 cells/ml and left to be incubated overnight at 37°C, CO_2 so that cells would be between 85-95% confluent at the time of transfection.

The following day, shRNA stock was prepared in HAM-F-12K media at a concentration of 1.2µg/50µl per well and mixed gently. Alongside to this, 2µg/50µl per well of Lipofectamine 2000 was prepared and both preparations were left to incubate at room temperature for 5 minutes before combining the diluted DNA and Lipofectamine 2000 allowing complexes to form at room temperature for 30 minutes. During which, media from the wells of the overnight cultures were removed and replaced with 100µl of the complexes containing the DNA/Lipofectamine solutions which were added dropwise to the wells whilst on a rocking platform and then incubated at 37°C, 5% CO2 for 6 hours, after which a further 900µl of pre-warmed HAM-F-12K media was added and left to incubate. Following 48hrs of transfection,

media from the wells was removed and fresh media containing 25µg/ml of Blasticidin selective antibiotic and observed over the following days.

2.2.7.2 Clonal selection and Cell Sorting:

Following 3-4 days post-transfection, cells were trypsinised and removed from wells and centrifuged at 400 g, RT for 3 minutes and resuspended in complete culture media. The cells were counted using trypan blue exclusion method and seeded into 96-well flat bottom plates at concentrations of 10 cells, 1 cell and 0.33 cells/well with the addition of 25 µg/ml blasticidin. Plates were observed over the following weeks and growing clones tested for T21 gene expression using qRT-PCR and immunofluorescent staining over several passages to ensure stable transfection.

Following culture of clones positively expressing GFP post-transfection, cells were trypsinised and removed from wells and centrifuged at 400 g, RT for 3 minutes and resuspended in culture media. Cells then underwent cell sorting using a MoFlo XDP High-Speed Cell Sorter under sterile conditions to enable re-culture of GFP positive cells. Following sorting, cells were gently centrifuged 300 g, RT for 3 minutes and seeded into flasks. Cells were supplemented with antibiotics 2 days post-cell sorting.

2.2.8 Investigation into T21 knockdown on cell signaling

2.2.8.1 Proteome Profiler Arrays

Mitogen activated protein kinase (MAPK) Proteome Profiler kits were obtained from R&D systems. These were used in conjunction with T21-stable shRNA transfectant lines. These kits were used as according to the manufacturer's protocol. Prior to testing, protein concentration of cells was assessed under manufacturer's advice and performed using the total protein assay protocol described in 2.2.4.1. After secondary antibody application, arrays were washed as stated in the protocol. ECL reagents were used to lightly wash each membrane and they were developed using the same technique as for immune blots described in 2.2.4. Following this, membranes were placed into a CCD camera chamber cooled to -25oC for development prior to the image being captured. Spot densitometry to test intensity was performed using ImageJ software.

CHAPTER III

CHARACTERISATION OF T21 AND CEP290 AT THE MOLECULAR LEVEL AND PROTEIN EXPRESSION

3 CHAPTER THREE: Characterisation of T21 and CEP290 at the molecular level and protein expression

3.1 Introduction

Since T21 (testis clone 21) has been identified to be a potential target antigen for immunotherapy, it has been considered for further investigations to approve its reliability as an effective tumour biomarker. The consecutive investigations of T21 have been substantially utilised in prostate cancer, focusing on the current understanding of its observed gene sequence similarities with a centrosomal protein called CEP290. Furthermore, T21 has required additional validations in terms of molecular characterisation and its functional attributes and, subsequently, determining its possible role in cancer. The preceding studies on T21 as a novel prostate cancer antigen were reported in previous publication (Miles et al., 2007; Miles et al., 2012).

T21 was first identified in 2007 by Miles et al as member of the Tumour Associated Antigens' family (TAAs) using a modified SEREX screening of cDNA libraries that were constructed from normal human testicular mRNA, expressed in plasmids and probed with allogeneic sera from prostate cancer patients (Miles et al., 2007) (Figure 3.1.1).

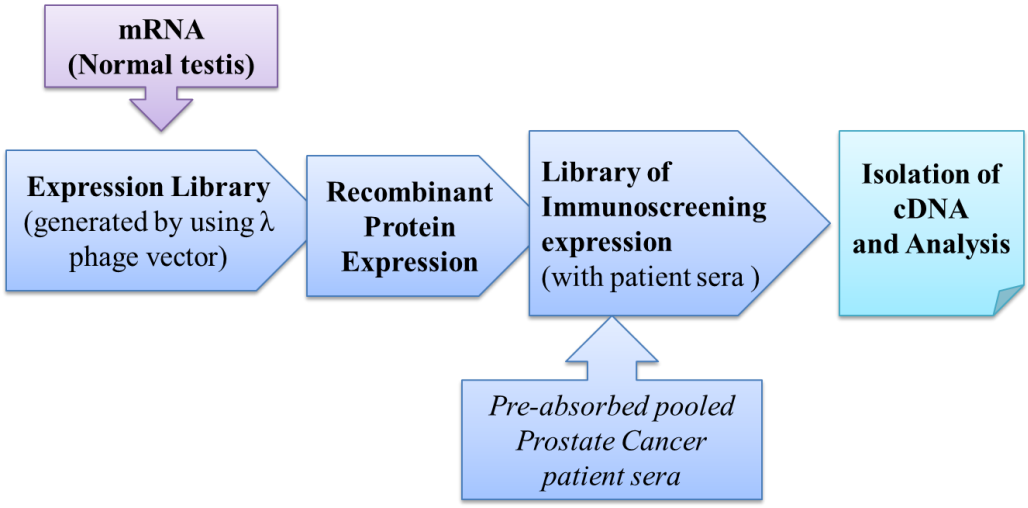


Figure 3.1. 1 Modified SEREX approach used to identify T21.
A normal testicular cDNA library was screened with allogeneic sera from prostate cancer patienst, from which positive clones were isolated and analysed.

In brief, an allogeneic testicular cDNA expression library (purchased from Clontech, USA) was screened using pooled prostate cancer patients' serum; pooled sera from four patients

having prostate cancer were used for screening. The reactive clones were isolated and subjected to two further rounds of screening in order to enhance their monoclonality. Which was then excised *in vivo* and subsequent restriction mapping and sequencing of the clones were performed on the cDNA. Further molecular analyses were subsequently performed to investigate its potential as an immunotherapeutic antigen. Therefore, PCR experiments were performed using testicular cDNA with the amplified T21 cloned sequence. In 2001, *In silico* analysis revealed the location of the T21 DNA sequence on chromosome 12q21.33 and further sequence analysis showed the full nucleotide sequence to be 2797bp in length, encoding a novel 524 amino acid protein. Structurally, T21 was found to consist of 19 exons with 12 exons covering the coding region of the gene. Expression analysis studies, using both conventional and qRT-PCR demonstrated the aberrant expression of T21 mRNA in various human neoplasms including gastric, kidney, and prostate cancer tissues and cell lines. Mild expression in benign prostatic hyperplasia (BPH) was observed for T21 but low or no expression was observed in other normal tissues examined (Miles et al., 2007).

Further investigations on T21 focused on determining the expression of T21 mRNA and protein in cell lines and in healthy, benign and cancerous tissues (Dr Alistair Rogers, MD Thesis 2009). The findings supported the hypothesis that T21 could be defined as a cancer-testis antigen due to the restricted expression profile in a number of malignancies and testis and the ability of T21 to induce an immune response in the tumour bearing host. Furthermore, T21 appeared to show increased expression according to tumour grade and stage and its role in cell proliferation suggest its relevance to prostate cancer development (Miles et al., 2012). The project also revealed homology between T21 and CEP290.

CEP290, also named cancer/testis antigen 87 (CT87), and CTCL tumor antigen se2-2, monoclonal antibody 3H11 antigen, is a 93.2kb gene located on chromosome 12q21.32; it spans 54 exons and forms a large protein product with a predicted molecular weight of 290kDa (Nagase et al., 1997). It was first identified from proteomic screening of a human lymphoblast cell line. Although the exact role of CEP290 protein is still largely unknown, it has been shown to play a role in centrosome organisation and basal bodies, cilia function and has been shown to associate with microtubule-based transport proteins including centrin, gamma-tubulin, Kinesin-like protein KIF3A, ninein and pericentrin (Chang et al., 2006). It has also been shown to interact with PCM1 a trafficker of centrosomal proteins,linked with the maintenance of spindle pole integrity maintenance prior to and during cell mitosis (Coppieters et al., 2010).

In 1997, Nagase and colleagues identified from human brain cDNA library sequences, KIAA0373 as a partial homologue of CEP290 (Nagase et al., 1997). Later, in 2001, another partial homolog of CEP290 was found expressed in a gastric cancer cell line library and termed 3H11Ag. This Ag was determined using monoclonal antibody (MAb) 3H11 which can bind specifically to different cancer cells obtained from different tissues. The nucleotide sequence analysis of the cDNA frame of 3H11 indicated that there was no highly homologous gene in the GenBank library. They identified a transcript of approximately 2.3kb in size and a 70 kDa encoded- protein from the generated sequence obtained (Chen and Shou, 2001). Subsequently, Andersen and colleagues in 2003 carried out proteomic investigations of the centrosomal fractions of CEP290 during interphase (G1/S phase) in human lymphoblastic KE37 cells.

Furthermore, there are more than 100 mutations associated with the CEP290 gene that have been identified mainly as cilia related disorders such as Joubert syndrome, Senior-Loken syndrome (Sayer et al., 2006; Valente et al., 2006), Leber congenital amaurosis (den Hollander et al., 2006), and Bardet-Biedl syndrome (Leitch et al., 2008). Although the role of CEP290 in cancer in unknown, homologues of CEP290 have been found in some forms of cancers such as CTCL tumour antigen SE2-2 / AF273044.1 which were identified as a tumour antigen in leukaemia and melanoma cell lines and, also 3H11 (mentioned above) in gastric cancer cell lines (Eichmuller et al., 2001; Chen & Shou, 2001).

The main objectives of this part of the project were:

1- To investigate the expressions levels of T21 and CEP290 mRNA and protein in cancer cell lines.

2- To investigate the effect of CEP290 knockdown on T21 mRNA expression.

3- To investigate the effect of CEP290 and T21 knockdown on cell proliferation.

3.2 Results:

3.2.1 T21 and CEP290 mRNAs and protein expression in cancer cell lines:

Previous findings demonstrated sequence similarities between T21 and CEP290 transcripts. Therefore, it was crucial to investigate the differences between the two molecules at the mRNA and protein levels. Quantitative RT-PCR has been used to investigate the expression of CEP120 and T21 at the mRNA level. Immunofluorescence and immunoblotting were used to investigate their expression at the protein level.

In order to compare the expression of T21 and CEP290 expression at the mRNA level, a more sensitive approach was adopted to quantify mRNA using quantitative real time quantitative PCR. Primers designed within the unique region were used to specifically amplify T21. They were optimised to achieve maximal efficiency and the relative abundance value of specific gene expression was determined by dividing the value derived by that of averaged housekeeping genes (Pfaffl, 2001). Two different housekeeping genes were used in this experiment, hypoxanthine guanine phosphoribosyl transferase 1 (HPRT-1) and TATA-box binding protein (TBP), as they both displayed low variability across samples and were therefore used for calculating relative gene expressions in all subsequent analyses. Quantitative RT-PCR experiments were performed following extraction of mRNA from a panel of cell lines derived from melanoma, breast and head and neck cancers. Here, to avoid DNA contamination (and possible amplification of intronic CEP290) of the mRNA, RNA spin columns were used instead of routine acid guanidinium thiocyanate-phenol-chloroform extraction using STAT-60.

CEP290 primers were designed within the upstream untranslated 5’ prime region of T21 up to and including the unique region. In light of the genomic alignment to CEP290, it was clear that the verification of the 3’ and 5’ end sequences of T21 would allow for a more complete transcriptional interpretation (Dr Amanda Miles, PhD Thesis 2004). The presence of the unique stop codon was confirmed by PCR with forward primers designed within the unique region and reverse primers designed to include the unique stop sequence codon. (Dr Suman Malhi, PhD Thesis 2013) (Figure 3.2.1.1)

(CEP290):ATTTGAAGTCCTCGTTCCACGCCTTCTCATCATCCTGAACACCGAGCTC
TGGGACTCCGGCGGAGAATCTAAACGTAAAGCATCACCCACGGTCGTGAACTGT
AGGCTCTCCTGGCATCCGGGATCTTATTCTGGCCTTGGCGGAGTTGGGGATGGTG
TCGCCTAGCAGCCGCTGCCGCTTTGGCTTGCTCGGGACCATTTGGCTGGACCCAG
AGTCCGCGTGGAACCGCGATAGGGATCTGTCAGGGCCCGCGGCCGGGTCCAGCT
TGGTGGTTGCGGTAGTGAGAGGCCTCCGCTGGTTGCCAGGCTTGGTCTAGAGGTG
GAGCACAGTGAAAGAATTCAAGATGCCACCTAATATAAACTGGAAAGAAATAAT
GAAAGTTGACCCAGATGACCTGCCC**(CEP290_primer_F)CGTCAAGAAGAACTG**
GCAGATAATTTATTGATTTCCTTAT**CCAAGGTGGAAGTAAATGAGC(CEP290_pr**
imer_R)TAAAAAGTGAAAAGCAAGAAAATGTGATACACCTTTTCAGAATTACTCA
GTCACTAATGAAGATGAAAGCTCAAGAAGTGGAGCTGGCTTTGGAAGAAGTAGA
AAAAGCTGGAGAAGAACAAGCAAAATTTGAAAATCAATTAAAAACTAAAGTAAT
GAAACTGGAAAATGAACTGGAGATGGCTCAGCAGTCTGCAGGTGGACGAGATAC
TCGGTTTTTACGTAATGAAATTTGCCAACTTGAAAAACAATTAGAACAAAAAGAT
AGAGAATTGGAGGACATGGAAAAGGAGTTGGAGAAAGAGAAGAAAGTTAATGA
GCAATTGGCTCTTCGAAATGAGGAGGCAGAAAATGAAAACAGCAAATTAAGAA
GAGAGAACAAACGTCTAAAGAAAAAGAATGAACAACTTTGTCAGGATATTATTG
ACTACCAGAAACAAATAGATTCACAGAAAGAAACACTTTTATCAAGAAGAGGGG
AAGACAGTGACTACCGATCACAGTTGTCTAAAAAAAACTATGAGCTTATCCAAT
ATCTTGATGAAATTCAGACTTTAACAGAAGCTAATGAGAAAATTGAAGTTCAGA
ATCAAGAAATGAGAAAAAATTTAGAAGAGTCTGTACAGGAAATGGAGAAGATG
ACTGATGAATATAATAGAATGAAAGCTATTGTGCATCAGACAGATAATGTAATA
GATCAGTTAAAAAAAGAAAACGATCATTATCAACTTCAAGTGCAGGAGCTTACA
GATCTTCTGAAATCAAAAAATGAAGAAGATGATCCAATTATGGTAGCTGTCAAT
GCAAAAGTAGAAGAATGGAAGCTAATTTTGTCTTCTAAAGATGATGAAATTATTG
AGTATCAGCAAATGTTACATAACCTAAGGGAGAAACTTAAGAATGCTCAGCTTG
ATGCTGATAAAAGTAATGTTATGGCTCTACAGCAGGGTATACAGGAACGAGACA
GTCAAATTAAGATGCTCACCGAACAAGTAGAACAATATACAAAAGAAATGGAAA
AGAATACTTGTATTATTGAAGATTTGAAAAATGAGCTCCAAAGAAACAAAGGTG
CTTCAACCCTTTCTCAACAGACTCATATGAAAATTCAGTCAACGTTAGACATTTT
AAAAGAGAAAACTAAAGAGGCTGAGAGAACAGCTGAACTGGCTGAGGCTGATG
CTAGGGAAAAGGATAAAGAATTAGTTGAGGCTCTGAAGAGGTTAAAAGATTATG
AATCGGGAGTATATGGTTTAGAAGATGCTGTCGTTGAAATAAAGAATTGTAAAA
ACCAAATTAAAATAAGAGATCGAGAGATTGAAATATTAACAAAGGAAATCAATA
AACTTGAATTGAAGATCAGTGATTTCCTTGATGAAAATGAGGCACTTAGAGAGC
GTGTGGGCCTTGAACCAAAGACAATGATTGATTTAACTGAATTTAGAAATAGCA
AACACTTAAAACAGCAGCAGTACAGAGCTGAAAACCAGATTCTTTTGAAAGAGA
TTGAAAGTCTAGAGGAAGAACGACTTGATCTGAAAAAAAAAATTCGTCAAATGG
CTCAAGAAAGAGGAAAAAGAAGTGCAACTTCAGGATTAACCACTGAGGACCTGA
ACCTAACTGAAAACATTTCTCAAGGAGATAGAATAAGTGAAAGAAAATTGGATT
TATTGAGCCTCAAAAAT**(T21):**ATGAGTGAAGCACAAT**(T21_primer_F)CAAAGAA**
TGAAATCATAGCACAGGAATTCTTGATCAAAGAAGCAGAGTGTAGAAATGCAG
ATATAGAGCTTGAACATCA**CAGAAGCCAGGCAGAACAGAA(T21_primer_R)**TGA
ATTTCTTTCAAGAGAAC……

Figure 3.2.1. 1 Genomic sequence of the new designed primers used for CEP290 and T21.

Subsequently, further investigations into T21 as a biomarker of prostate cancer could be aided by analysing mRNA and protein expression profiles in comparison to CEP290 in order to elucidate its function and involvement, if any, in cancer. In summary, the discovery of the unique T21 sequence and its translation into protein holds the possibility of a potential prostate cancer biomarker and further study as to its immunogenicity could present a viable immunotherapeutic target and may suggest its incorporation into a cancer vaccine.

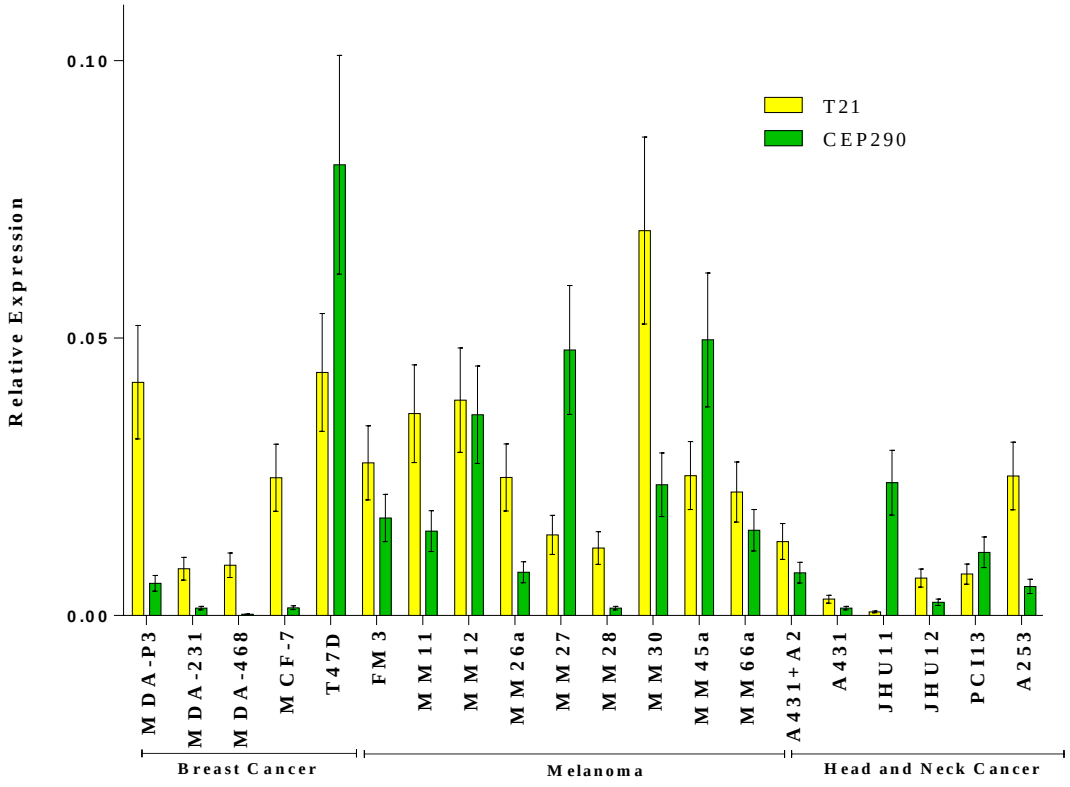


Figure 3.2.1. 2 qRT-PCR for T21 and CEP290 mRNA expression in human cancer cell lines.
Real time PCR expression of CEP290 and T21 mRNAs in various cancer cell lines derived from breast, melanoma and head and neck origin. Experiment was carried out three times in duplicate (n=3) represented with standard error of mean with data calculated by ΔC_t *and represented as relative gene expression.*

T21 and CEP290 mRNAs expressions were detected in most of cancer cell lines assessed, T21 expression was remarkably higher in breast (MDA-P3 and MCF-7), metastatic

melanoma (MM30) and head and neck cancer cell line (A253) (Figure 3.2.1.2). In the prostate cancer cell lines DU145, PC3 and LNCaP (all derived from disseminated tumour cells at metastatic secondary sites), CEP290 expression was higher than T21. In addition, T21 expression was lower in LNCaP, OPCT-1 clone (T21high/CD44high) and OPCT-2 (Figure 3.2.1.3).

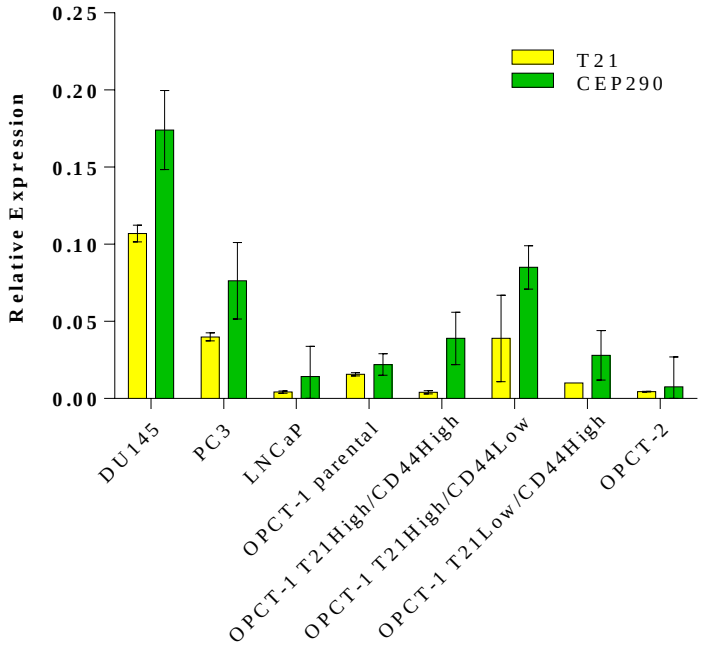


Figure 3.2.1. 3 qRT-PCR for T21 and CEP290 mRNAs expression in prostate cancer cell lines.
Experiment was performed three times in duplicate (n=3) represented with standard error of mean with data calculated by ΔCt and represented as relative gene expression.

To compare the expression of T21 and CEP290 at the protein level, , immunofluorescent staining using prostate cancer cell lines (PC3, DU145, OPCT-1 parental) were performed with polyclonal antibodies against T21 and CEP290 (Figure 3.2.1.2). Although fluorescent immunostaining of T21 and CEP290 showed a cytoplasmic co-localisation of the two proteins, this raised a question about the specificity of T21 antibody. Consequently, it was essential to use an antibody that recognizes specifically the unique-region of T21. This antibody (Pacific Immunology, USA) was designed and optimised to differentiate between

T21 and CEP290 expression using fluorescent immunostaining and Western blotting. T21 was expressed in the cytoplasm of DU145, PC3 and LNCAP cells as tested by immunofluorescence staining. In the LNCaP cell line, T21 was also expressed in the membrane, which opens up the possibility to target T21 by using antibody based on immunotherapy (Figure 3.2.1.4). The specificity of this antibody was also tested by immunoblotting, where it recognised a unique band of approximately 55kDa when using LNCaP cell lysates and a prominent band at 57kDa in PC3 and DU145 lysates. Interestingly, immunoblotting of these cell extracts using antibody against CEP290 showed a different expression pattern, confirming further the specificity of the new T21 antibody (Figure 3.2.1.6).

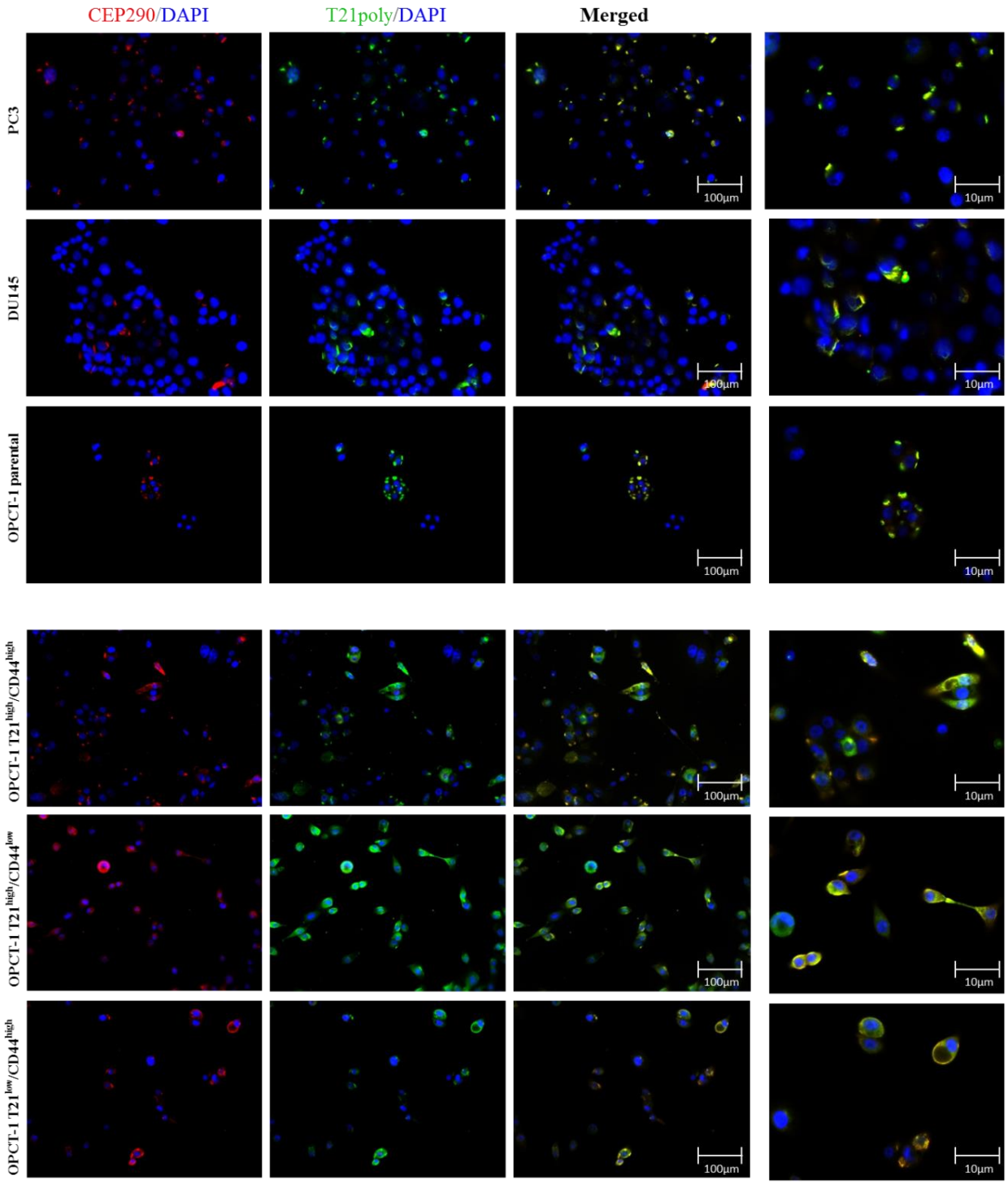


Figure 3.2.1. 4 Fluorescent immunostaining composites comparing T21poly and CEP290 antibodies localisation in prostate cancer cell lines (PC3, DU145, OPCT-1 parental and OPCT-1 clones).

Cells were stained with CEP290 antibody (red) and T21poly antibody (green) and nuclear staining achieved using DAPI (blue). Centrosomal localisation was observed using CEP290 was antibody similar to expression when using T21poly antibody. Representative images using objective magnification x10 & x20 (n=3).

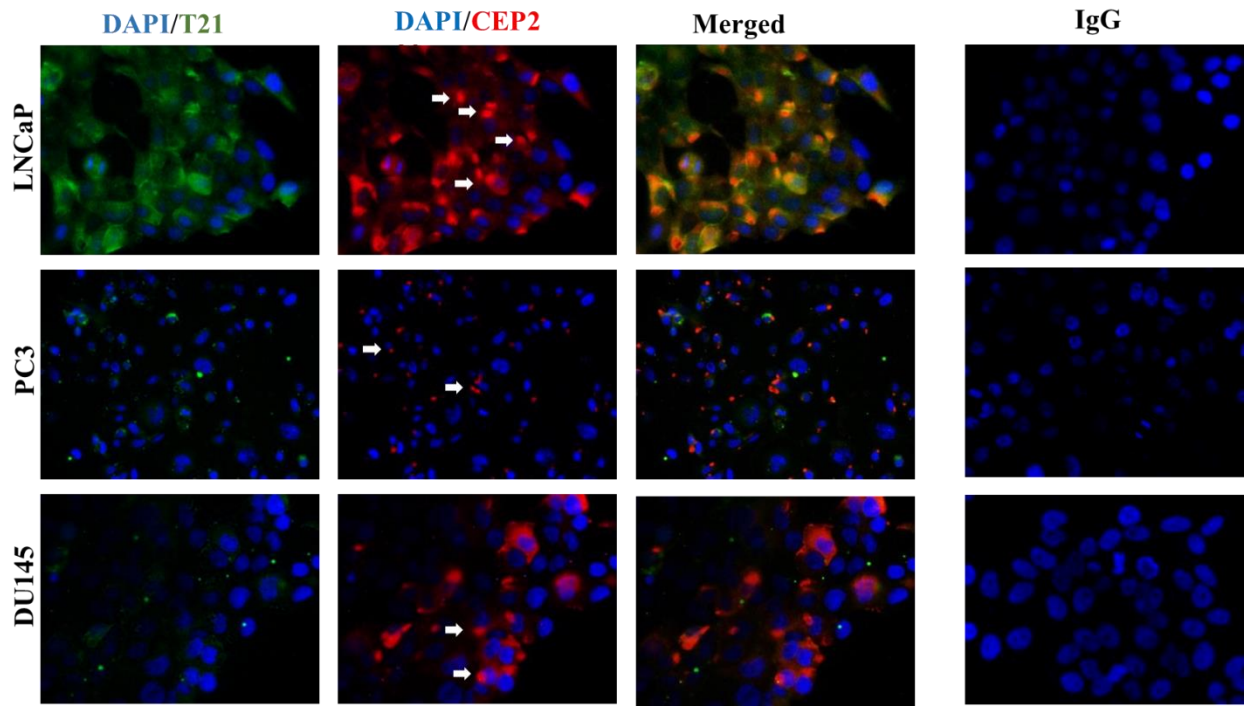


Figure 3.2.1. 5 Immunofluorescence composites comparing T21and CEP290 expression in prostate cancer cell lines (LNCaP, PC3 and DU145 cell lines) using T21 unique region-specific antibody and CEP290 antibody.

Cells were stained with CEP290 antibody (red) and T21unique antibody (green). The nuclei were stained with DAPI (blue). Centrosomal localisations were observed using CEP290 antibody and were absent when using T21unique antibody. Representative images using objective magnification x20 (n=3).

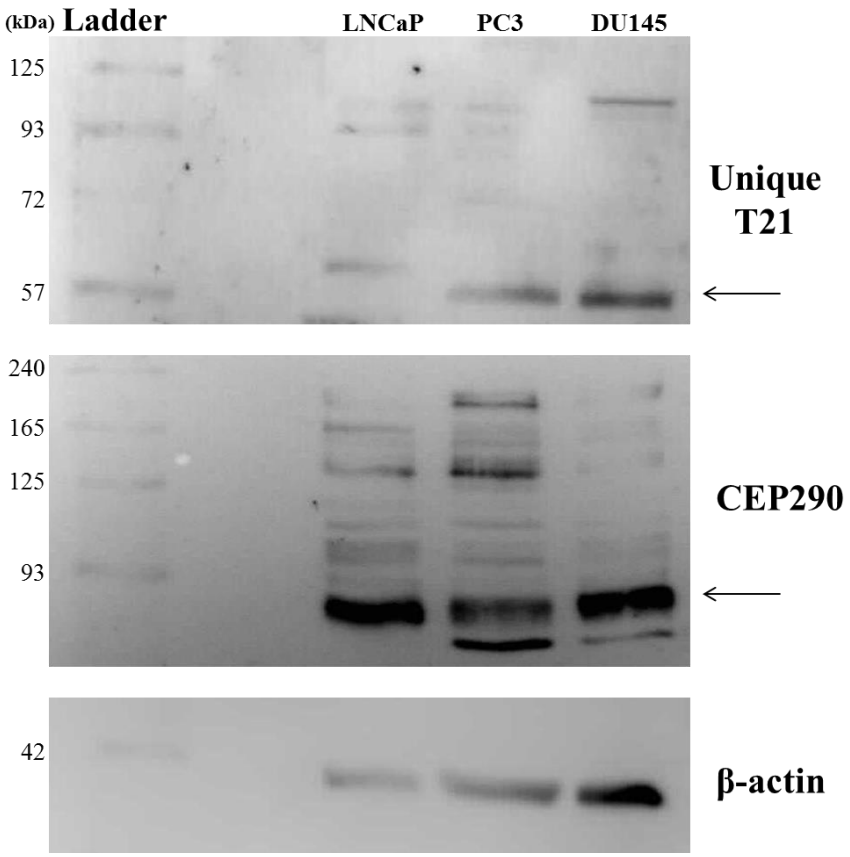


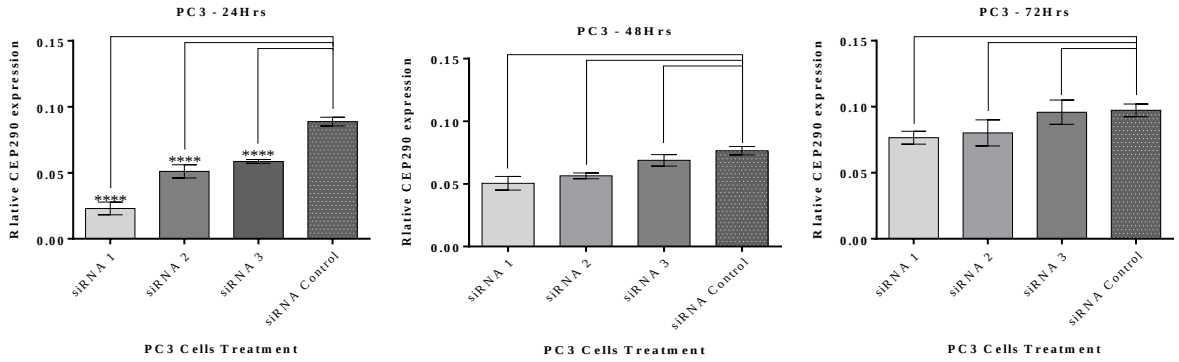
Figure 3.2.1. 6 Western blot analysis using T21unique and CEP290 antibodies.
Immunoblotting protein expression in prostate cancer cell lines (LNCaP, PC3 and DU145). Protein expression of these prostate derived cell lines showing several discreet bands (57 kDa for T21unique antibody and 90 kDa for CEP290 antibody) performed using western blotting and using β-actin (45 kDa) as representative protein loading control (n=2). (A different antibody with 290kDa for CEP290 is currently used "ongoing work")

3.2.2 Effect of transient gene silencing of CEP290 on T21 expression

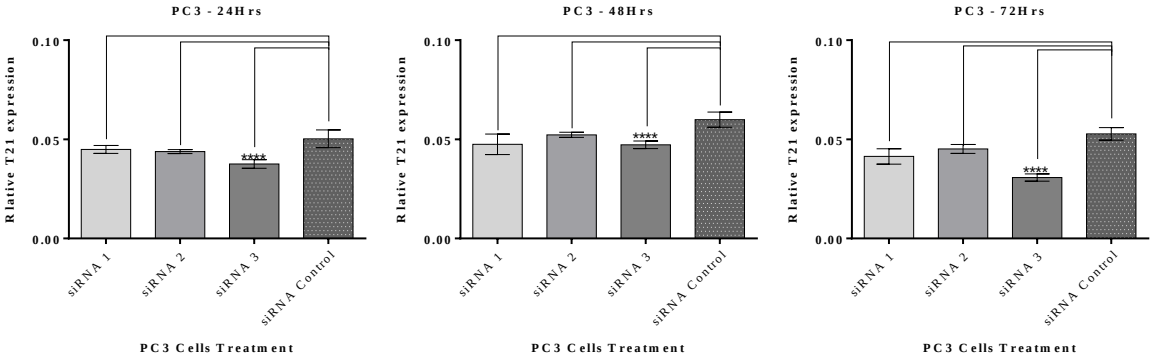
PC3 and DU145 prostate cancer cell lines express high levels of CEP290 and T21 mRNAs (Figure 3.2.1.3). Therefore, these cell lines were used to investigate the effect of CEP290 knockdown on T21 expression. Three siRNA oligonucleotides were designed at 5' sequences of CEP290, synthesised and transfected together with the control siRNA into PC3 and DU145 using Interferin, a non-liposomal amphiphile transfection reagent.

Total RNA was extracted from the cells after 24, 48 and 72 hours post transfection using RNA spin columns. The total level of CEP290 and T21 mRNAs were assessed by qRT-PCR using CEP290 and T21 specific primers (Figure 3.2.2.1 and 3.2.2.2 (As). CEP290 siRNA1 was the most effective in silencing CEP290 in PC3 (78% inhibition) after 24 hours while the remaining two siRNA molecules (siRNA1 and siRNA2) achieved 61% and 58% knockdown efficiency, respectively (Figure 3.2.2.1 (C)). In DU145, CEP290 siRNA1 was also the most effective in silencing CEP290 with 72% after 24 hours while the other two (siRNA1 and siRNA2) produced 62% and 59% knockdown efficiency (Figure 3.2.2.2 (C)). However, the transient gene silencing of CEP290 using siRNA1 and 2 has no significant knockdown of T21 expression at 24, 48 and 72 hours. However, siRNA 3 did affect T21 expression in PC3 cells over 24, 48 hours (Figure 3.2.2.1). For DU145 cells siginificant silencing of CEP290 was achieved 24 hours post transfection for all siRNAs, but siRNA 3 was not specific for CEP290; and also demonstrated the ability to silence T21 at 24 and 48 hours (Figure 3.2.2.2)

(A)



(B)



(C)

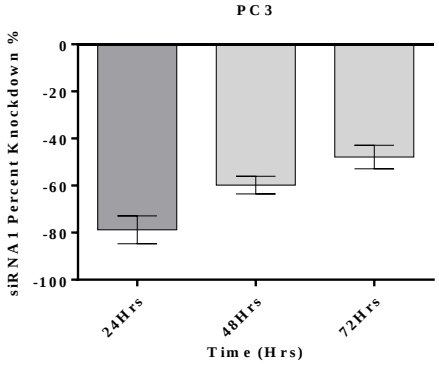
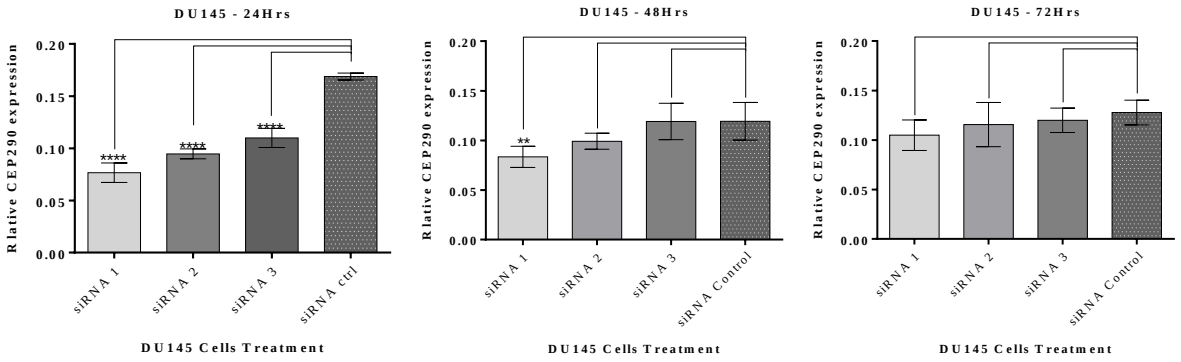


Figure 3.2.2. 1 CEP290 silencing of PC3 (CEP290 and T21 positive expression prostate cancer cell line) following CEP290 specific siRNA transfection.
(A) Time course performed by real time PCR showing CEP290 siRNA1, siRNA2, and siRNA3 silencing over time 24, 48 and 72 hours. (B) Real time PCR analysis showing the relative expression of T21 in PC3 mRNA knockdown following transfection with CEP290 siRNA1, siRNA2, and siRNA3 silencing over time 24, 48 and 72 hours. (C) Time course performed by

*real time PCR showing the percentage of the obtained relative expression of CEP290 post CEP290 siRNA1 silencing over time. Experiments were carried out three times in duplicate (n=3) represented with standard error of mean with data expressed as percentage change to the negative siRNA control. Statistical significance (*p<0.05) (**p<0.01) (***p<0.001) (****p<0.0001) as determined by student's t test.*

(A)



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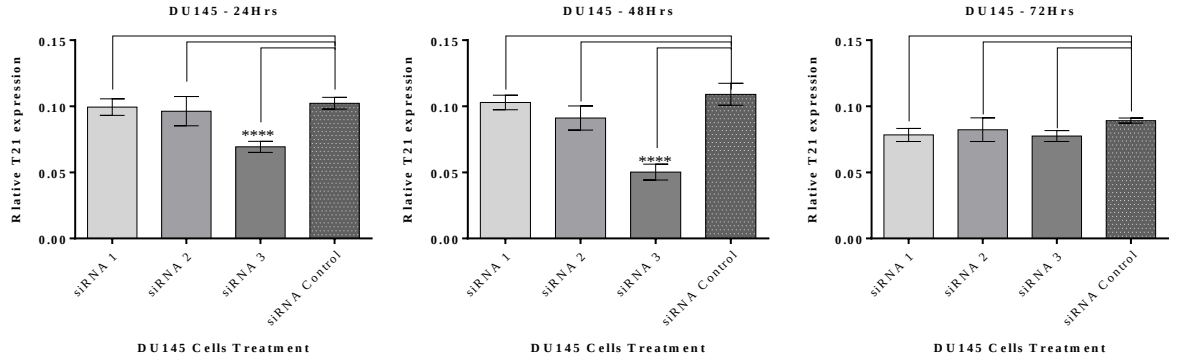


(C)

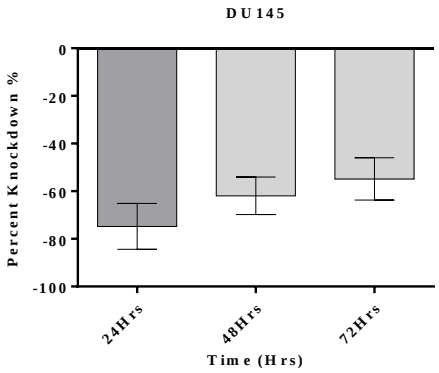


Figure 3.2.2. 2 CEP290 and T21 knockdown of a strong CEP290 and T21 genes expression prostate cancer cell line (DU145) following CEP290 specific siRNA transfection.

(A) Time course performed by real time PCR showing CEP290 siRNA1, siRNA2, and siRNA3 silencing over time 24, 48 and 72 hours on DU145 cells. (B) Real time PCR analysis of T21 mRNA relative expression following transfection of DU145 cell line with CEP290 siRNA1, siRNA2, and siRNA3 silencing over time 24, 48 and 72 hours. (C) Time course performed by real time PCR showing the effect (in percentage) of CEP290 siRNA1 silencing over time on

*DU145 cells. Experiments were carried out three times in duplicate (n=3) represented with standard error of mean with data expressed as percentage change to the negative control siRNA. Statistical significance (*p<0.05) (**p<0.01) (***p<0.001) (****p<0.0001) as determined by student's t test.*

In conclusion, it is shown that gene silencing of CEP290 appears to be specific and its expression is independent of T21.

3.2.3 Effect of CEP290 knockdown on cellular proliferation

The previous findings reported T21 as prostate cancer antigen which belongs to cancer-testis antigens (CTAs) family with highly restricted expression patterns in testis tissue and various malignancies, but not in other normal tissues. T21 mRNA also over-expressed in prostate cancer compared with benign glands and increased T21 expression was positively correlated with pathological stage of disease (Miles et al., 2007; Miles et al., 2012). Moreover, based on the *in silico* analysis T21 has a close association with part of CEP290. To determine whether CEP290 and T21 share functional characteristics and to give an insight into their relationship and T21 in cancer progression, cell proliferation was assessed following CEP290 gene silencing.

CEP290 is known as nephrocystin 6 and is active in centrosomes and the connecting cilium of photoreceptors. Although the functional roles of CEP290 domains in the molecular physiology of CEP290 are largely unknown, although the BPNLS domain has been shown to permit the partial localisation of CEP290 to the nucleus, where it interacts and modulates the activity of Activating Transcription Factor 4 (ATF4) (Sayer et al., 2006). Furthermore, a C-terminal myosin-tail homology domain may provide a fundamental structural base for the myosin motor and could promote the microtubule-associated transport of CEP290 to centrosomes (Chang et al., 2006). CEP290 also interacts with a major component of centriolar satellites (pericentriolar material 1 (PCM1)), and the cilia regulator centrosomal protein 110 (CP110). CEP290 knockdown leads to disruption of the subcellular distribution of PCM1 and protein complex formation with PCM1 and is associated with disorganisation of the cytoplasmic microtubule network. This suggested that CEP290 is required for ciliogenesis. CEP290 is also important for the ciliary targeting of Rab8, a small GTPase that is regulated by Bardet-Biedel Syndrome (BBS) protein complex (the BBSome) in ciliogenesis (Kim et al., 2008).

To assess the effect of the transient knockdown of CEP290 on prostate cancer cell proliferation ^{3}H thymidine incorporation proliferation assays was perforemed. The ^{3}H Thymidine incorporation was assessed following CEP290 siRNA silencing. Briefly, $5x10^{4}$ cells/well were cultured in 24-well plates and transfected with the designed CEP290 siRNAs and at 24, 48 and 72 hours. ^{3}H thymidine (^{3}H-Thy) uptake was assessed 18 hours after adding thymidine, the media was removed and the cells were washed and transferred into 96-well

round bottom plates. Plates were then harvested using a cell harvester onto 96 Uni/Filter scintillation plates and were counted using a Top-Count scintillation counter.

The ^{3}H Thymidine incorporation (proliferation) assay showed significant reductions in cellular proliferation at 24, 48 and 72 hours post CEP290 siRNA transfection, particularly for PC3 cell proliferation. In contrast, DU145 showed no significant reductions in cellular proliferation at 24, 48 and 72 hours following CEP290 siRNA treatment. The three CEP290 siRNAs were assessed in comparison to cells growing normally (without transfection) in order to ensure that these findings were due to a reduction in CEP290 and not a consequence of the transfection procedure used. All of the above experiments were performed on cells that had undergone transient transfection to confirm that the time coarse transfection had the same effect on cell proliferation. However, these results are inconclusive since for PC3 cells addition of control siRNA reduced cell proliferation to same extent as CEP290 siRNA and DU145 proliferation was unaffected by siRNAs' treatment.

(A) Effect of CEP290 knockdown on PC3 cell proliferation

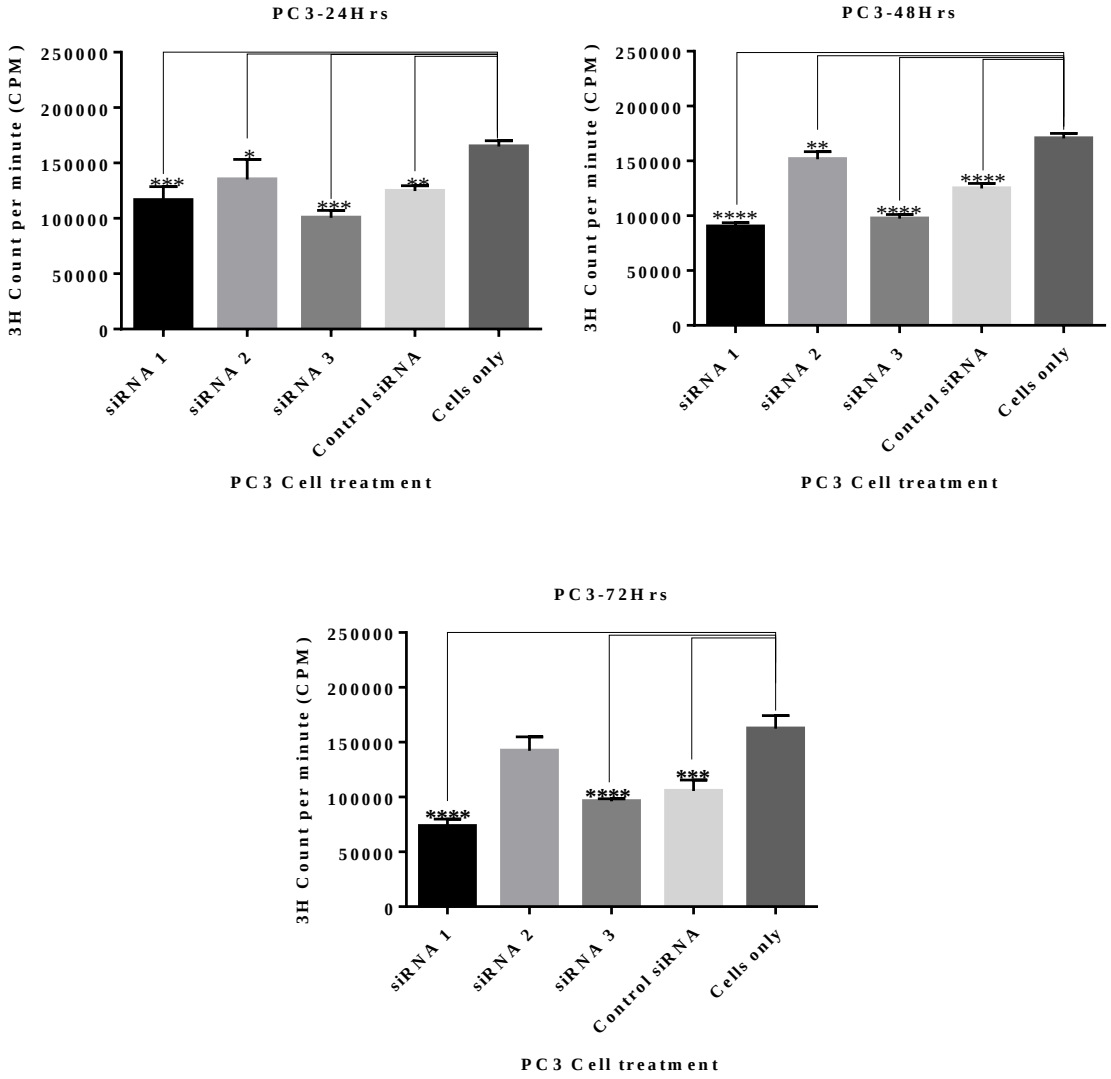


Figure 3.2.3. 1 ^{3}H Thymidine incorporation (proliferation) assay to assess the effect of CEP290 siRNA treatment on PC3 cells.
*Measurements were taken over time (24, 48 and 72 hours). Statistical significance (*p<0.05) (**p<0.01) (***p<0.001) (****p<0.0001) as determined by student's t test.*

(B) Effect of CEP290 knockdown on DU145 cell proliferation

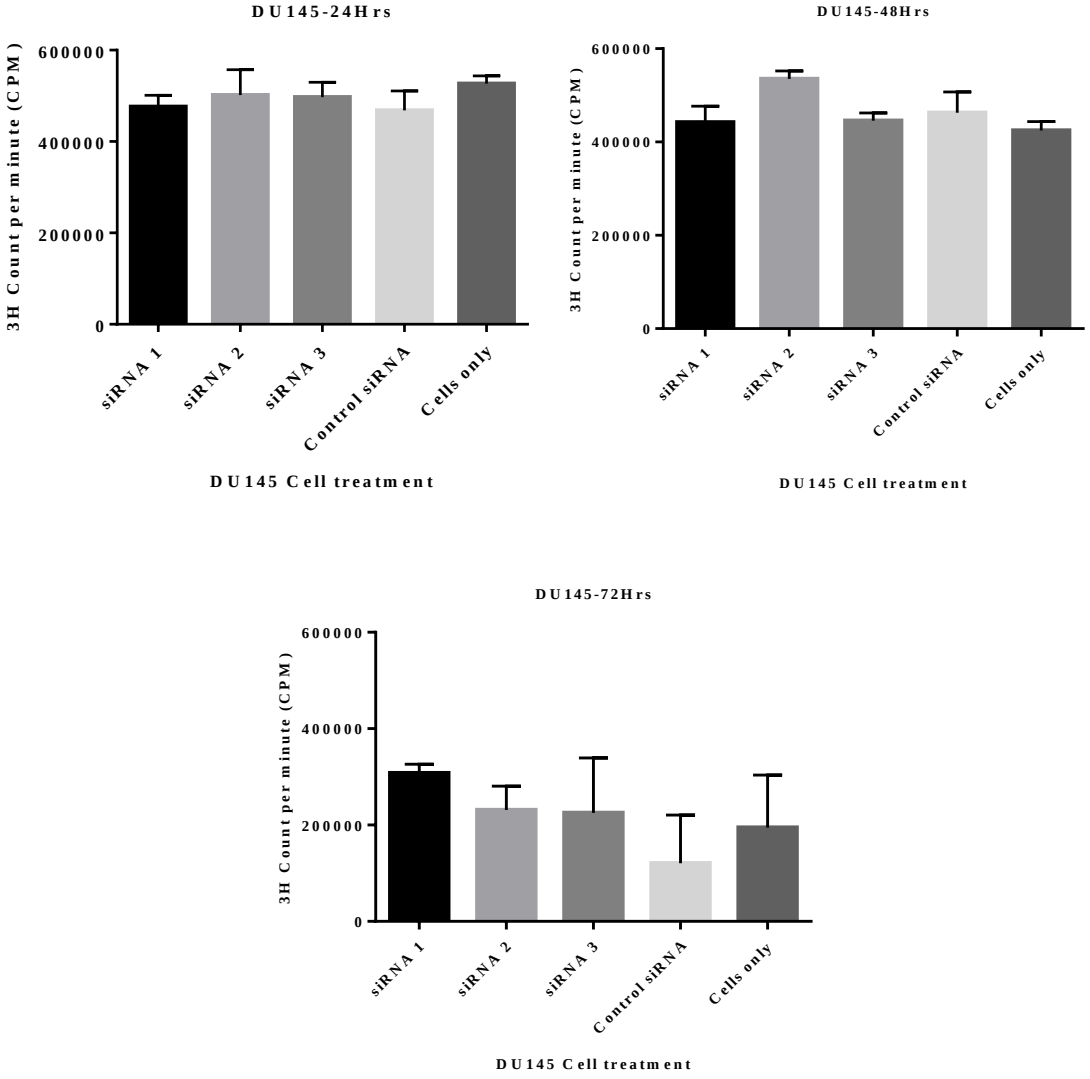


Figure 3.2.3. 2 ^{3}H Thymidine incorporation (proliferation assay) to assess the effect of CEP290 siRNA treatment on DU145 cells.
*Measurements were taken over time (24, 48 and 72 hours). Statistical significance (*p<0.05) (**p<0.01) (***p<0.001) (****p<0.0001) as determined by student's t test.*

3.3 Discussion:

T21 is a novel prostate Tumour-Associated Antigen (TAA) and has been shown to elicit a humoral immune response in prostate cancer patients (Mile et al. 2007; Mile et al. 2012). T21 has also been shown to be over-expressed in malignant glands of prostate cancer compared to benign glands and stroma at both the mRNA level and protein expression. T21 shares significant similarity with a Centrosomal Protein called CEP290. This large protein has been implicated in several cilia related syndromic disorders such as Joubert syndrome (Sayer et al, 2006; Valente et al, 2006). Consequently, it was necessary to determine the differences between these two molecules to facilitate further studies on the role of T21 in prostate cancer tumourigenesis. Quantitative RT-PCR using specific primers to CEP290 and T21, showed that T21 is a separate product, different from CEP290, however the levels of expressions of these two molecules in tissues and cell lines did not always correlate, which made the analysis difficult. Many researchers have reported on the fact that the levels of expression of RNA products do not necessarily reflect the level of expression of the corresponding protein products. CEP290 itself may indeed be a cancer associated protein leading to anti-CEP290 antibody production, which has been identified in gastric (Chen & Shou, 2001), CTCL tumours and also leukaemia and melanoma cell lines (Eichmuller et al., 2001).

To investigate CEP290 and T21 expression further, a custom made polyclonal antibody was used to study T21 protein expression. For CEP290 protein expression, a commercial antibody was purchased and used. Unfortunately, immunofluorescence studies using the polyclonal antibody, failed to show specificity for T21 protein as this antibody appears to also recognise CEP290. Therefore a new antibody directed against the unique region of T21 was produced. Remarkably, this antibody recognised specifically T21 both by immunofluorescence and immunoblotting. The fluorescent immunostaining with the unique T21 antibody confirmed that T21 did not localise to the centrosomes (Figure 3.2.1.3). Following immunofluorescent staining of the prostate cancer cell lines with the new (specific) T21 antibody there was a clear demonstration of cytoplasmic staining in all three cell lines which is consistent with the predicted cellular localisation of this protein. From these results it can be inferred that T21 is unlikely to share a functional role similar to that of CEP290 at the centrosomal sites during cell proliferation.

An important question that until now has remained unanswered was the nature and relationship between T21 and CEP290. To date mRNA levels failed to distinguish any

associations in expression of the two protein and so far western blot analysis concluded a seemingly lower expression of T21 compared to CEP290 in prostate cancer cell lines.

Therefore, CEP290 knockdown was performed using siRNAs in order to assess its effect on mRNA T21 expression. CEP290 was efficiently silenced in PC3 and DU145 using siRNA and the transfected cells were observed by microscopy for changes in cell behaviour and morphology during in vitro culture. In summary, cells treated with CEP290 siRNAs did not exhibit any noticeable changes when compared with control siRNA or interferon treatment other than a seemingly reduced capability to proliferate. Following this observation, experiments assessing cell proliferation were performed using ^{3}H thyimidine incorporation parallel to assesse mRNA T21 knockdown following CEP290 siRNAs transfection. mRNA T21 reduction was observed only with CEP290 siRNA 3, in contrast to CEP290 siRNA 1 and 2 that silenced CEP290 within 24 hours but showed no effect on T21 mRNA levels, suggesting independent expression of these two genes.

As CEP290 has been found to be involved in centrosomal function, it could naturally be concluded that its presence would be in some way be linked to proliferating cells. This was evident in earlier studies using T21/CEP290 siRNA in prostate cancer cells; following reanalysis of affymetrix data for CEP290 pathway markers, genes known to be implicated in CEP290 pathways were significantly modified following T21/CEP290 siRNA transfection. As a consequence of the previous investigation into T21 and its more recently confirmed association with CEP290, it was critical to establish the link between CEP290 and T21 with regard to cellular proliferation post CEP290 knockdown to determine further the potential role (if any) of T21 in cancer progression. The results were inconclusive since the control siRNA reduced PC3 proliferation similarly to CEP290 siRNA and expression with DU145 did not show loss of proliferation. This aspect of the study requires further investigation and optimisation (Figure 3.2.3.1 and 3.2.3.1.2).

The emerging evidence for T21's role in prostate cancer and also the fact that T21 is a novel transcript as demonstrated in this part of study infers that T21 has the potential to be a target for cancer therapy. Since the molecular function of this novel transcript (T21) remains undetermined, subsequent chapters will focus on elucidating some of the key pathways that T21 is implicated in.

CHAPTER IV

THE EFFECT OF T21 KNOCKDOWN ON KEY CELLULAR PATHWAYS THROUGH THE ANALYSIS OF GENE EXPRESSION PROFILES OBTAINED BY NEXT GENERATION SEQUENCING (NGS)

4 CHAPTER FOUR: The effect of T21 knockdown on key cellular pathways through the analysis of gene expression profiles obtained by Next Generation Sequencing (NGS)

4.1 Introduction

As discussed in the previous chapter, T21 and CEP290 were shown to be different protein products as demonstrated through the study of their mRNAs and proteins using molecular and cellular approaches. Further confirmation was achieved by silencing CEP290 using specific siRNA that showed no significant impact on the unique sequence of T21. Therefore, despite the shared sequence similarity between T21 and CEP290, the function and interactions of T21 are likely to be distinct. The expression of T21 has been reported to be highly associated with the pathological stage of tumour progression and this aspect of the present study was designed to investigate the potential role of T21 in prostate cancer.

In 2001 the first sequencing of the human genome was reported by an international consortium, which provided extensive resources and a basis for further research into the regulatory pathways of cell behaviour (Lander et al, 2001; Venter et al, 2001). Since then, genomic research has expanded dramatically, leading to a revolution in the development of new technologies with high-throughput capability. For instance, during the past decade, microarray-array based experiments have been extensively applied for research into the cancer genome and transcriptomes, which has provided further insight into the molecular genetics associated with breast cancer (Sotiriou and Pusztai, 2009). Nonetheless, microarray-based expression profiling alongside genomic hybridisation data analysis has encountered crucial limitations. The profiling of microarray data provides a semi-quantitative analysis that is restricted due to the nature of the probes, which can affect both sensitivity and specificity. On the other hand, comparative genomic hybridisation and Single Nucleotide Polymorphism (SNP) provides abundant information on abnormal gene copy number(s), but without knowledge of structural genomic abnormalities and nucleotide base-pair mutations (Tan et al, 2007). Consequently, automated sequencing tools were developed to further characterize genomic structure and function, providing an extensive amount of information in a relatively short time frame with qualitative and quantitative data on any single gene expression, down to the single nucleotide level. Among, these tools, Next Generation Sequencing (NGS) is certainly a revolutionary and effective technology that has been developed, providing unprecedented parallel sequencing power, which encompasses several different methodologies for characterization of complex genetic disorders (including cancer) at their

genomic, transcriptomic and epigenomic levels (Venter et al, 2003; Margulies et al, 2005; Shendure and Hanlee, 2008; and Reis-Filho, 2009).

The recent development of NGS technologies has provided a platform for performing low cost high-throughput sequencing. Two of the common NGS platforms currently used in research and clinical labs are the Life Technologies Ion Torrent Personal Genome Machine (PGM) and the Illumina MiSeq which have been extensively used to investigate the molecular characteristics of disease. These platforms have similar basic methodology in terms of template preparation, sequencing, imaging, and data analysis. However, each platform has unique aspects in how sequencing is accomplished (Figure 4.1.1) (Metzker, 2010; Grada and Weinbrecht, 2013). NGS, nonetheless, poses a considerable challenge related to data storage, analysis and solutions management. Therefore, this powerful technology requires further advanced bioinformatics tools that are essential for successful NGS applications (Zhang et al, 2011).

This part of the study has focused on analysing the effect of T21 knockdown on whole genome expression in PC3 prostate cancer cells using Next Generation Sequencing (NGS). The data obtained were analysed using bioinformatics tools to identify the genes differentially expressed in T21 knockdown and its corresponding control. Moreover, potential important pathways were identified from NGS data analysis. Further investigations of these pathways using stable knockdown of T21 (shRNA) was subsequently performed to analyse changes in protein expression using proteome profiler arrays (performed and discussed in Chapter 5).

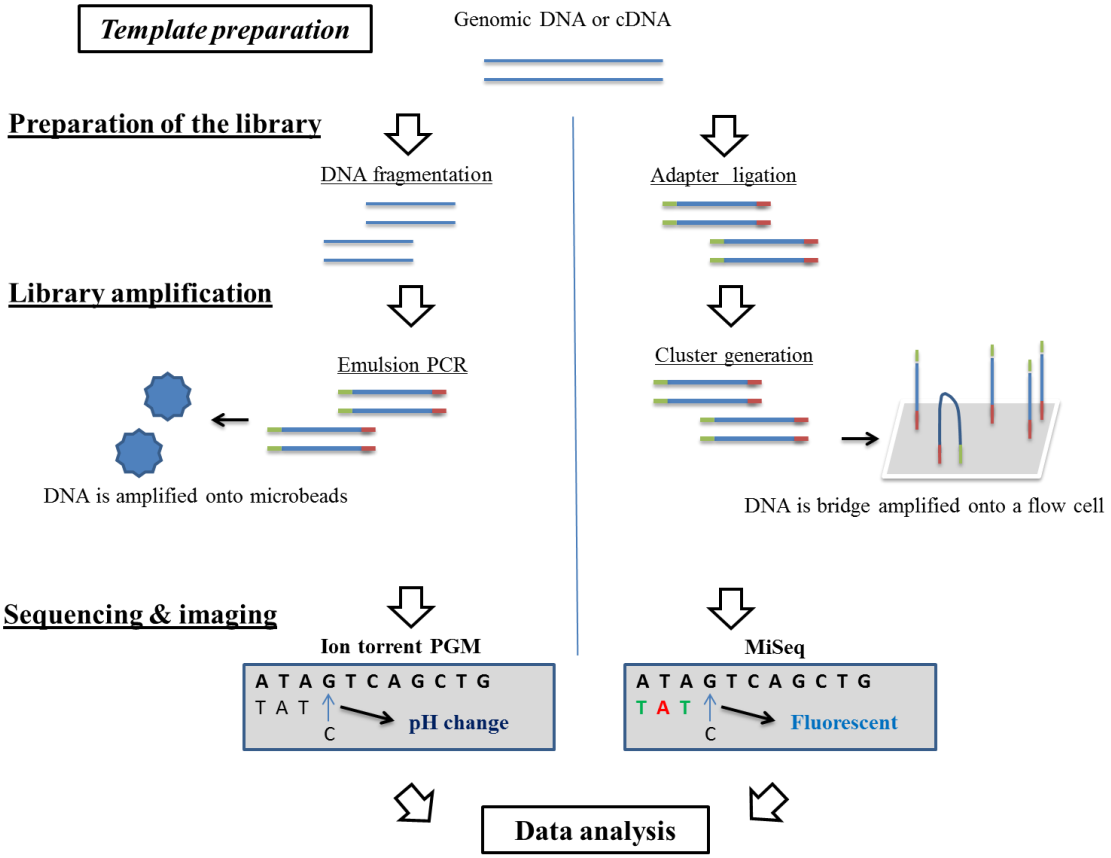


Figure 4.1. 1 Next-generation sequencing (NGS) methodology for the most two common platforms (Ion Torrent Personal PGM and the Illumina MiSeq).

(Adapted from Grada A and Weinbrecht K, 2013).

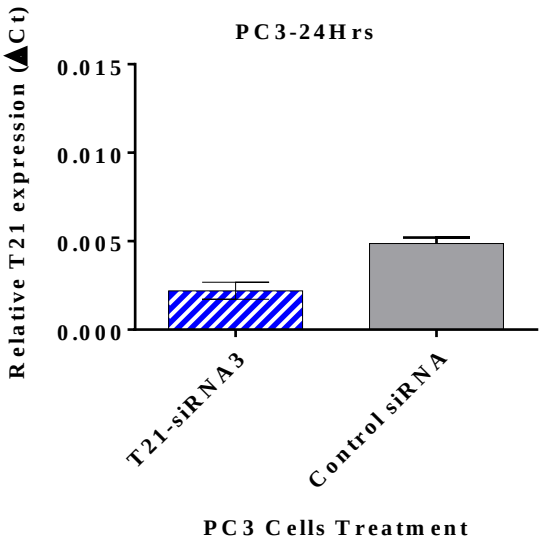
4.2 Results:

4.2.1 T21 silencing using small interfering RNA (siRNA):

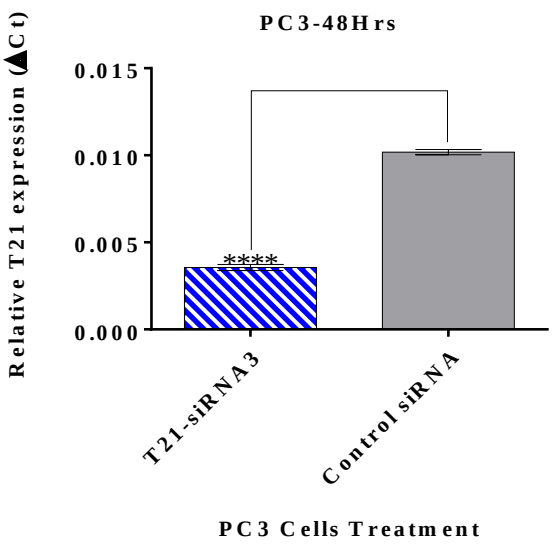
T21 silencing is an experimental technique that was performed in order to understand the role of T21 in prostate cancer. First of all, three small interfering RNAs (siRNAs) were designed within the unique region of T21 and used to silence gene expression in PC3 prostate cancer cell line. Then, total RNA was extracted from cells using RNA spin columns after 24, 48 and 72 hours post T21 silencing and assessed at the mRNA level by qRT-PCR using T21 specific primers. The data showed that T21-siRNA 3 had a significant effect in silencing T21 after 48 hours (Figure 4.2.1.1). siRNA 1 and 2 knockdown did not have significant effects at 24, 48 and 72 hours post-transfection.

In parallel, of assessing the efficiency of T21 mRNA knockdown, investigation of the effect of T21 knockdown on cellular proliferation was assayed using ^{3}H thymidine incorporation assay over period of time (24, 48 and 72 hours). Interestingly, the data demonstrated a significant reduction of the proliferation observed in PC3 cells transfected with T21 siRNA 3 and when compared to control siRNA after 48 and 72 hours (Figure 4.2.1.2). No significant effect on PC3 cell proliferation was observed after 24 hours post-transfection.

(A)



(B)



(C)

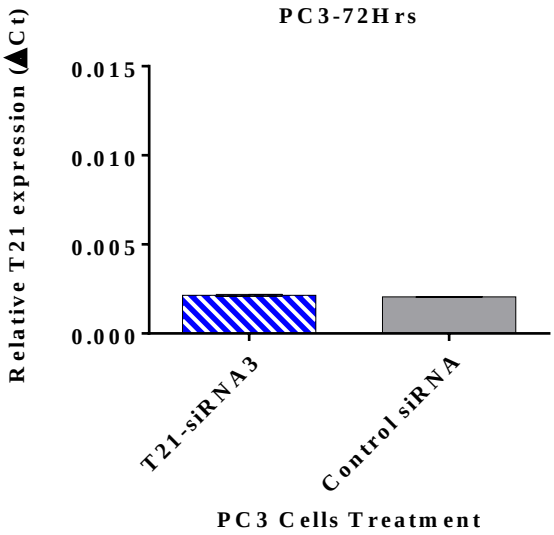


Figure 4.2.1. 1 T21 knockdown of a T21 expression using a prostate cancer cell line (PC3).

*Time course was performed using quantitative real time PCR showing T21 siRNA silencing over time (A) 24 hours, (B) 48 hours and (C) 72hours. Experiments were carried out three times in duplicate (n=3). Results are given as the standard error of the mean, normalised for relative expression of T21-siRNA and negative control siRNA. Statistical significance is indicated (****p<0.0001 for 48 hours) as determined by Student's t test.*

(A) (B)

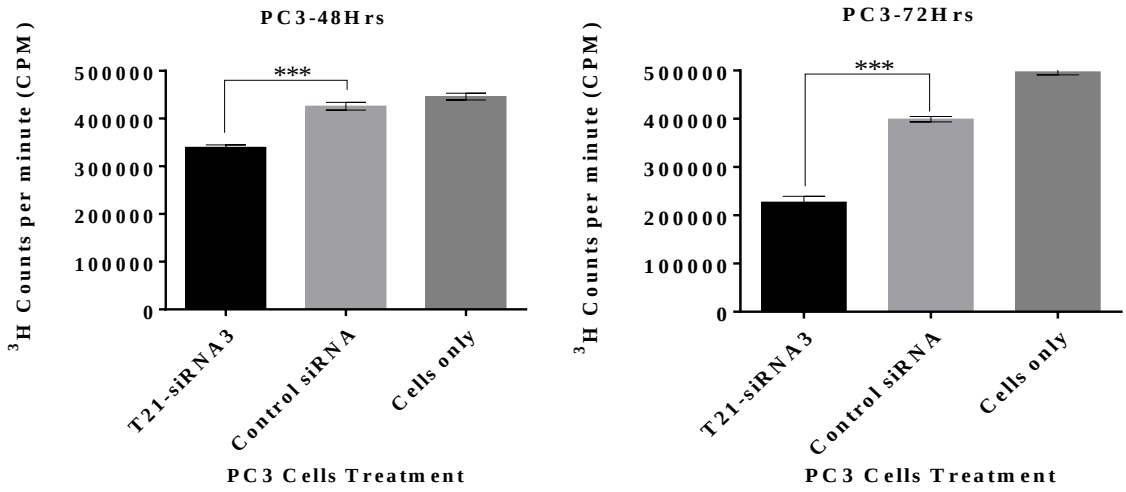


Figure 4.2.1. 2 Cellular proliferation assay following T21 specific siRNA 3 transfection in PC3 cells.

*Time course performed using a ^{3}H thymidine incorporation assay to demonstrate reduced PC3 cell proliferation following T21- siRNA silencing over time (A) 48 and (B) 72 hours. Experiments were carried out in triplicate wells (n=3) on three separate occasions represented with standard error of the mean. Statistical significance indicated (***p<0.0004 for 48 hours and ***p<0.0003 for 72 hours) as determined by Student's t test.*

4.2.2 Investigating the effect of T21 knockdown on key cellular pathways through the analysis of gene expression profiles obtained by Next Generation Sequencing (NGS)

This study has focused on elucidating the role of T21 in the regulation of key cellular pathways in prostate cancer. Following successful T21 silencing in the PC3 prostate cancer cells (was previously performed by Drs. Amanda Miles and Morgan Mathieu), Next Generation Sequencing (NGS) was used to determine the effect of T21 silencing on gene expression. The cells treated with either control siRNA or T21 siRNA were lysed using RNA STAT-60 and the total RNA was extracted using isopropanol precipitation. The RNA quality was assessed using a RNA 6000 pico chip kit (Agilent) and an Agilent 2100 Bioanalyzer (Agilent). RNA samples with RNA integrity number (RIN) values between 8 and 10 were stored at -80°C until analysis by next generation sequencing (NGS). RNA samples with RIN values below 8 were discarded.

Next generation sequencing of total RNA samples were outsourced and carried out by the Gene Service from Source Bioscience. Libraries were created by producing a series of randomly fragmented RNA to which two adapters were linked. These libraries were then denatured to single molecules and randomly hybridised to the surface of a flow cell to form clusters. Once the latter were formed, the flow cell was sequenced on the Illumina Genome Analyser IIx platform using 38bp single end reads. Alignment of the reads against the human genome was performed by the Bioinformatics Service from Source Bioscience. Technical repeats were merged and logarithmic (fold-change) values and p values were calculated to determine significant differences (Table 4.2.2.1 A and B).

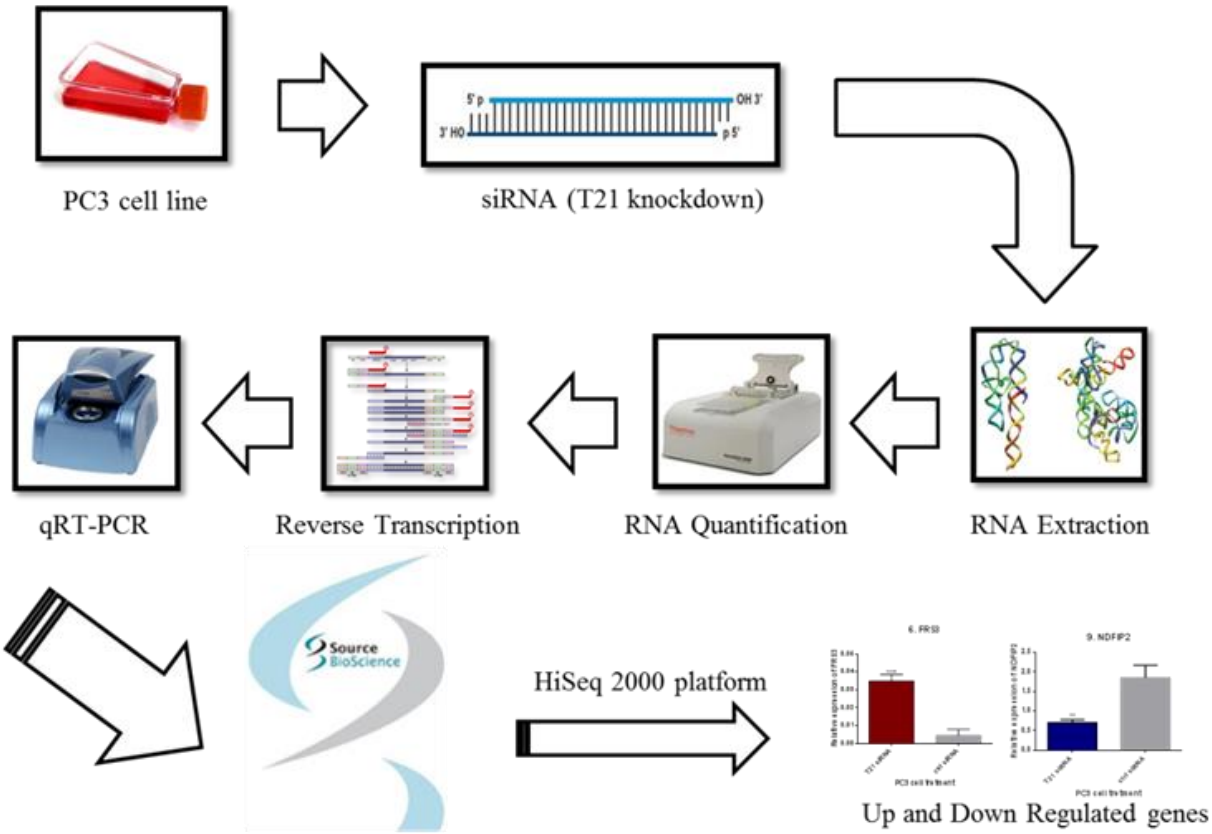


Figure 4.2.2. 1 Workflow illustrating the steps performed for Next Generation Sequencing (NGS) sample preparation.

In brief, hundreds of millions of DNA fragments were sequenced simultaneously. Sequencing was performed by extending one base at a time in the chemistry process, based on reversible single base extensions. Four fluorescently reversible dideoxynucleotides (ddA, ddC, ddG and ddT) are introduced in the flow cell simultaneously. These bases compete with each other to bind to the template. Natural competition ensures the highest accuracy. Following the chemistry, the instrument takes a picture for each added base and saves the image in a tiff format. The fluorophore is then cleaved and the 3' hydroxyl end is de-protected for the next reversible single base extension. This process is done in many cycles and each cycle includes the chemistry step as well as the imaging step. For each cycle, hundreds of tiff images are generated. Specialised RTA (Real-Time Analysis) software loaded onto the platform automatically converts scanned images into digital intensity signals, and interprets these signals as nucleotide bases. Over a specified number of cycles, these bases are combined to generate millions of lines of raw sequence data, or "reads" as shown in tables (Table 4.2.2.1 A and B). These data were used and categorised according to the potential pathways of each genes and its involvement in tumourigensis.

(A)

test_ID	Gene	Locus	Sample_1	Sample_2	Status	Value_1	Value_2	ln(fold_ change)	test_stat	p_value	Significant
XLOC_038816	**MAPK6**	chr15:52311416-52358462	PC3_ control	PC3_ siRNA	OK	219.79	103.84	-0.749777	6.29648	3.04E-10	Yes
XLOC_034098	**CALM3**	chr19:47104565-47114039	PC3_ control	PC3_ siRNA	OK	776.38	372.08	-0.73554	10.8804	0	Yes
XLOC_026153	**HIPK2**	chr7:139246315-139720123	PC3_ control	PC3_ siRNA	OK	636.09	325.71	-0.669348	10.1078	0	Yes
XLOC_029074	**RAP2A**	chr13:98086475-98120244	PC3_ control	PC3_ siRNA	OK	376.56	199.03	-0.637602	6.8739	6.25E-12	Yes
XLOC_022135	**BCL10**	chr1:85731463-86043933	PC3_ control	PC3_ siRNA	OK	165.7	99.402	-0.511033	6.72187	1.79E-11	Yes
XLOC_038894	**MAP2K1**	chr15:66679154-66790146	PC3_ control	PC3_ siRNA	OK	441.56	270.41	-0.490364	7.53079	5.05E-14	Yes
XLOC_023352	**TP53BP2**	chr1:223967595-224033674	PC3_ control	PC3_ siRNA	OK	170.58	104.93	-0.485883	3.04592	0.00232	Yes
XLOC_025750	**CDK6**	chr7:92234234-92546501	PC3_ control	PC3_ siRNA	OK	140.67	90.605	-0.439898	3.0797	0.002072	Yes
XLOC_029013	**NDFIP2**	chr13:80051498-80130210	PC3_ control	PC3_ siRNA	OK	203.44	132.6	-0.428058	3.29346	0.00099	Yes
XLOC_017277	**CEP290**	chr12:88429267-88535084	PC3_ control	PC3_ siRNA	OK	31.688	17.128	-0.615205	2.95058	0.003172	Yes

(B)

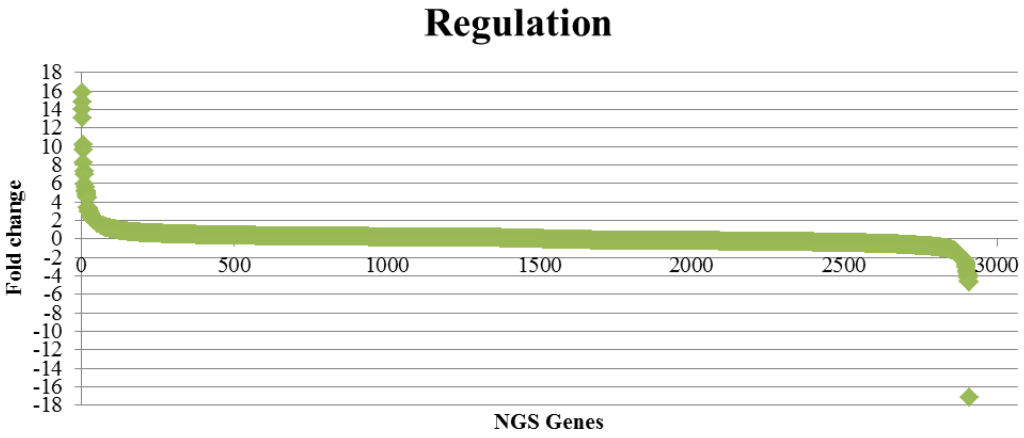
test_ID	Gene	Locus	Sample_1	Sample_2	Status	Value_1	Value_2	ln(fold_ change)	test_stat	p_value	Significant
XLOC_005051	**CHD8**	chr14:21853357-21905404	PC3_control	PC3_siRNA	OK	100.554	186.034	0.615233	-4.96775	6.77E-07	Yes
XLOC_043823	**TP53INP2**	chr20:33284721-33302432	PC3_control	PC3_siRNA	OK	89.767	145.21	0.480951	-3.43546	0.000592	Yes
XLOC_014239	**DUSP14**	chr17:35441922-36244363	PC3_control	PC3_siRNA	OK	94.724	161.27	0.532127	-12.8714	0	Yes
XLOC_043316	**IGFBP5**	chr2:217536827-217858722	PC3_control	PC3_siRNA	OK	66.188	113.43	0.538673	-3.25888	0.001119	Yes
XLOC_034522	**ZBTB7A**	chr19:4045216-4066816	PC3_control	PC3_siRNA	OK	43.151	77.388	0.584114	-3.07444	0.002109	Yes
XLOC_012925	**FRS3**	chr6:41737913-41757879	PC3_control	PC3_siRNA	OK	12.646	24.5	0.661342	-10.5106	0	Yes
XLOC_010527	**RAB6B**	chr3:133502876-133614680	PC3_control	PC3_siRNA	OK	1.1811	2.4189	0.716824	-4.52293	6.10E-06	Yes
XLOC_037757	**FGF1**	chr5:141689991-142077635	PC3_control	PC3_siRNA	OK	156.83	329.66	0.742906	-28.8754	0	Yes
XLOC_035591	**PDZD2**	chr5:31639516-32122047	PC3_control	PC3_siRNA	OK	14.21	33.148	0.847034	-5.91178	3.38E-09	Yes
XLOC_034165	**BCL2L12**	chr19:50162828-50177172	PC3_control	PC3_siRNA	OK	141.25	376.91	0.981467	-15.9198	0	Yes

Table 4.2.2. 1 Examples of genes down regulated and up regulated genes obtained by NGS following T21 knockdown in PC3 cell line.
(A) Down Regulated and (B) Up Regulated Genes. Selection was based on a minimal 2 fold-change of relative expression to the control and the published literature indicating the role of the gene specific in cancer or its involvement in processes known to be altered in cancer cells.

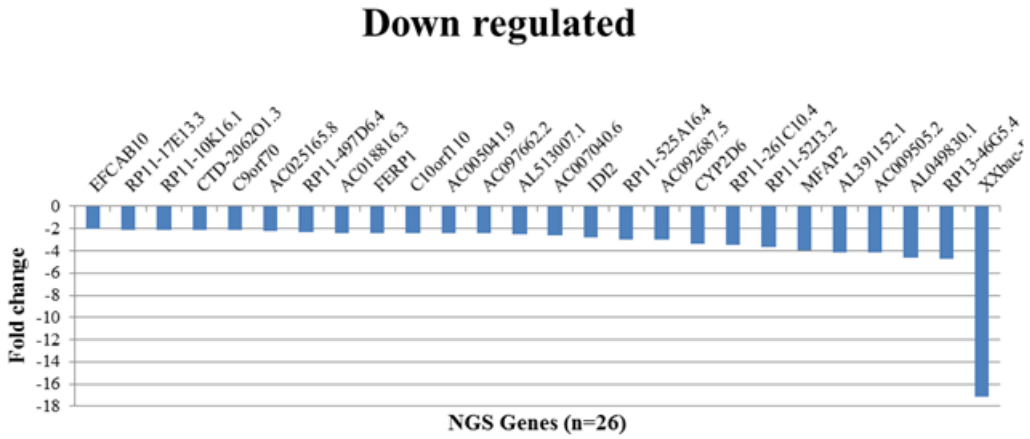
Threshold cut off of values above (up-regulated expression) and below (down-regulated expression) 2 fold-changes were applied to determine significant gene regulation changes. Although a complete bioinformatics analysis and experimental validation of gene expression was performed, genes that were either significantly down regulated (26 genes) or up regulated (197 genes) following T21 siRNA knockdown were used for further analysis (Figure 4.2.2.2). Following a more focused examination of gene functions and literature searches, a more refined shortlist of 20 genes in total (10 up regulated and 10 down regulated genes) that were cancer-associated were selected for more detailed examination.

The functions of genes undergoing significant changes of expression were then obtained online using a variety of web tools such as OMIM (http://www.ncbi.nlm.nih.gov/omim/), Uniprot (http://www.uniprot.org/), and GeneCards (http://www.genecards.org/).

In summary, the down and up regulated genes were chosen based on their potential pathways and their features involved in each pathways in order to understand their role in T21-mediated tumour progression. For instance, cell cycle pathways include several genes such as MAPK6, CALM3 and CDK6 which were shown from NGS data as down regulated genes as a result of T21 knock down. ZBTB7A, in contrast, has a role in cell cycle pathways as a repressor of the CDKN2A gene transcription and represented by NGS data as an up regulated gene following T12 silencing. Other genes known to be involved in apoptosis were also significantly down-regulated or up-regulated such as HIPK2, BCL10 and BCL2L12, a member of BCL family, which play a role in regulating apoptosis. RAS oncogene family and MAP kinase pathways were also found present in the NGS data as either down-regulated or up regulated upon T21 knockdown. The list of selected genes based on their potential pathways are summarised in Table 4.2.2.2 (A) and (B) for the down and up regulated genes respectively.



(A)



(B)

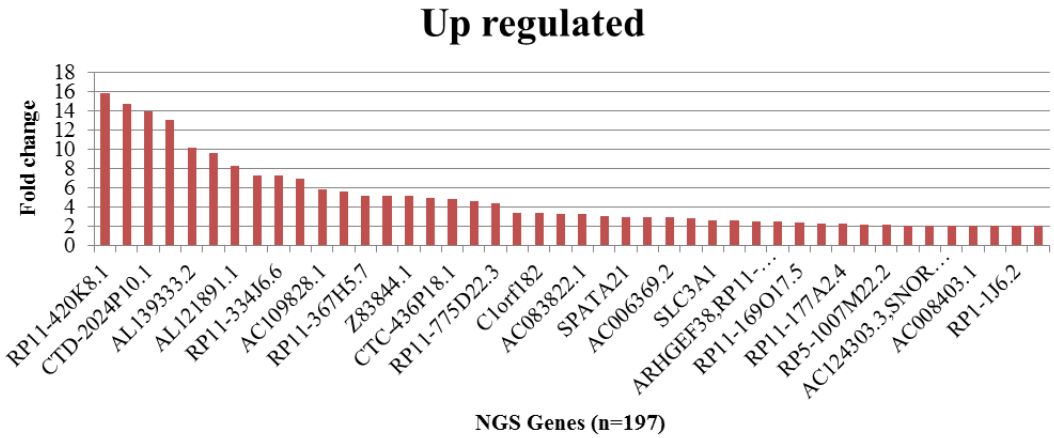


Figure 4.2.2. 2 Bioinformatics analysis of cellular pathway(s) that are altered following knockdown of T21 using bioinformatics tools.

The data obtained from NGS following T21 knockdown experiment in PC3 prostate cancer cell line were used for the Several genes which were categorised (A) Down-regulated and (B) Up-regulated; and only those with a significant fold change were selected.

(A)

Gene	Pathway?	Down regulated when T21 knockdown
MAPK6	cell cycle	Ser/Thr protein kinase may promote entry in the cell cycle
CALM3	cell cycle	regulates the centrosome cycle and progression through cytokinesis
HIPK2	apoptosis	Inhibits cell growth and promotes apoptosis
RAP2A		RAP2, member of RAS oncogene family
BCL10	apoptosis	Promotes apoptosis
MAP2K1		Activates ERK1 and ERK2 MAP kinases
TP53BP2	apoptosis	apoptosis-stimulating protein of p53
CDK6	cell cycle	involved in the control of the cell cycle
NDFIP2		May modulate EGFR signaling
CEP290	cell cycle	Activates ATF4-mediated transcription

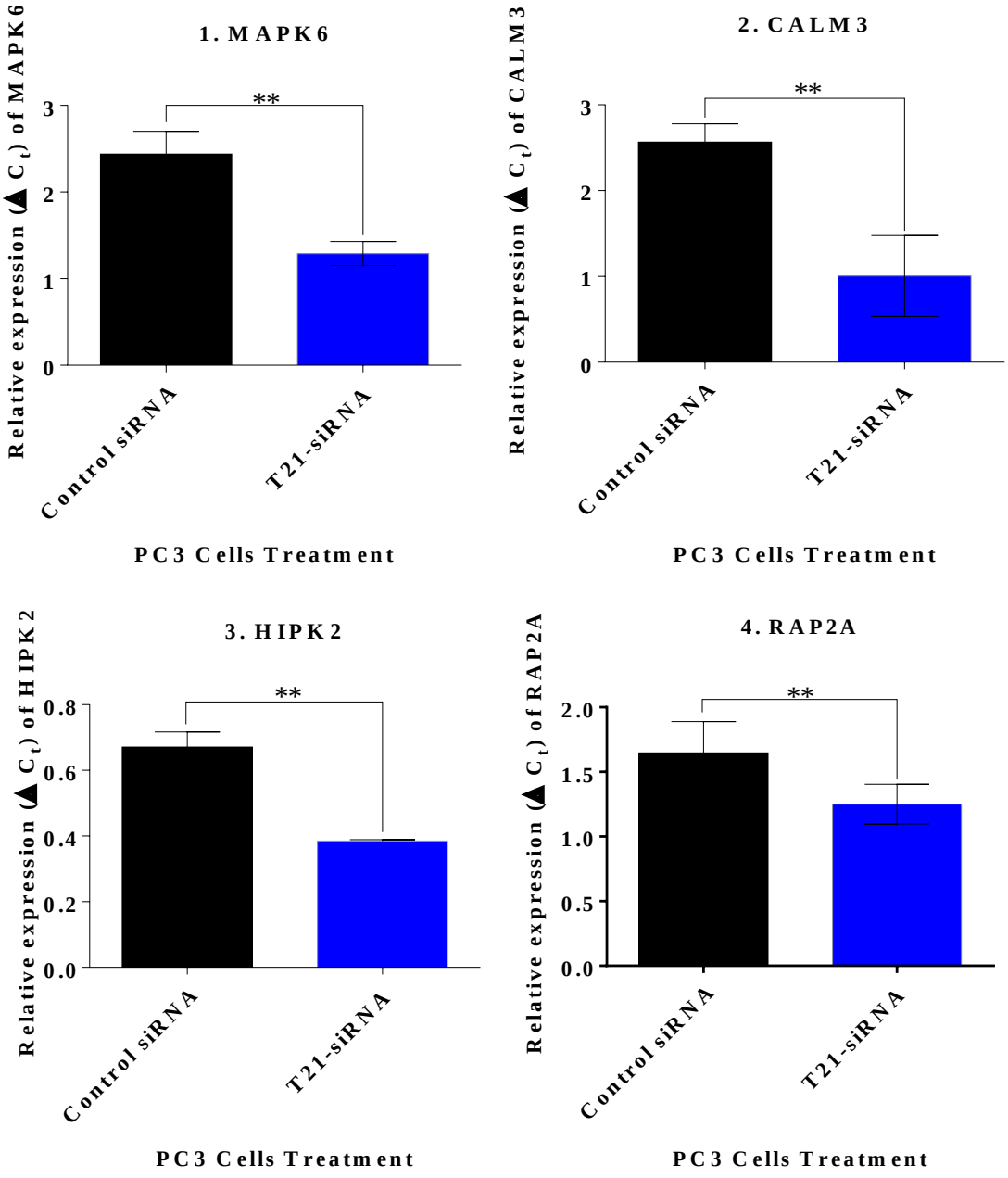
(B)

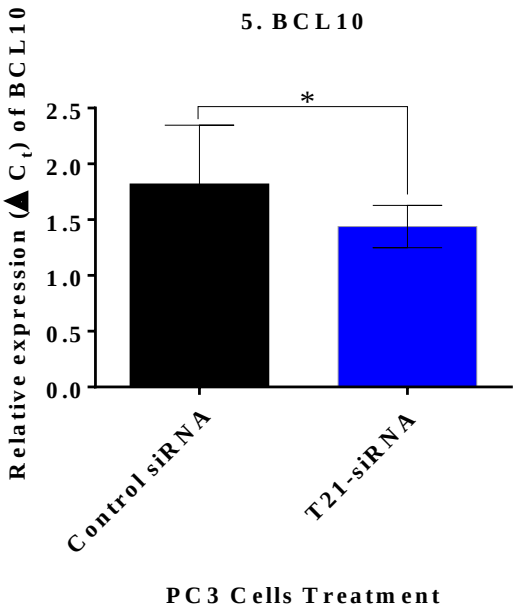
Gene	Pathway?	Up regulation when T21 knockdown
CHD8	proliferation	Suppresses p53/TP53-mediated apoptosis, negative regulator of Wnt signaling pathway
TP53INP2	p53 pathway	tumor protein p53 inducible nuclear protein 2
DUSP14	Cell growth regulation	Involved in the inactivation of MAP kinases. Dephosphorylates ERK, JNK and p38 MAP-kinases
IGFBP5	cell growth/survival	IGF-binding proteins prolong the half-life of the IGFs
ZBTB7A	cell cycle	Specifically represses the transcription of the CDKN2A gene.
FRS3	ras pathway	function in linking FGF receptor stimulation to activators of Ras
RAB6B	ras pathway	RAB6B, member RAS oncogene family
FGF1	cell cycle/mets	The heparin-binding fibroblast growth factors play important roles in the regulation of cell survival, cell division, angiogenesis, cell differentiation and cell migration.
PDZD2	cell cycle and immune activation	Up-regulated in primary prostate tumors and may be involved in the early stages of prostate tumourigenesis.
BCL2L12	anti/pro apoptosis	BCL2 family members form hetero- or homodimers BCL family act as anti or pro-apoptotic regulators

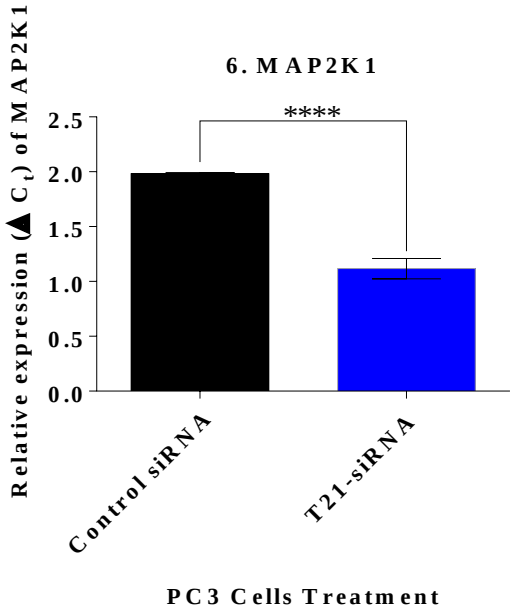
Table 4.2.2. 2 List of selected genes classified into different groups corresponding to their function within the cell.
Gene selection was based on a minimal 2 fold-change (FC) of expression to control and published literature indicating the role of the gene specific to cancer or its involvement in processes known to be altered in cancerous cells.

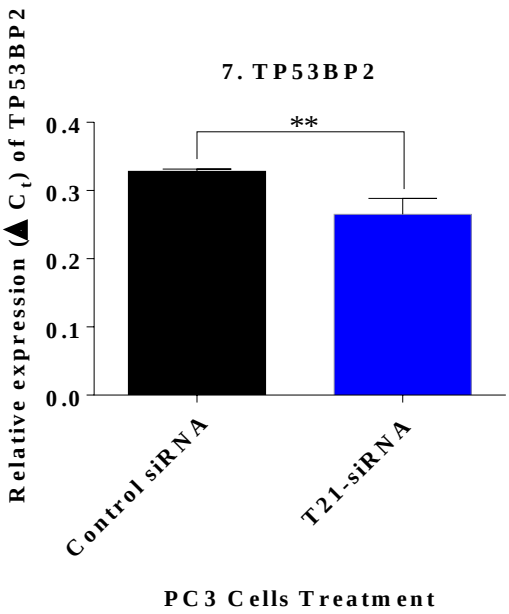
To confirm the results obtained by NGS, specific primers for the selected genes which were found down-regulated and up regulated genes were designed and optimised (list of primers -

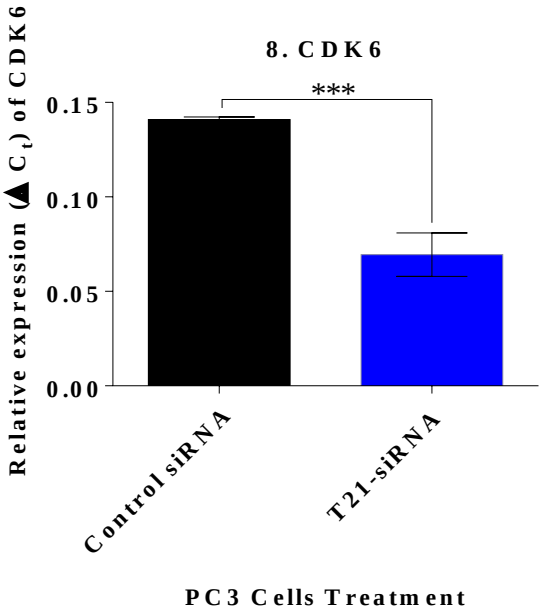
Chapter 2) for qRT-PCR and using the PC3 prostate cancer cell line mRNA treated with siRNA (T21 vs control). The mRNAs were obtained from the treated cells after knockdown 24 to 48 hours post-transfection of siRNAs. As a control, relative expressions of these genes were normalised to the House Keeping Genes HKGs (TBP-1 and HPRT-1). The results obtained by qRT-PCR confirmed and validated the results obtained by NGS for the selected down-regulated and up-regulated genes (Figure 4.2.2.3 and Figure 4.2.2.4).




5. BCL10
*
Relative expression (◭ Ct) of BCL10
2.5
2.0
1.5
1.0
0.5
0.0
Control siRNA
T21-siRNA
PC3 Cells Treatment


6. MAP2K1
****
Relative expression (◭ Ct) of MAP2K1
2.5
2.0
1.5
1.0
0.5
0.0
Control siRNA
T21-siRNA
PC3 Cells Treatment


7. TP53BP2
**
Relative expression (◭ Ct) of TP53BP2
0.4
0.3
0.2
0.1
0.0
Control siRNA
T21-siRNA
PC3 Cells Treatment


8. CDK6
***
Relative expression (◭ Ct) of CDK6
0.15
0.10
0.05
0.00
Control siRNA
T21-siRNA
PC3 Cells Treatment

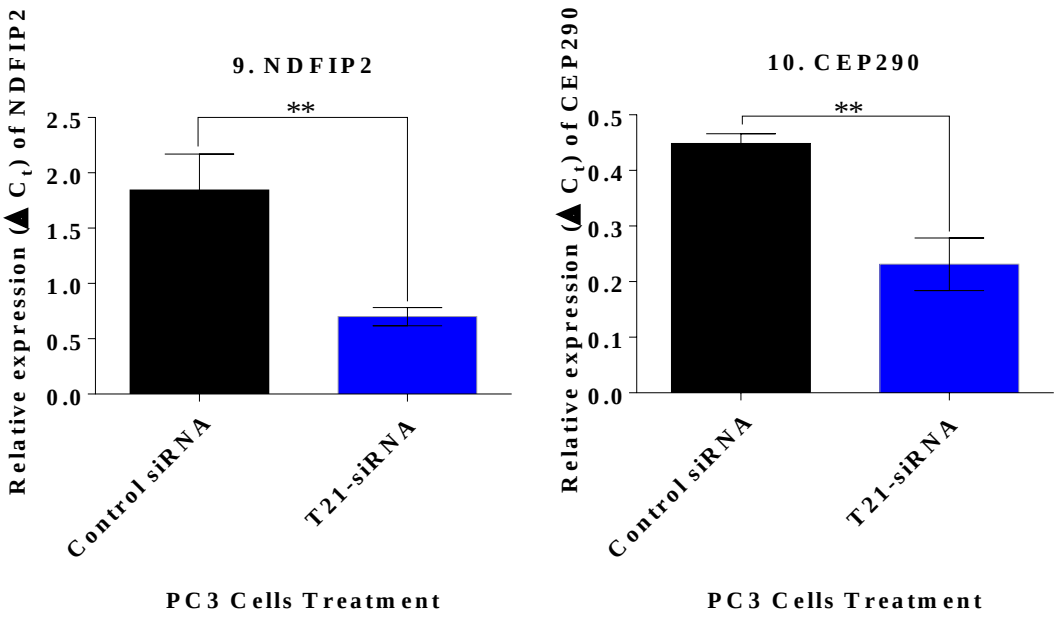
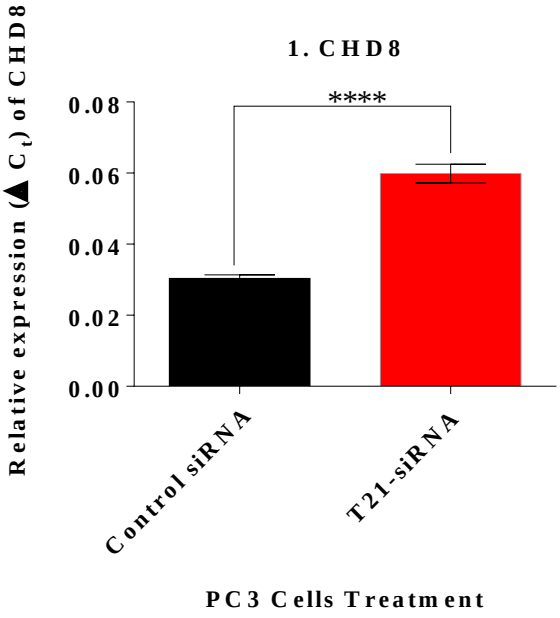
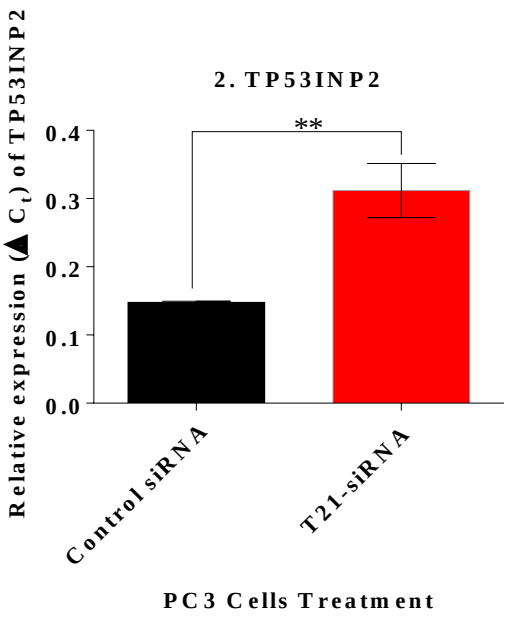
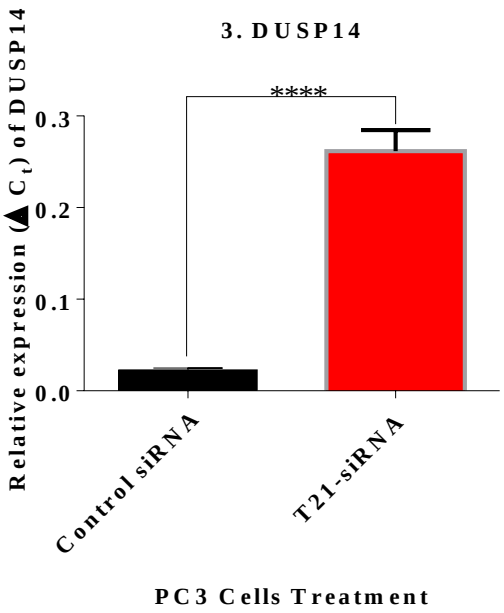
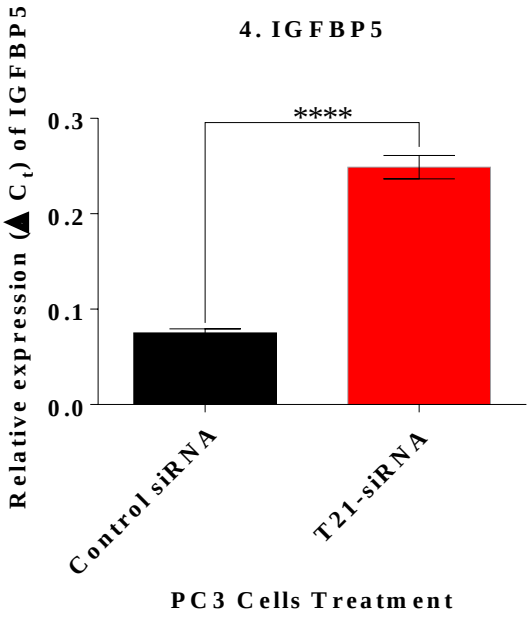


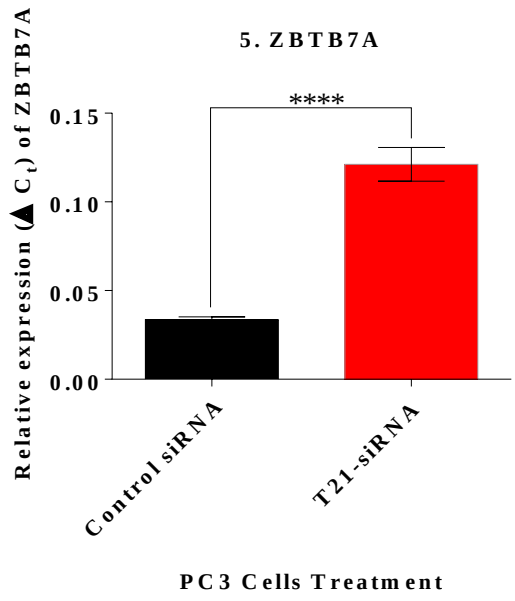
Figure 4.2.2. 3 qRT-PCR analysis of the down regulated genes, selected from NGS data. *These genes were down regulated as a result of T21 knockdown using siRNA in PC3 cell line. The relative expression (ΔCt) of each gene was normalised to House Keeping Genes (HKGs) TBP-1 and HPRT-1 primers. Statistical significance indicated (****p<0.0001, ***p<0.001, **p<0.01) was determined by Student's t test.*

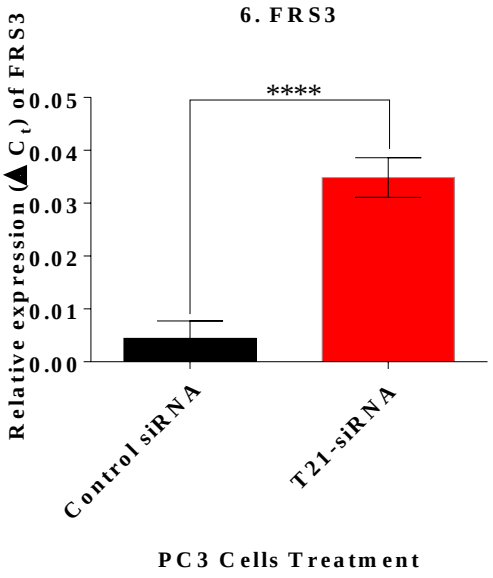

1. CHD8
****
0.08
0.06
0.04
0.02
0.00
Relative expression (▲ Ct) of CHD8
Control siRNA
T21-siRNA
PC3 Cells Treatment

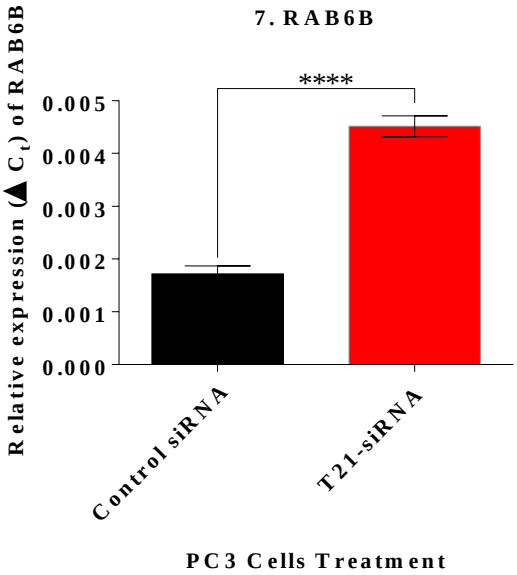

2. TP53INP2
**
0.4
0.3
0.2
0.1
0.0
Relative expression (▲ Ct) of TP53INP2
Control siRNA
T21-siRNA
PC3 Cells Treatment

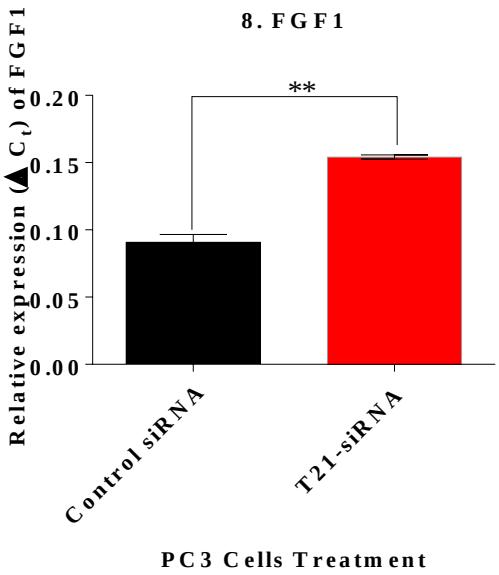

3. DUSP14
****
0.3
0.2
0.1
0.0
Relative expression (▲ Ct) of DUSP14
Control siRNA
T21-siRNA
PC3 Cells Treatment


4. IGFBP5
****
0.3
0.2
0.1
0.0
Relative expression (▲ Ct) of IGFBP5
Control siRNA
T21-siRNA
PC3 Cells Treatment


5. ZBTB7A
****
0.15
0.10
0.05
0.00
Relative expression (◭ Ct) of ZBTB7A
Control siRNA
T21-siRNA
PC3 Cells Treatment


6. FRS3
****
0.05
0.04
0.03
0.02
0.01
0.00
Relative expression (◭ Ct) of FRS3
Control siRNA
T21-siRNA
PC3 Cells Treatment


7. RAB6B
****
0.005
0.004
0.003
0.002
0.001
0.000
Relative expression (◭ Ct) of RAB6B
Control siRNA
T21-siRNA
PC3 Cells Treatment


8. FGF1
**
0.20
0.15
0.10
0.05
0.00
Relative expression (◭ Ct) of FGF1
Control siRNA
T21-siRNA
PC3 Cells Treatment

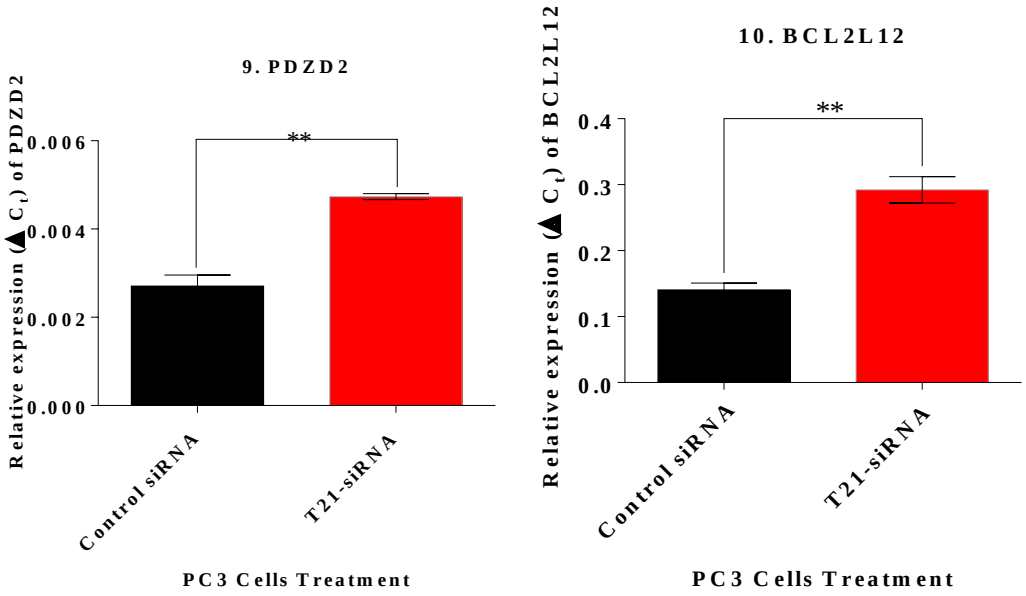


Figure 4.2.2. 4 qRT-PCR validations of the selected up-regulated genes, selected from NGS data.

*These genes were up-regulated following T21 siRNA transfection of PC3 cells. The relative expression (ΔCt) of each gene was performed by using qRT-PCR and normalised to House Keeping Genes (HKGs) TBP-1 and HPRT-1 primers. Statistical significance (****p<0.0001, ***p<0.001, **p<0.01) was determined by Student's t test.*

4.3 Discussion:

In this chapter experiments were performed using gene silencing techniques to determine the potential role of T21 in cancer progression. Firstly, three siRNA oligonucleotides designed within the unique region of T21 were synthesised and transfected into PC3 cells. Only one siRNA led to the knockdown of T21 (T21-siRNA3) achieving the most significant knockdown in expression of both mRNA and protein between 24 hour and 48 hours post-transfection. No noticeable changes occurred in cell morphology; however cells treated with T21 siRNA were seemingly hindered in their capacity to proliferate. Cellular proliferation assays using ^{3}H Thymidine incorporation confirmed this observation after 48 and 72 hours following T21-siRNA transfection. Furthermore, other knockdown experiments were performed using T21 specific shRNA transfection, which was employed to study the long term effects of T21 silencing on transfected cells and used for proteome profiler arrays in chapter 5. As the transient knockdown of T21 in PC3 cells using T21-siRNA was more reliable at this stage, it has been used to confirm the NGS data. The results indicated that several pathways important for cell proliferation and survival, centrosome and structural rearrangement and cell cycle control were affected. As an example, some MAPK pathways were clearly down-regulated upon T21mRNA knockdown such as MAPK6 (Mitogen Activated Protein Kinase 6) and MAP2K1 (Mitogen-Activated Protein Kinase Kinase 1). MAPK6 and MAP2K1 are members of the mitogen activated protein kinase (MAPK) signalling network which represents another molecular cascade that has been shown to be vastly modified in tumour cells. Much like the PI3K pathway, MAPK is involved in a series of events governing cell behaviour, ranging from proliferation through to differentiation, apoptosis, migration and cell transformation (Huang et al, 2004; Kim and Choi, 2010). Whereas PI3K is a single line of communication, MAPK consists of a series of different pathways that interact with one another to bring about their action (Qi et al., 2005).

The MAPK pathways essentially are used to link the extracellular signals to the machinery that control cellular processes such as growth, proliferation, differentiation, migration and apoptosis (Dhillon et al., 2007). The MAPK signalling abnormalities may affect most, if not all, of these processes and play a critical role in the development and progression of cancer. Selected down regulated genes were chosen from NGS data that showed significant fold-changes by using bioinformatics tools for modeling the MAPK pathways. MAPK6, mitogen-activated protein kinase 6, for instance, represented as Ser/Thr protein kinase which appears

to have a role in promoting the cell cycle. MAP2K1, in addition, as a member of MAPK family has a role in activation of the ERK pathway through ERK1 and ERK2 MAP kinases. The ERK pathway is deregulated in approximatly one third of all human cancers (Dhillon et al., 2007). Briefly, ERK1 and ERK2 are activated due to phosphorylation by MEK1 and MEK2 which are themselves activated when phosphorylated by Raf-1, B-Raf and A-Raf . Numerous extracellular signals can activate ERK signalling and the corresponding pathways whereby growth factors and mitogens activate ERK signalling in particular are correlated to cancer (Downward, 2003). MAPK pathway modulation has also been reported to influence chemotherapeutic drugs and may enhance or decrease drug efficacies (Boldt et al., 2002).

RAS proteins, on the other hand, have a role in controlling signalling pathways as key regulators of several aspects of normal cell growth and malignant transformation. RAS can gain abnormalities due to activating mutations in the RAS genes in most human tumours as well as alterations within upstream or downstream signalling components. Thus, the RAS pathway can be targeted in order to inhibit tumour growth, survival and metastasis and has been considered for immunotherapeutic intervention (Downward, 2003). The RAP2A gene is down regulated, as shown by NGS as a consequence of T21 knockdown in PC3 cells and is a member of RAS oncogene family. It can be inferred therefore that T21 is involved in maintaining and/or enhancing the expression of genes important in cancer. Interestingly, other pathways involved in signalling transduction events were up-regulated following T21 downregulated. As an example, DUSP14 is a dual-specificity cysteine-based protein-tyrosine phosphatase that belongs to the DUSPs family of molecules involved in the de-phosphorylation of cellular kinases implicated in proliferative and survival transduction signalling such as ERK, JNK and p38 MAP kinases pathways (Patterson et al., 2009). IGFBP5 (insulin-like growth factor binding protein 5), is another pro-proliferation and pro-survival molecule involved in promoting the function of the Insulin Growth Factor IGF-1. IGFBP serves as a carrier for IGF-1 which prolongs its half-life in a cell and tissue specific manner and therefore enhances its pro-proliferation and pro-survival functions (Beattie et al., 2006). FRS3 (Fibroblast Growth Factor Receptor Substrate 3) acts as an adapter that links FGF and NGF receptors to downstream signalling pathways and may be responsible through this mechanism of the activation of the MAP kinase signalling pathways (Dixon et al., 2006).

In addition, it also observed an up-regulation of those genes that would inhibit the response to DNA damage and those that modulate immune responses. Conversely, genes involved in regulatory pathways were suppressed in the presence of T21 and included RAS family

members, cell stress/DNA repair pathways and the PI3K-Akt pathway. Although a full and through analysis is essential to elucidate pathways that may involve T21 activity, the data presented here indicate that T21 appears to be involved in promoting tumourogenesis and therefore could represent an attractive target for therapy.

It was observed that cyclin-dependent kinase 6 (CDK6) was down regulated following T21 knockdown; CDK6 is involved in cell cycle progression and differentiation (Grossel et al., 2006). Moreover, TP53BP2 which is an apoptosis- stimulating protein of tumour suppressor protein 53 (p53), was down regulated upon T21 silencing. The data presented here also indicates the up regulation of genes that are involved, for instance, in the promotion of tumourogenesis through cell proliferation and cell cycle control such as MAPK6, or overriding DNA damage pathways and promoting cell survival like HIPK2 occurs as a result of T21 silencing. The up regulation of genes such as CALM3 and RAB3IP may be indicative of a more specific role T21 plays in centrosomal and structural rearrangement during mitosis. Moreover, genes that were suppressed following T21 silencing play regulatory roles in tumour progression through regulation of RAS family (RAB6B, FRS3), cell stress/DNA repair pathways (CHD8) (Nishiyama et al., 2009) or genes that are involved in the Akt pathway. Although the molecular characterisations of NGS data were selected and successfully validated using qRT-PCR outcomes, further investigations at the protein level are required in order to confirm a functional role. Therefore, proteome profiler arrays were used to further address this issue (chapter 5).

CHAPTER V

EXPRESSION OF T21 AND ITS IMPACT ON CELL SIGNALLING PATHWAYS IN CANCER

5 CHAPTER FIVE: Expression of T21 and its Impact of Cell Signalling Pathways in Cancer

5.1 Introduction:

Cancer is a complex cellular disorder characterised by disruptions in signaling pathways that control cell homeostasis and functions. Cell behaviour is governed by complex cascades of molecular events that instruct the cell to react to external signals within the environment and according to their lineage and differentiation status. Therefore, alterations in normal cell signaling pathways will affect the function of other proteins, including transcription factors, which may alter the expression of targeted genes. An array of signaling networks include interactions by a diverse and extensive numbers of molecules and these networks might operate as a single cascade or multiple pathways to determine specific cellular activities.

Tumorigenesis requires deregulation of at least at least six cellular processes (Johnson et al., 1996) in order to acquire the capabilities of cell proliferation signal independency, apoptosis evasion, anti-growth signal insensitivity, unlimited replicative potential, the ability to invade and metastasis and to promote and maintain angiogenesis for nutrient supply (Hanahan and Weinberg, 2000). Moreover, it has been conclusively demonstrated that MAPK signaling abnormalities are involved in most if not all these processes, and play important roles in cancer development and progression (Downward, 2003; Wellbrock et al., 2004; Kolch, 2005; Bradham and McClay, 2006; Galabova-Kovacs et al., 2006; Kohno and Pouyssegur, 2006; Torii et al., 2006).

Mitogen-activated protein kinase (MAPK) pathways are activated by extracellular signals mediated by growth factors, cytokines, stress proteins or mitogens which subsequently modulate and control crucial cellular processes such as growth, proliferation, differentiation, migration and apoptosis (Dhillon et al, 2007). Signaling via the MAPK pathways involving multiple kinase phosphorylation events, as shown in Figure 5.1.1. Six fundamental groups of MAPKs have been identified to date in mammals; extracellular signal-regulated kinase (ERK)1/2, ERK3/ 4, ERK5, ERK7/8, Jun N-terminal kinase (JNK)1/2/3 and the p38 isoforms α/β/ϒ (ERK6)/δ (Schaeffer and Weber, 1999; Chen et al., 2001; Kyriakis and Avruch, 2001; Krens et al., 2006).

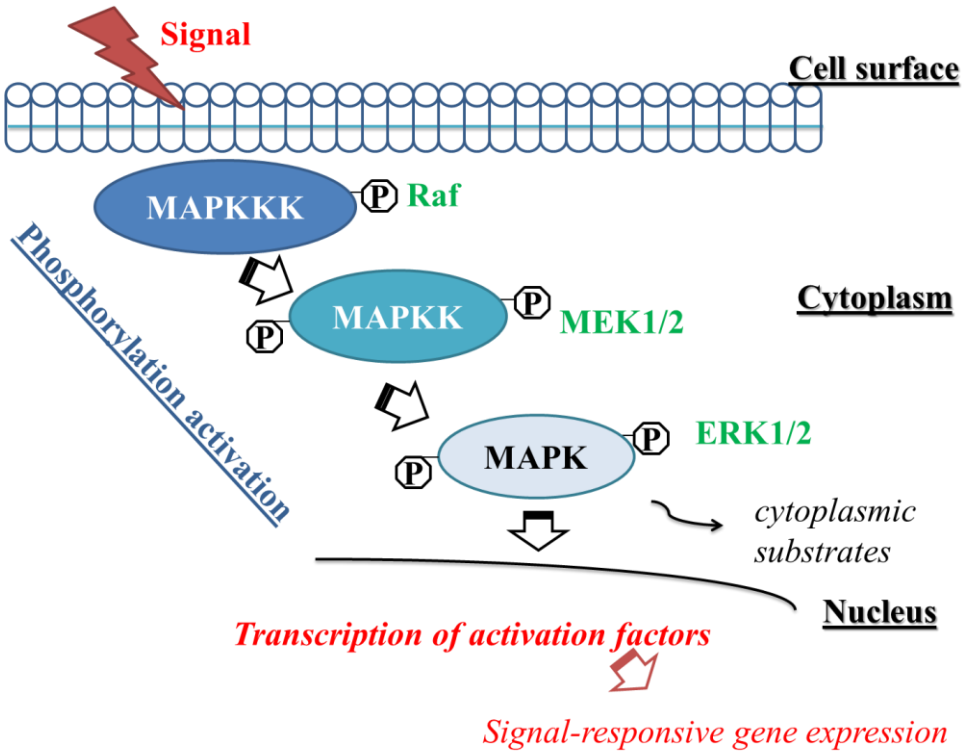


Figure 5.1. 1 Schematic diagram of a MAPK cascade.
It represents phosphorylation of MAP kinase kinase kinase (MAPKKK) which in turn phosphorylates MAP kinase kinase (MAPKK), which then phosphorylated MAP kinase (MAPK) (Adapted from Neill et al. 2001).

The PI3k (Phosphoinositide 3-kinase) signaling pathway is dominant major signaling cascade that acts downstream of growth factor receptor tyrosine kinases (RTKs) (Cantley, 2002). PI3K plays a role in regulating the lipid second messenger phosphatidylinositol-3, 4, 5-triphosphate (PIP3) production at the cell membrane. Consequently, PIP3 leads to the activation of a broad range of downstream targets such as the activation of the serine-threonine protein kinase Akt. The PI3K-Akt signaling pathway of PI3K-Akt has a role in conducting and regulating various cellular processes including, survival, cell growth and apoptosis that are altered during tumourigenesis. The involvement of PI3K/Akt pathway in oncogenesis has been extensively investigated and has become a prime target for cancer therapies (Vivanco and Sawyers, 2002; Chang et al, 2005). Moreover, Akt is a primary mediator of PI3K-initiated signaling and has a number of critical downstream substrates such as the cAMP-response-element-binding protein (CREB) signaling which has been reported to be associated with malignant transformation (Son J et al, 2010). CREB is activated as a result

of Akt phosphorylation and leads to glycogen synthase kinase-3 (GSK-3) inactivation which promotes cell survival and cell cycle progression (Figure 5.1.2) (Nicholson and Anderson, 2001). GSK-3 phosphorylates several transcription factors such as c-Myc, c-Jun, and c-Myb and the translation factor eIF2B (Plyte et al., 1992; Welsh et al., 1996). The uncontrolled frequent signaling through the PI3K pathway can be due to PTEN loss as a result of chromosomal deletion (Blanco-Aparicio et al., 2007), which in turn affects phosphorylation leading to post-translational modifications that affect protein activity. Furthermore, PTEN loss also increases PIP3 production and modifies Akt activity (Maehama and Dixon, 1998; Chow and Baker, 2006) leading to an increase in cellular proliferation and gene transcription and inhibition of apoptosis by inhibiting the activity of other proteins, for example BAD (Datta et al, 1997).

The changes in cell signaling that occur in cancer is significantly influenced and determined by dominant and recessive genetic events, mediated by oncogenes and tumour suppressor genes respectively. Subsequently, proto-oncogenes and tumour suppressor gene are significantly modified in cancer, leading to the production of proteins with post-translational modifications. For example, RAS and B-RAF point mutations will lead to a significant increase in cellular proliferation via either the PI3K or MAPK pathways (Bos, 1989; Davies et al., 2002). In addition altered p53 activity/function, which occurs as a result of point mutation, will reduce its capability of mediating cell cycle arrest and apoptosis, thus further contributing to increase cell proliferation (Levine and Oren, 2009).

The data reported in the previous chapter highlighted the involvement of T21 in tumour cell proliferation. These studies also indicated that various genes involved in the regulation of various cellular processes are affected by T21 down regulation. To investigate further the effect of T21 on cell proliferation, this aspect of the research focused on the analysis of kinase pathways in a setting where T21 expression was knocked down by shRNA transfection.

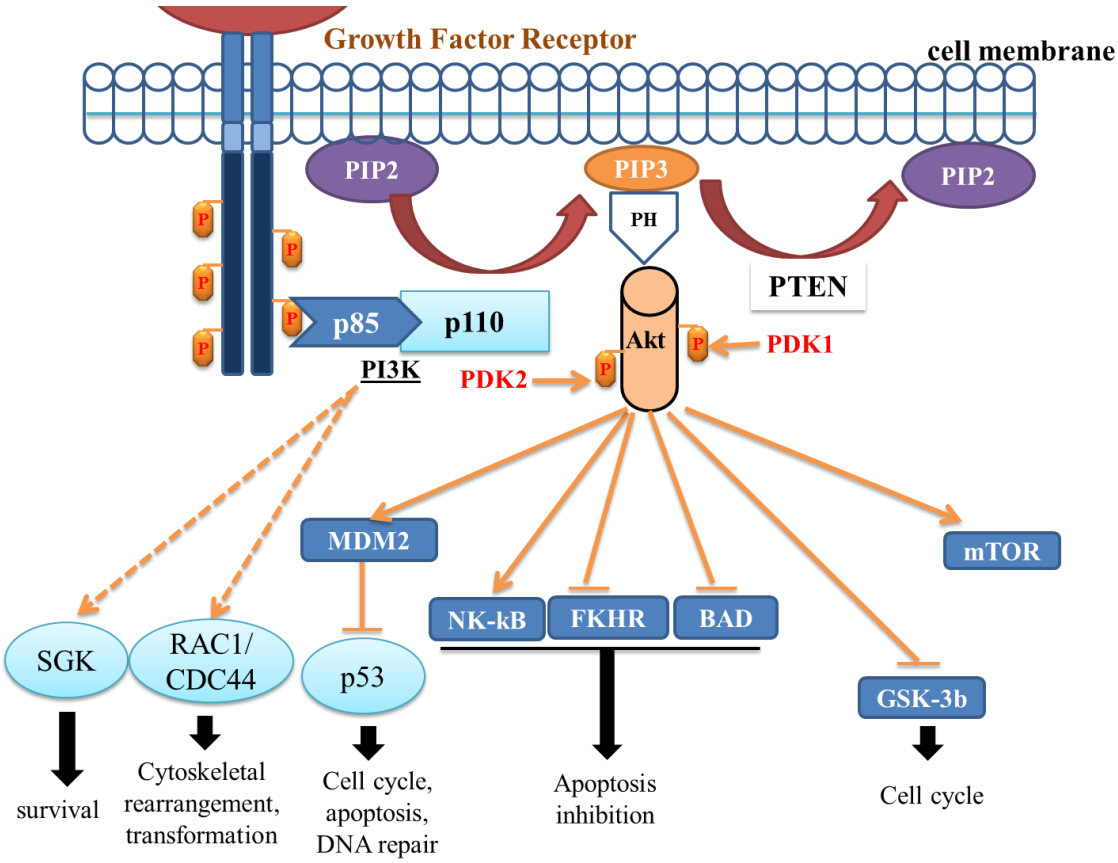


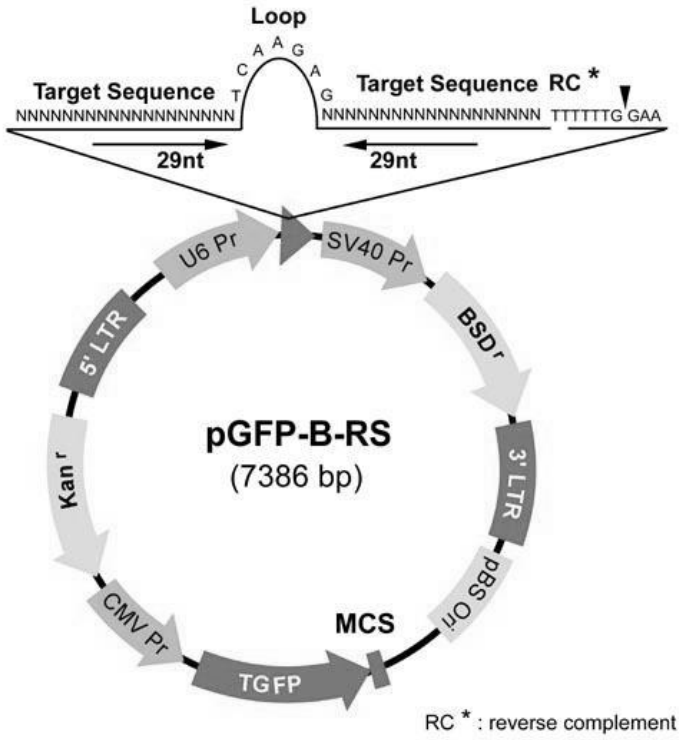
Figure 5.1. 2 a schematic representation of the PI3K/Akt/mTOR pathway.

(Adapted from Vara et al, 2004)

5.2 Results

5.2.1 Stable knockdown of T21 using short hairpin RNA (shRNA):

In order to study the effect of long term (stable) silencing of T21 on cellular proteins and their activity, short hairpin RNA (shRNA) for stable transfection was used. As shown in the previous chapter, T21 siRNA3 caused a significant knockdown of T21 in PC3 prostate cancer cells, therefore shRNA was customized to include this sequence; the pGFP-B-RS mammalian expression vector containing the T21-specific sequence (purchased from Origene Technologies) along with a scramble cassette control vector (Figure 5.2.1.1) were constructed. PC3 cells were transfected with either T21-shRNA or control vector as described in Chapter 2.



T21 unique region shRNA - Top strand sequence (5' – 3') (sense, loop and antisense)
5'-GCACAGGAATTCTTGATCA TCAAGAG
TGATCAAGAATTCCTGTGC-3'

Figure 5.2.1. 1 pGFP-B-RS mammalian expression vector (Origene Technologies).
Vector contains a Blastacidin resistance gene used to select following cell transfection as well as GFP reporter gene. A non-effective 29mer scrambled shRNA cassette in pGFP-B-RS vector was also purchased from Origene Technologies as a negative control.

On the following day of the transfection, the majority of PC3 cells expressed GFP (Green Fluorescence Protein) (Figure 5.2.1.2). Then, the transfected cells were grown in media supplemented with Blasticidin (selective antibiotic) over one passage before single colonies were achieved through limiting dilution assays. Although T21 protein expression was indeed decreased after shRNA transfection, the effects of the silencing were short-lived and T21 expression returned to that of the control cells after a number of passages (data not shown). Therefore, further refinement of the transfection methodology was undertaken, including the antibiotic concentration used, timing of fresh antibiotic supplements to the media and further cell sorting using flow cytometry and FACS sorting in an attempt to establish a more stably transfected cell line. In brief, following culture of clones positively expressing GFP, the expanded colonies that demonstrated the most stable significant knockdown compared to control cells were sorted for GFP positive expressing cells using a MoFlo XDP High-Speed Cell Sorter (Beckman Coulter) (method descripted in Chapter 2). The obtained clones were returned to culture. Once the cells were sufficient in number, mRNA was extracted and reverse transcribed to synthesise cDNA in order to assess for the expression of T21 using qRT-PCR. Further sorted clones were cultured to be used for investigating protein expression using immunoblotting technique.

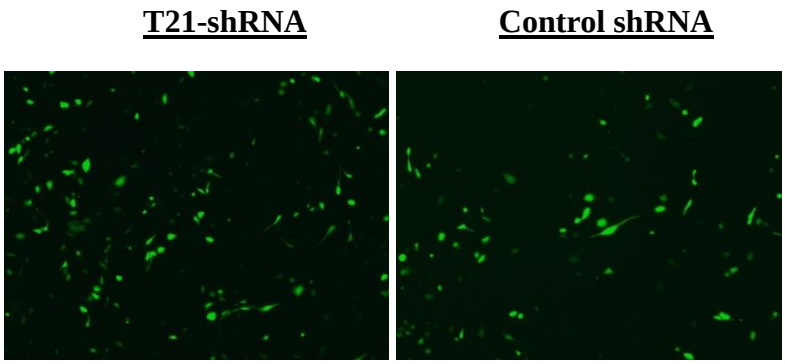


Figure 5.2.1. 2 Immunofluorescence images demonstrating GFP (Green Fluorescence Protein) expression in PC3 cells.
Immunofluorescence images of PC3 cells showing GFP expression one day following T21 specific shRNA transfection.

Following shRNA transfection, clones were selected for further sorting of GFP-positive cells and qRT-PCR was performed. The analysis of qRT-PCR showed a significant T21 knockdown following transfection of the PC3 cell line with pGFP-B-RS vector containing T21 shRNA (Figure 5.2.1.3).

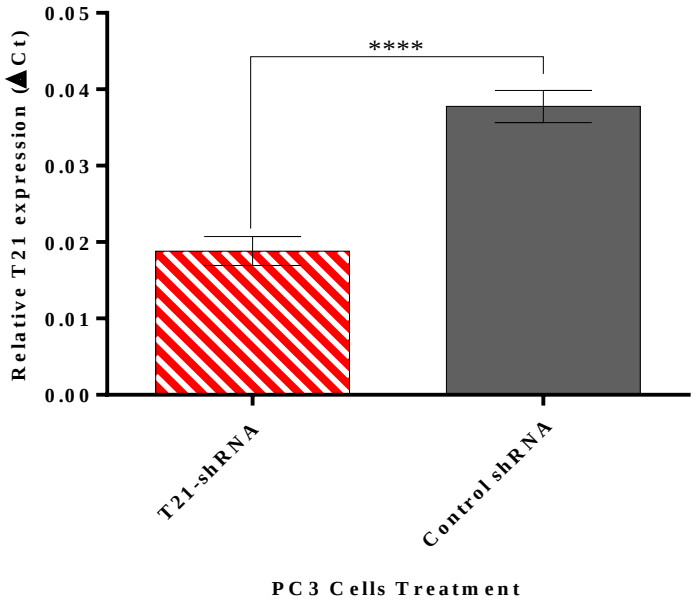


Figure 5.2.1. 3 qRT-PCR of T21 stable knockdown of the PC3 prostate cancer cell line following T21 specific shRNA transfection.
Experiments were carried out three times in triplicate (n=3). The results are given as the standard error of mean with relative expression of T21 shRNA in PC3 to the control shRNA.

In addition, T21 protein expression in PC3 cells lysates by western blotting following T21 specific shRNA transfection demonstrated a significant decrease compared to control shRNA. The protein expression in PC3 lysate treated with T21 shRNA showed no band detectable at approximately 57kDa, whereas this band was apparent in control shRNA treated cells (Figure 5.2.1.4).

These results confirmed the silencing of T21 protein expression following T21 shRNA silencing and these samples were subsequently used to investigate changes in protein kinase activity using proteome profiler arrays.

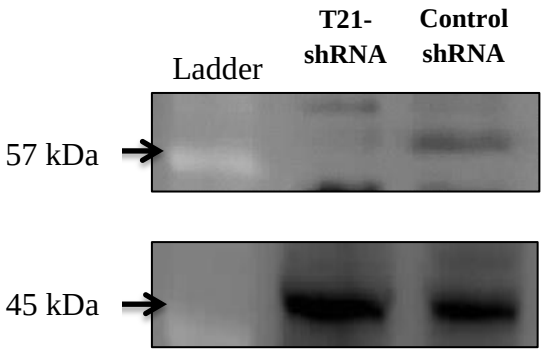


Figure 5.2.1. 4 Western blot analysis of T21 protein expression following T21 specific shRNA transfection.

Protein expression following culture of PC3 clones post T21-shRNA transfection compared to control cells. Above: T21 identified (57 kDa band). Below: β-actin (45 kDa Band), representative protein loading control (n=2).

5.2.2 The effect of stable T21 knockdown on MAPK proteome profiler

MAPK proteome arrays (R & D Systems) specific for the MAPK and PI3K pathways were used to determine the effect of T21 knockdown on their phosphorylation status. Therefore, the relative levels of phosphorylated forms of specific signaling proteins involved in these pathway cascades were investigated. Protein lysates were extracted from the T21 and control PC3 cells treated with shRNAs, diluted and mixed with a cocktail of biotinylated detection antibodies. The samples were then incubated with nitrocellulose membranes spotted in duplicate with antibodies against different kinases and their substrates (Table 5.2.2.1). Streptavidin-Horseradish Peroxidase and chemiluminescent detection reagents were added for signal detection. Representative images of the arrays are shown in Figure 5.2.2.2. Every two spots seen in the image represents a duplicate of phosphorylation status corresponding to kinases or kinase substrates. Both the positive and negative controls for each array are highlighted.in green and red respectively

(A)

Akt1	HSP27	p38 β
Akt2	JNK1	p38 δ
Akt3	JNK2	p38 ϒ
Akt pan	JNK3	p53
CREB	JNK pan	p70 S6Kinase
ERK1	MKK3	RSK1
ERK2	MKK6	RSK2
GSK-3 α/β	MSK2	TOR
GSK-3 β	p38 α	

(B)

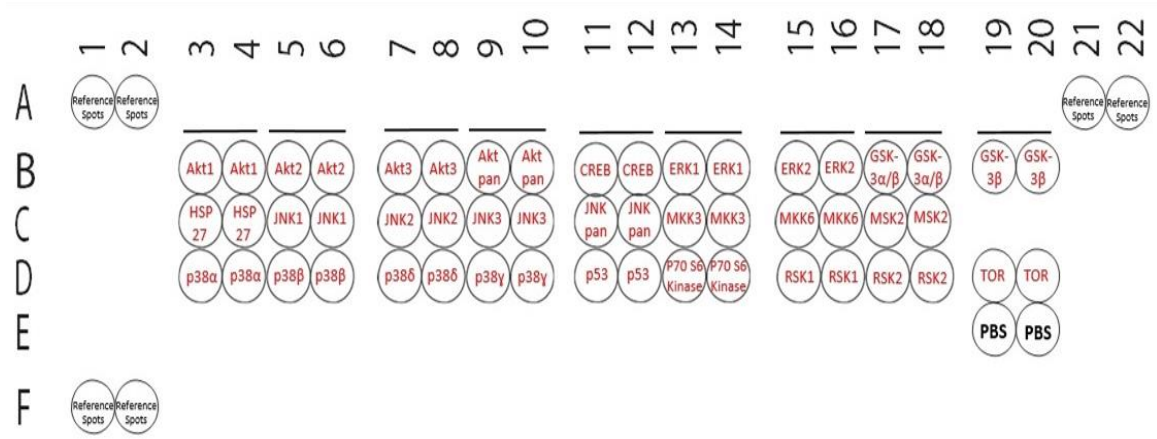
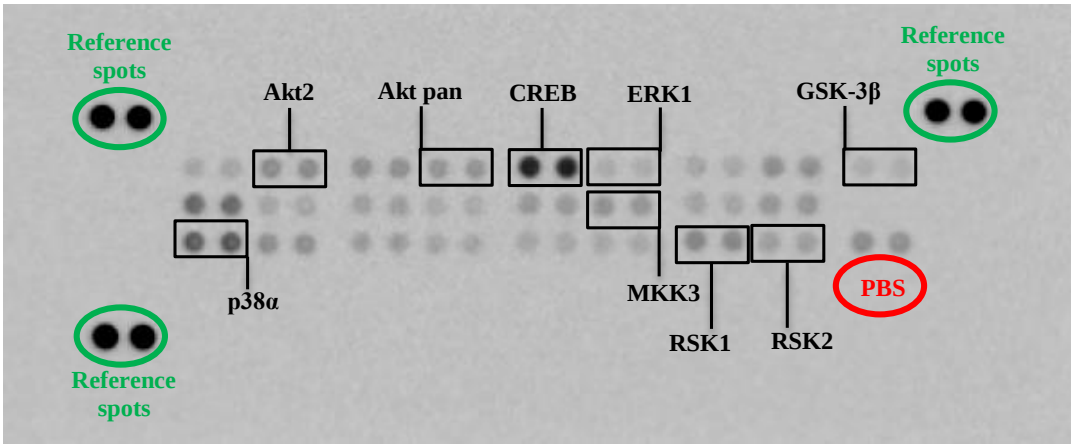


Table 5.2.2. 1 List of 26 antibodies included sequentially in the Human Phospho-MAPK Array.

(A) List of the antibodies. (B) Human Phospho-MAPK Proteome Profiler array coordinates in duplicate to detect the relative phosphorylation of these kinases in a single sample.

The data from the MAPK array showed various reductions in the duplicated spots of kinases and their substrate protein expressions. p38α and CREB in particular showed a recognised protein expression reduction in PC3 cells after T21 knocked down with shRNA. Moreover, RSK1 and RSK2 were reduced in their protein expression with T21 silencing compared to control shRNA treated cells. Further slight reductions were observed for Akt2, Akt pan, ERK1, MKK3 and GSK-3β upon T21 silencing.

Control shRNA



T21 shRNA

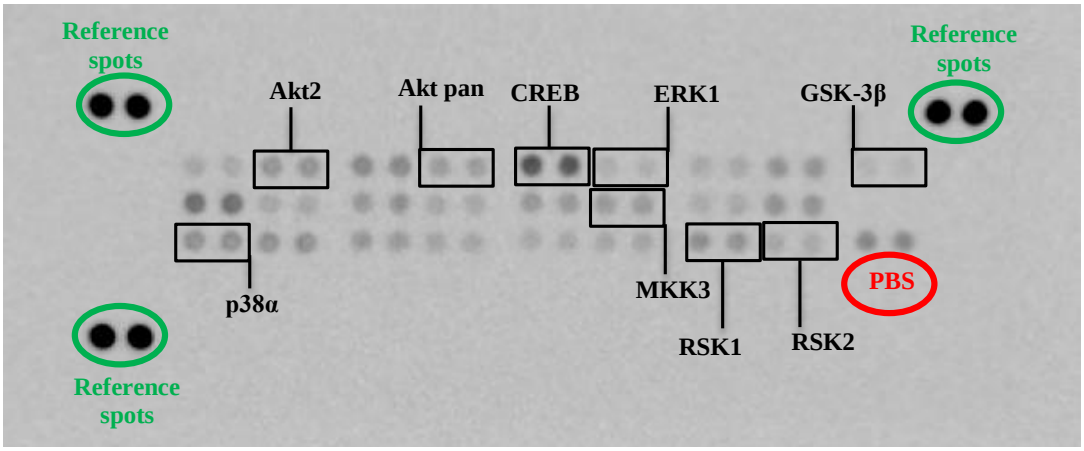


Figure 5.2.2. 1 Human Phospho-MAPK Proteome Profiler array images.
Protein expression obtained from Control shRNA and T21 shRNA cells lysates (n=2). Duplicate spots represent the capture region for a specific signaling protein and its repeat. Positive controls are highlighted in green (Reference spots) and negative controls in red (PBS).

The images were subjected to densitometry analysis to obtain accurate levels of protein phosphophorylation. The average of these readings is shown in Figure 5.2.2.2. As highlighted here, there appeared to be a difference in the levels of certain phospho-proteins known to be tumour associated. In particular, it showed that when T21 protein was reduced, there was a decrease in the levels of both Akt2 and GSK-3β proteins, both of which are involved in the PI3K signaling pathway. In comparison to ERK-1, there was no noticeable drop in the level of expression of phosphorylated ERK-2 under these conditions. For the former two proteins, this would suggest that when T21 is expressed by tumours, then there would be an increase in

the level of their phosphorylated form, suggesting possible post-translational modification resulting from T21 knockdown expression.

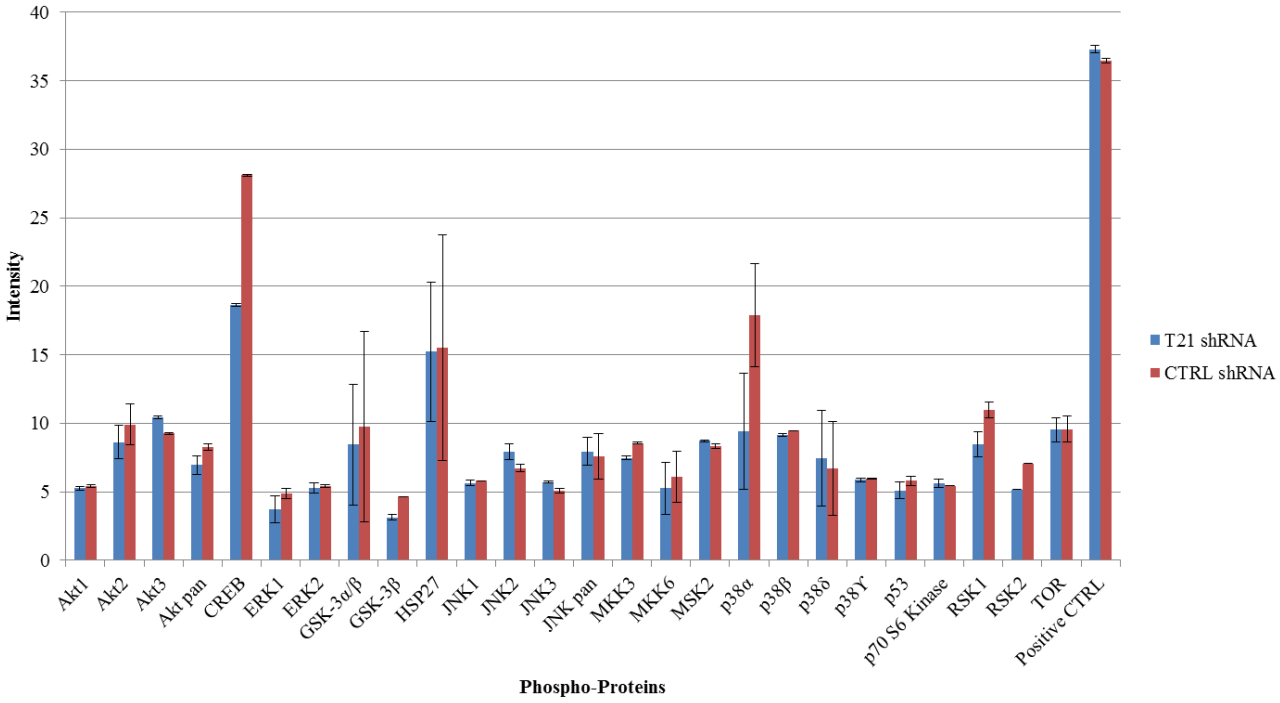


Figure 5.2.2. 2 Densitometry readings of the images from the Phospho-MAPK Arrays shown in Figure 5.2.2.1.
Duplicate spots represent the capture region for a specific signaling protein and its repeat versus positive controls (n=2).

As a result, there appeared to be a difference in the levels of certain phospho-proteins, as a consequence of T21 silencing. Specifically, it seemed that when T21 was decreased, there were decreases in the levels of Akt2, CREB, GSK-3β and RSK1 when using stable knockdown. In addition MSK2, ERK2 levels were decreased following transient T21 knockdown using siRNA. Conversely, it was noted that there was a slight in increase in the levels of Akt3, JNK2 and JNK3 when T21 shRNA were used and only PSK1 was slight increases as a result of transient knockdown under the same conditions (data of T21 siRNA knockdown on MAPK proteome profiler not shown).

5.3 Discussion:

As described at the beginning of the previous chapter, T21 siRNA 3 silenced T21 expression at the mRNA level in PC3 to a significant degree at 48 hours. Therefore, it was crucial to assess the ability of this assay whether T21 shRNA transfection could achieve silencing and also decrease T21 protein expression. shRNA was therefore customised using the pGFP-B-RS mammalian expression vector containing the T21-specific sequence (purchased from Origene Technologies) along with a scramble cassette control vector as (described in methodology section of chapter 2). PC3 cells were then treated with T21 shRNA and control shRNA and T21 expression levels confirmed by qRT-PCR technique to demonstrate T21 knockdown at the gene expression level. Subsequently, once T21 protein knocked down had been achieved, further analysis of the phosphorylation status of proteins associated with the MAPK pathway and its substrates was undertaken.

PC3 cells treated with T21 shRNA and control shRNA were lysed and western bolting was carried out. The protein expression of T21 showed successful silencing of a band at approximately 57kDa compared to the control shRNA, which was the molecular mass corresponding to the previously determined size of T21 previously obtained in chapter 3 using western bolting. As T21 was silenced and confirmed at the protein expression level, the lysed PC3 cells treated with T21 and control shRNA were applied to a MAPK proteome phospho-protein profiler array. These experiments were performed to determine the effect of T21 expression / knockdown on various proteins involved in cancer development and progression. The MAPK proteome profiler array was chosen because of the importance of these pathways in cancer and to confirm the results of NGS data analysis in chapter 4.

Both NGS and MAPK proteome profiler array data obtained in the present study and knowledge of their corresponding pathways has given new insight to our understanding the role of T21 in cancer. For instance, from the MAPK array data, increased phosphorylation of the PI3K effectors Akt and GSK-3 is links to enhanced cell proliferation. It was also shown that the impact of T21 expression on proteins involved in cell cycle progression, such as MAPK6 and FGF1, were validated by the NGS data. Collectively this infers that T21 represents a pivotal control over pathways that are intimately involved in cancer cell survival and growth, as demonstrated here by altered expression of members of the MAPK pathway.

MAPK array images demonstrated differing protein expression patterns for MAPK kinases. P38 α and CREB showed a significant lower protein expression following T21 knocked down compared to control shRNA. p38 MAPKs (α, β, γ, and δ) are members of the MAPK family which are activated due to a variety of environmental stresses and the presence of inflammatory cytokines. In MAPK cascades, the membrane-proximal component is a MAPKKK, typically a MEKK or a mixed lineage kinase (MLK). The MAPKKK phosphorylates and activates MKK3/6 and p38 MAPK kinase. MKK3/6 can also be activated directly by ASK1, which is stimulated by apoptotic stimuli. p38 MAPK is involved in regulation of HSP27, MAPKAPK-2 (MK2), MAPKAPK-3 (MK3), and several transcription factors including ATF-2, Stat1, the Max/ Myc complex, MEF-2, Elk-1, and indirectly CREB via activation of MSK1 (Figure 5.3.1) (Plotnikov et al., 2011; Huang etal., 2010; Cargnello and Roux, 2011).

In addition, CREB has a role in activating the transcription of target genes in response to a wide range of stimuli, including neurotransmitters, hormones, growth factors, synaptic activity, stressors and inflammatory cytokines. These stimuli activate a variety of intracellular signaling pathways, leading to activation of a number of protein kinases such as PKA, Ca2+/CaMKIV, and MAPK (figure 5.3.2) (Tardito et al., 2006).

These complex molecular networks operate to determine cell behaviour and to the best of my knowledge this is the first time that T21 has been ascribed a definitive role in cancer cell growth regulation. Thus, the application of gene silencing techniques has allowed its potential role in cancer to be better defined and suggests that T21 is potentially a main player in the cancer process.

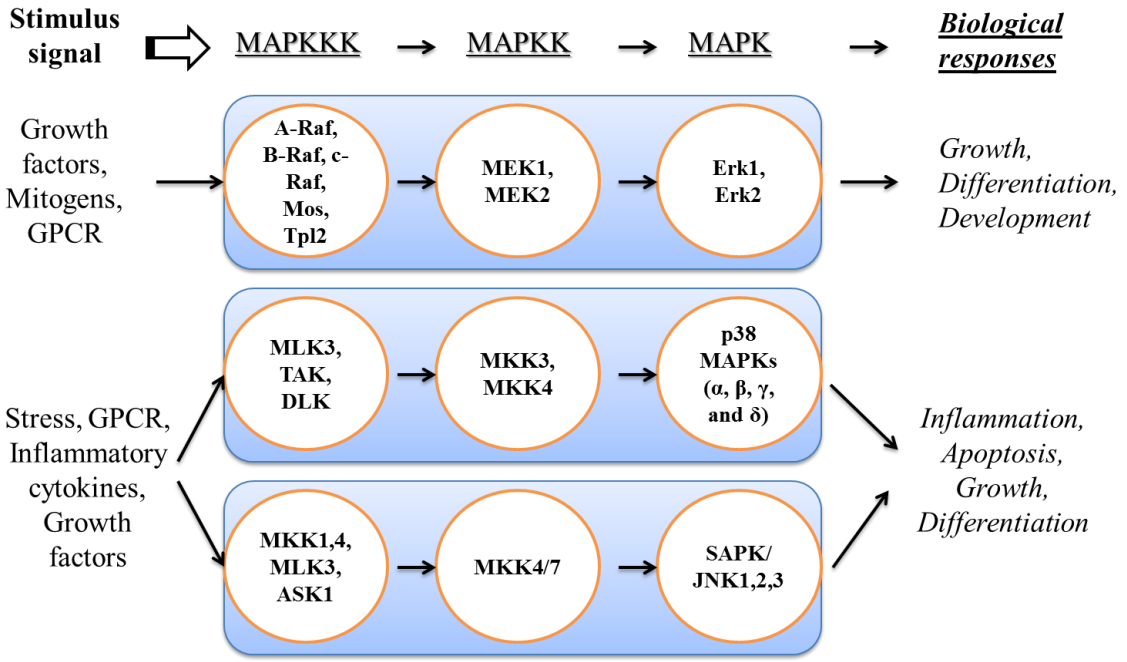


Figure 5.3. 1 The MAP kinase signaling cascades during phosphorylation of MAPKKK to MAPKK to MAPK.

In 2014, Barrantes and Nebreda reviewed recent progress in understanding how the p38 MAPK signalling pathway participates in the different steps of metastasis in order to understand the roles of p38 MAPKs in invasion and metastasis suggesting that tumour cells need to modulate p38 MAPK activity levels to successfully metastasise (Barrantes and Nebreda, 2014).

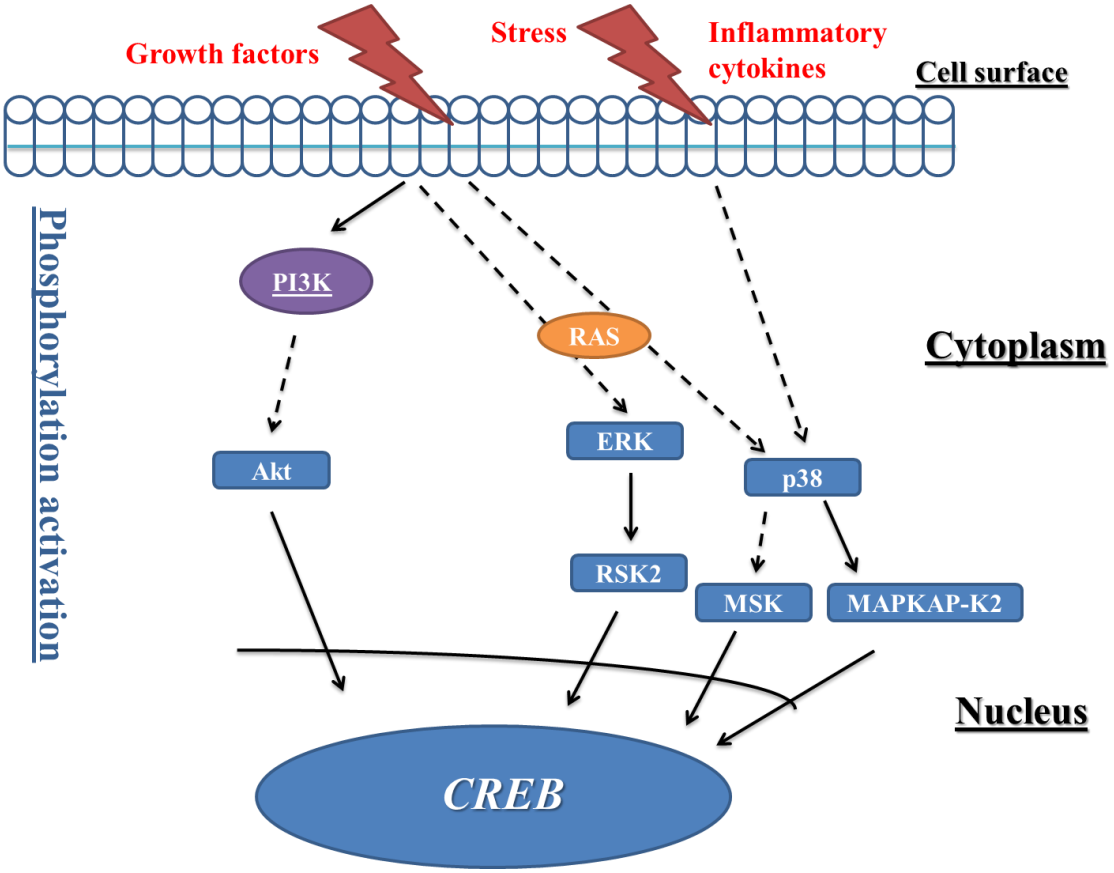


Figure 5.3. 2 Schematic representation of the signaling pathways modulating CREB activation.

(adapted from De Cesare et al. (1999)

CHAPTER VI

GENERAL DISCUSSION

6 CHAPTER SIX: General Discussion

6.1 Introduction and rationale for the study

T21 was identified as a prostate-associated tumour antigen using a modified SEREX expression cloning method and has been shown to elicit a humoral immune response in prostate cancer patients. It has also been shown to be over-expressed in malignant glands of prostate cancer compared to benign glands and stroma at the mRNA level. In addition, T21 expression has been associated with tumour stage and correlated with increasing Gleason grade and PSA recurrence (Miles et al. 2007, Miles et al. 2012). Previous studies suggested that T21 was a prostate cancer antigen of potential immunotherapeutic importance and therefore worth further characterisation and investigation. As T21 fundamentally shares remarkable similarity with the Centrosomal Protein CEP290, which has been implicated in several cilia related syndromic disorders such as Joubert syndrome, it was considered imperative to determine the differences and similarities between these two molecules and to facilitate further studies on the role of T21 in prostate cancer progression, especially at the molecular level.

The precise molecular function of this novel molecule (T21) in cancer was unknown and this constituted the basis of my thesis. The study focused on elucidating the role of T21 in the regulation of cellular pathways involved in prostate cancer. Following successful knockdown (silencing) of T21 mRNA and protein, gene expression profiling, by Next Generation Sequencing (NGS), made it possible to analyse the genome of human prostate cancer cells treated with interfering RNA. Genes that were either up or down regulated in the presence of T21 provided evidence that T21 may share functional activity and interact with these various genes.

To further investigate the role of T21 in the regulation of cellular pathways during prostate tumourigenesis, a proteome profiling approach was performed to investigate MAPKinase pathways. The mitogen activated protein kinase (MAPK) signaling network represents another cascade that are significantly modified in tumour cells. There are several MAPK pathways that are involved in a series of cell processes including proliferation, differentiation, apoptosis, migration and cell transformation. They consist of different pathways that interact with one another to bring about their action. What is clear from the results of this present

study is that T21 does indeed affect the expression of a wide variety of genes, including many associated with MAPK signaling and is therefore likely to play a central role in the cancer process.

6.2 T21 and CEP290 mRNAs and protein expression in cancer cell lines:

The recognition that the centrosomal protein CEP290 has a significant degree of homology to T21 has raised important questions with regards to its molecular and protein expression in prostate cancer cells. In order to address these questions, CEP290 was assessed alongside T21 to understand the relationship between the two molecules at their molecular level, including protein expression. The qRT-PCR data specific for CEP290 and T21 showed different expression profiles using newly designed, specific primers. Furthermore, CEP290 silencing was performed to investigate its effect on T21 expression which was assessed using qRT-PCR and to determine the relationship between CEP290 and T21. This approach led to the investigation of CEP290 knockdown on prostate cancer cell proliferation.

Various cancer cell lines (including prostate cancer cell lines) were screened using CEP290 and T21 specifically designed primers. The results illustrated different molecular characteristic between them. CEP290 and T21 are two different products that are expressed differently at the mRNA level. The successful development of RNA interference techniques meant that functional assays on prostate cell lines could be performed. The assessment of CEP290 knockdown and its effect on T21 in prostate cancer using siRNA revealed interesting data. CEP290 knockdown using specific siRNAs showed no significant effects on T21 expression (qRT-PCR data in chapter 3). This approach was also used to assess the impact of CEP290 transient knockdown on cell proliferation. Following CEP290 silencing in PC3 and DU145, apoptotic cells were observed over time (24, 48 and 72 hours) in culture (data not shown) and proliferation of ^{3}H thymidine incorporation seems to be slightly reduced in PC3 cells upon CEP290 silencing, compared to the siRNA controls.

The results obtained above were further confirmed using a T21 antibody which recognizes specifically the unique (exon) region of T21. By immunoblotting, this antibody recognised a unique band of approximately 55kDa when using LNCaP cell lysates and a band at 57kDa in PC3 and DU145 lysates. This difference in the molecular weights is most likely due to post-translational modifications that tend to positively charge the protein hence a difference in the protein molecular weight (e.g. Phosphorylation, sumoylation). Interestingly, immunoblotting with CEP290 antibody using these cell extracts showed a different expression pattern which confirmed that T21 is a separate protein product from CEP290 protein. This was one of the main aims of the study and so gave credence to further investigations on the function of T21.

Taken together, the mRNA and protein expression data shown here, depicts T21 as a different product from CEP290 and strongly argues the case for T21 involvement in promoting tumour proliferation. Other published evidence has provided preliminary data showing that T21 and CEP290 are differentially expressed, suggesting that the T21 unique region antibody compared to the CEP290 cross –reacting antibody can distinguish between the two proteins (Miles et al. 2012). Alternative splicing is a common post transcriptional process that can be used to generate multiple transcript variants from a single gene and can play an important role in cellular development, differentiation and neoplasia (Black, 2003). Thus, this is the likely explanation for the transcription of members of the CEP290 family of variant proteins, including T21. This aspect of study has enhanced our understanding of the expression and role of T21 in prostate cancer cell lines. T21 may therefore be considered as a target of interest that would be considered as a useful biomarker for immunotherapeutic applications when increased T21 protein may be detectable in serum or urine from prostate cancer patients (Miles et al., 2012). This has not been investigated and represents an obvious avenue for further study, using for example highly sensitive ESI TOF-TOF mass spectrometry to confirm the presence of T21 in as a secreted product in blood or urine.

6.3 Investigating the role of T21 in prostate cancer tumourigenesis:

Having confirmed that T21 is differently expressed at the RNA and protein level in prostate cancer cell lines, the next logical question was to attempt to address the functional significance of T21 in tumourigenesis. With regards to characterising antigens as potential markers or therapeutic targets, it was clear that T21 had promise and studies were therefore initiated to investigate its function using siRNA and shRNA silencing techniques. T21 siRNA 3 showed a significant ability to silence T21 in PC3 cells, as confirmed by qRT-PCR data analysis 48 hours following the transfection. Moreover, cellular proliferation, assayed using ^{3}H Thymidine incorporation, showed a reduction in cell proliferation after 48 and 72 hours following T21-siRNA silencing. This suggests a potential role for T21 in cancer proliferation. Therefore, two different approaches were utilised in order to understand more precisely the role of T21 in cancer cell signaling pathways: Next Generation Sequencing (NGS) and Proteome profiler arrays.

Next Generation Sequencing (NGS) data had been previously obtained as a result of T21 knockdown in PC3 cells. These data indicated that T21 functions by regulating several cellular pathways involved in tumourigenesis. The expression of a number of genes was affected by T21 silencing; specifically, T21 siRNA knock down significantly up regulated (197 genes) or down regulated (26 genes). Following a more focused examination of gene function and literature searching, a more refined shortlist of 20 genes that had roles in the processes involved in cancer were chosen to illustrate and confirm the general changes in mRNA expression and to extrapolate potential functional attributes of T21 and the role they might play in the cancer progression.

The data obtained from NGS following T21 knockdown experiment in PC3 prostate cancer cells was used for bioinformatics modelling of cellular pathway(s) that were altered following knockdown of T21. Several genes were categorised as up-regulated and down-regulated and were selected based on a significant fold change in expression. These genes were classified into different groups corresponding to their function within the cell (involvement in cell proliferation, apoptosis, cell adhesion, cell cycle and cell proliferation). The selection of a panel of genes to be included for further studies was based on a minimal 2 fold-change when compared to their expression in the control group. This selection also relied on published literature in the field of cancer where those genes were reported to be altered in cancer cells.

Functions of genes undergoing significant changes of expression were obtained using a variety of web tools such as OMIM or GeneCards. Subsequently, a list of up regulated and down regulated genes were selected according to their potential pathways and the NGS data was validated by using qRT-PCR and specific primers. Primers for up and down regulated genes were obtained and optimised, then, qRT-PCR was performed using PC3 prostate cancer cell mRNA generated under optimal conditions: treating with siRNA (T21 vs control) as a transient knockdown after 48 hours. The qRT-PCR results confirmed and validated the results obtained by NGS for selected up and down regulated genes, providing further evidence that T21 may not only share common protein functions with them, but also that T21 is a central player in regulating the expression of these genes.

The data indicated the up regulation of genes were involved, for instance, in the promotion of tumourigenesis through cell proliferation and cell cycle control such as MAPK6 (Hoeflich et al., 2006). Overriding DNA damage pathways and promoting cell survival, for example, through the tumor suppressor homeodomain-interacting protein kinase-2 (HIPK2) which is essentially a nuclear protein and is involved prominently within two fundamental cellular functions involving cell cycle/apoptosis regulation and transcription steering, is of significance and appears to involve, at least in part, the activity of T21 (Kondo et al., 2003; Wiggins et al., 2004). HIPK2 has also has been identified as an interacting protein with p53, that is phosphorylated by HIPK2 at Ser46 leading to activation of pro-apoptotic target genes (D'Orazi et al., 2002; Hofmann et al., 2002).

The up regulation of genes such as CALM3 may be indicative of a more specific role for T21 in centrosomal and structural rearrangement during mitosis. CALM3 has been previously detected within significant under-expression of genes in colorectal cancer, providing further evidence of its implicating in cancer development at this site (Mlakar et al., 2009). Moreover, genes that were suppressed play regulatory roles during tumour progression, through regulation of RAS family members (RAB6B, FRS3) or those genes that involved in MAPK and Akt pathways (Tomasovic et al, 2012). Cell stress/DNA repair pathways in the chromodomain helicase DNA-binding family member CHD8 can bind to p53 and suppress its transactivation activity by recruiting histone H1 during embryogenesis and has been shown to play a key role as a tumor suppressor in gastric cancer (Nishiyama et al. 2009; Sawada et al., 2013).

Therefore, the results from NGS data validation indicated an up regulation of some genes that promote tumourigenesis through various molecular networks, including cell proliferation and survival, centrosome and structural rearrangement and cell cycle control in addition to the up regulation of those genes that would inhibit the response to DNA damage and those that modulate immune responses. In contrast, genes involved in regulatory pathways were suppressed in the presence of T21 and included RAS family members, cell stress/DNA repair pathways and the PI3K-Akt pathway. Although a full and through analysis is essential to fully elucidate pathways that may involve T21 activity, the data presented here indicate that T21 appears to be involved in promoting tumourigenesis and therefore could represent an attractive target for therapy.

6.4 T21 role in Cell Signalling Pathways and Cancer:

Further knockdown experiments were performed using T21 specific shRNA transfection, which were used successfully to study what long term effects arise from silencing of T21 expression. Although the selected NGS data was successfully validated using qRT-PCR, further investigations at the protein level were performed in order to analyse potential signaling pathways and the effects of T21 knockdown in PC3 prostate cancer cells. Therefore, proteome profiler arrays were used for studying the protein expression and subsequently to analyse the interactions of T21 and their role in cancer progression. MAPK Proteome Profiler arrays were used in order to assess the effect of T21 knockdown on their phosphorylation status. Cells transfected in this manner represented the ideal way of further investigating T21.

Akt is a primary mediator of PI3K-initiated signaling and has a number of effective downstream substrates such as cAMP-response-element-binding protein (CREB) signaling which has been reported to be associated with malignant transformation (Son et al, 2010). The activation of CREB due to Akt phosphorylation leads to inactivation of glycogen synthase kinase-3 (GSK-3) that has a role in promoting cell survival and cell cycle progression (Nicholson and Anderson, 2001). T21 silencing by shRNA showed a noticeable, significant reduction in the expression of CREB compared to the control shRNA.

p38α MAPK, on the other hand, showed lower expression as a result of T21 knockdown and it has been approved previously that p38 MAPK signaling may be involved in various steps in metastasis and has a potential role in cancer invasion (Barrantes and Nebreda, 2014). The previous published findings suggested that p38α MAPK function may contribute to cell cycle defects and tumorigenesis. Also, p38α role was observed to induce terminal differentiation and inhibit the proliferation of rhabdomyosarcoma-derived cells which is one of the most common solid tumors of childhood, occurring as a consequence of defects in the differentiation of muscle precursor cells, which was attributed to deficiency in p38 MAPK activity (Puri et al., 2000). These potential pathways were altered as a result of T21 silencing as shown by NGS data analysis and proteome MAPK array data. This suggests a line of investigation that would allow us to further understand the actual role of T21 and verify its possible function as a tumour antigen.

6.5 Conclusion and Future Work

Immunotherapy provides a real promise for developing new therapies to target cancer and the identification of Tumour Associated Antigens (TAAs) is crucial in developing these targeted therapies. TAAs expression patterns are predominantly confined to tumours and healthy testis and placenta and would be re-expressed during cancer transformation to carry out specific roles in malignant cells. T21 as a tumour antigen and as a potential immunotherapeutic was identified by a modified SEREX technique and classified as Cancer-Testis Antigens (CTAs). Represents a promising means to be used as a potential immunotherapeutic target. Very little information is available about the role of CTAs in cancer cells. The present study was undertaken to increase our knowledge on T21 in cancer and more specifically in order to assess and understand its role in cancer development and progression.

From the data presented here, the evidence suggests that T21 is in fact a transcript variant of the centrosomal gene CEP290. T21 mRNA and protein was shown to be differentially expressed using designed primers targeting the T21 "unique region" sequence when compared to CEP290. Data collected also using immunofluorescence indicated that T21 was not localised to the centrosome, suggesting further that T21 activity is independent of that of CEP290. Although assessment of cell proliferation following T21 gene knockdown demonstrated an association between T21 and increased tumour cell proliferation, further investigations were required to determine the potential pathways that are associated with this process. T21 gene silencing studies have shown the possible involvement of this antigen in processes disrupted in cancer, in addition to its positive influence in cell proliferation.

The analysis and experimental verification of the NGS data by qRT-PCR described here strongly suggested a role for T21 in cell proliferation and survival, modulation of immune responses and suppression of regulatory processes involved in tumourigenesis. As a result, data collected and validated at this stage provided an argument to implicate T21 in a wider context and other aspects of malignant cell biology.

Further investigations were performed following T21 stable knockdown using shRNA and the use of proteome profiler arrays. The data obtained by use of a MAPK proteome profiler array showed various effects of T21 silencing on some MAPK proteins especially on p38α as well as CREB.

In summary, this study set out to characterise a SEREX-defined antigen T21 in order to ascertain its value as a diagnostic/prognositic marker through molecular expression analysis and functional studies and to further understand its role in the cancer process. A final objective was to determine its candidacy as a potential target for immunotherapy. The results presented here indicated that T21 is a CT antigen functionally involved in key signaling pathways important in cancer. Further studies should address in more detail the implications of these findings in the molecular signaling events that maintain and progress the malignant phenotype. Effective technology such as Genechip oligonucleotide microarray and mass spectrophotometry platforms could be used appropriately to confirm T21 as a target biomarker in accessible samples like blood and urine. Further confirmatory analysis of the MAPK and other pathways are suggested from the results obtained here and here is still the need to address the potential of T21 as an immune target for vaccine therapy. From the defined protein structure and given the identification of the unique (exon) stretch of amino acids, studies should be undertaken to define MHC restricted epitopes within this region and assess their ability to potentiate T cell mediated immunity together with the natural expression of immunogenic peptides associated with cell surface expressed MHC class I antigen.

CHAPTER VII

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7 REFERENCES

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Communications resulting from this study

National/ Regional Conferences Attended:

- The 6th Saudi Scientific International Conference (Brunel University in London, 2012)
- The 12th International Conference on Progress in Vaccination against Cancer "PIVAC-12" (Nottingham Trent University, 2012)
- Chemotherapy and the immune response in cancer (Royal Society of Medicine in London, 2012)
- School of Science and Technology 6th Research Conference (Nottingham Trent University, 2012)

Presentation:

- School of Science and Technology 6th Research Conference (Nottingham Trent University, 2012)

Posters Given:

- The 6th Saudi Scientific International Conference (Brunel University in London, 2012)
- School of Science and Technology 6th Research Conference (Nottingham Trent University, 2012)