MOLECULAR EXPRESSION AND IMMUNOTHERAPEUTIC POTENTIAL OF THE NOVEL TUMOUR ASSOCIATED ANTIGEN T21

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This thesis is dedicated to the memory of my Grandfather, Gurbachan Singh Dhillon

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ABBREVIATIONS

Ab	Antibody
ACT	Adoptive T Cell Therapy
ADCC	Antibody Dependent Cellular Cytotoxicity
Ag	Antigen
AIRE	Autoimmune Regulator protein
APC	Antigen-Presenting Cell
BLAST	Basic Local Alignment Search Tool
BM-DC	Bone Marrow Derived Dendritic Cell
BPH	Benign Prostatic Hyperplasia
^{51}Cr	Radioisotope Chromium 51
CARs	Chimeric Antigen Receptors
CEP290	Centrosomal Protein 290 kDa
CD	Cluster of Differentiation molecules
CDC	Complement Dependent Cytotoxicity
cDNA	Complementary Deoxyribonucleic Acid
CDS	Coding DNA Sequence
CLIP	Class II Associated Ii Peptide
CML	Chronic Myeloid Leukaemia
cpm	Counts per minute
CSC	Cancer Stem Cell
CT	Cancer/Testis antigen
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxicity T Lymphocyte Antigen 4
DAMPs	Damage Associated Molecular Patterns
DC	Dendritic Cell
DISC	Death Inducing Signalling Complex
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
ELISA	Enzyme-Linked Immunosorbent Assay
EliSpot	Enzyme-linked immunosorbent Spot assay
ER	Endoplasmic Reticulum
EST	Expressed Sequence Tag
FDA	Food and Drug Administration
GFP	Green Fluorescence Protein
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
^{3}H-thymidine	Titrated radioactive thymidine
HCC	Hepatocellular carcinoma
HEF	Hepatocyte Growth Factor
HIF-1α	Hypoxia Inducible Factor 1 alpha
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HPV	Human Papilloma Virus
HRP	Horseradish Peroxidise
HSP	Heat Shock Protein
IFA	Incomplete Freund's Adjuvant
IFNγ	Interferon gamma
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
IP	Immunoprecipitation
iROS	inducible Reactive Oxygen Species
LPS	Lipopolysaccharide

MØ	Macrophage
mAb	Monoclonal Antibody
MALDI TOF	Matrix-Assisted Laser Desorption/Ionisation Time of Flight
MDSC	Myeloid Derived Suppressor Cell
MHC	Major Histocompatibility Complex
MMP	Matrix Metalloproteases
mRNA	Messenger Ribonucleic Acid
NCBI	National Center of Biotechnology Information
NFκB	Nuclear Factor kappa beta
NGS	Next Generation Sequencing
NK	Natural Killer Cell
NKT	Natural Killer T Cell
OLP	Overlapping Long Peptide
PAMPs	Pathogen Associated Molecular Patterns
PBMC	Peripheral Blood Mononuclear Cell
PDGF	Platelet Derived Growth Factor
PIN	Prostatic Intraepithelial Neoplasia
Poly-IC	Polyinosinic-Polycytidylic Acid
PSA	Prostate Specific Antigen
qPCR	Semi-quantitative Polymerase Chain Reaction
RACE	Rapid Amplification of cDNA Ends
Rb	Retinoblastoma
RCC	Renal Cell Carcinoma
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RP	Recombinant Protein
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RT-Q-PCR	Real Time Quantitative Polymerase Chain Reaction
RT	Room Temperature
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SEB	Staphylococcal Enterotoxin B
SEREX	Serological Analysis of Recombinant cDNA Expression libraries
shRNA	short hairpin RNA
siRNA	small interfering RNA
T21	Testis clone 21
TAA	Tumour Associated Antigen
TAM	Tumour Associated Macrophage
TAP	Transporter Associated with Antigen Processing
TBI	Total Body Irradiation
TCR	T Cell Receptor
TGFβ	Transforming Growth Factor beta
TIL	Tumour Infiltrating Leukocyte
TLR	Toll-like Receptor
TNFα	Tumour Necrosis Factor alpha
TMA	Tumour Microarray
TME	Tumour Microenvironment
TRAIL	TNF Related Apoptosis Inducing Ligand
Treg	Regulatory T Cell
TSG	Tumour Suppressor Gene
T Th	T Helper Lymphocytes
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor
VitE	Vitamin E

ABSTRACT

The application of immunotherapeutic approaches to target tumour cells by harnessing the body's most important defence the immune system, could provide opportunities to oppose cancer. A prerequisite of tumour immunotherapy lies in the identification of antigens that are distinctively expressed in cancer and can elicit immune responses in the tumour-bearing host. Testis clone 21 (T21) was first identified as a promising prostate-associated tumour antigen identified using modified SEREX expression cloning showing restricted protein expression to normal testis and prostate, breast, kidney, ovary, melanoma, colon and stomach cancer among others. More recently, T21 was found to share significant sequence similarity with a centrosomal protein called CEP290. This study proposed to address the current understanding of T21 through analysis of gene sequence similarities observed with CEP290 and begin to extrapolate possible functional attributes of T21 and the role it may play in cancer progression. Furthermore this investigation aimed to evaluate T21 as a prospective prognostic indicator of prostate cancer and in addition, investigate T21 as a potential target for immunotherapy. *In silico* sequence analysis revealed that T21 contained a 93bp region, a consequence of an alternative splicing event resulting in the retention of a CEP290 intronic region making its translation to protein unique. This finding was supported by experimental observations using PCR and northern blotting. Using this T21 "unique region" sequence, T21 mRNA and protein was shown to be expressed in some normal tissues including prostate, stomach and spinal cord when compared to testis therefore T21 cannot be considered a cancer testis antigen as previously suggested. Evidence of T21 protein expression was shown in a number of cancer tissues, however a histological study demonstrated no significant discrimination between benign and prostate cancer patients. *In vitro* assessment of cell proliferation following *T21* gene knockdown indicated an association between T21 and increased tumour cell proliferation. Expression profiling of data obtained from Next Generation Sequencing (NGS) indicated T21 function was related to cell proliferation and survival, modulation of immune responses and suppression of regulatory processes involved in tumourigenesis. Collective evidences suggest T21 activity to be independent of that of CEP290. Finally, this study identified a novel immunogenic and naturally processed peptide target using a transgenic murine model, further verified using human cancer patient PBMCs. Future studies to determine the immunotherapeutic potential of T21 would focus on providing evidence of peptide-specific tumour killing targeting this novel antigenic sequence in the first instance.

CHAPTER 1

INTRODUCTION

1.1 Cancer

Cancer has remained one of the leading causes of morbidity and mortality worldwide. The latest global figures published by the World Health Organisation (WHO), state that an estimated one in eight deaths are attributed to cancer, second only to cardiovascular disease as the leading cause of death across the world. In 2008, there were an estimated 12.7 million new cancer cases with an estimated 7.6 million cancer-related deaths worldwide, making cancer responsible for more deaths than AIDS, tuberculosis and malaria combined. Current figures estimate that cancer is responsible for almost one in four (28%) of total deaths in the UK. Cancer Research UK statistics for 2010 reported a total of 157,275 deaths from cancer of which lung, colorectal, breast and prostate cancers accounting for almost half (46%). Decades of research towards earlier detection and improved treatments supported by public cancer awareness campaigns regarding risk factors and promoting healthier lifestyles have meant that cancer is no longer viewed as an inevitable death sentence. With projections for a burgeoning world population that continues to live longer than before have meant considerable investment to find more refined diagnostic aids and effective treatments to tackle the expected economical and social impact of cancer morbidities over the coming years.

1.1.1 Cancer is a multi-factorial disease

Cancer is a term broadly used to describe many diseases characterised by uncontrolled cell division arising from the sequential acquisition of key mutations in genes which enable the cell to break free of the regulatory control mechanisms that maintain homeostatic balance (Bertram, 2000). In normal cells, a stringent command over each cell proliferation, differentiation and programmed cell death event is achieved by the action of complex networks of molecular mechanisms responding to strict signalling instruction from other cells in their vicinity. This constantly dynamic interaction ensures that all tissues are proportionally and architecturally maintained in a manner suited to the body's requirements. Disturbances in these interactions which undermine the homeostatic balance are evident during the development of successful tumours through their defining features which are self-sufficiency in regard to growth signals and insensitivity to inhibitory ones, an infinite capacity for self-renewal, a resistance to apoptosis, sustained angiogenesis and invasion of tissue and metastasis. Four more recent concepts emerging as key to facilitating cancer are the tumours capability to reprogramme the cells energy metabolism, promote genomic stress, evade immune destruction and to promote inflammation, together these ten features typify the characteristic "hallmarks" of cancer illustrated in Figure 1.1 (Hanahan & Weinberg, 2011).

Once beyond the control of intrinsic and external control mechanisms, cancers can continually transform over many divisions accumulating further genetic mutations and gain novel functions that confer selective growth advantages over neighbouring normal cells. Crucially, tumours continue to survive and thrive due to their rapid clonal expansion acquiring random mutations and adapting to selective pressures of the surrounding microenvironment much in the fashion of Darwinian evolution (Bertram, 2000).

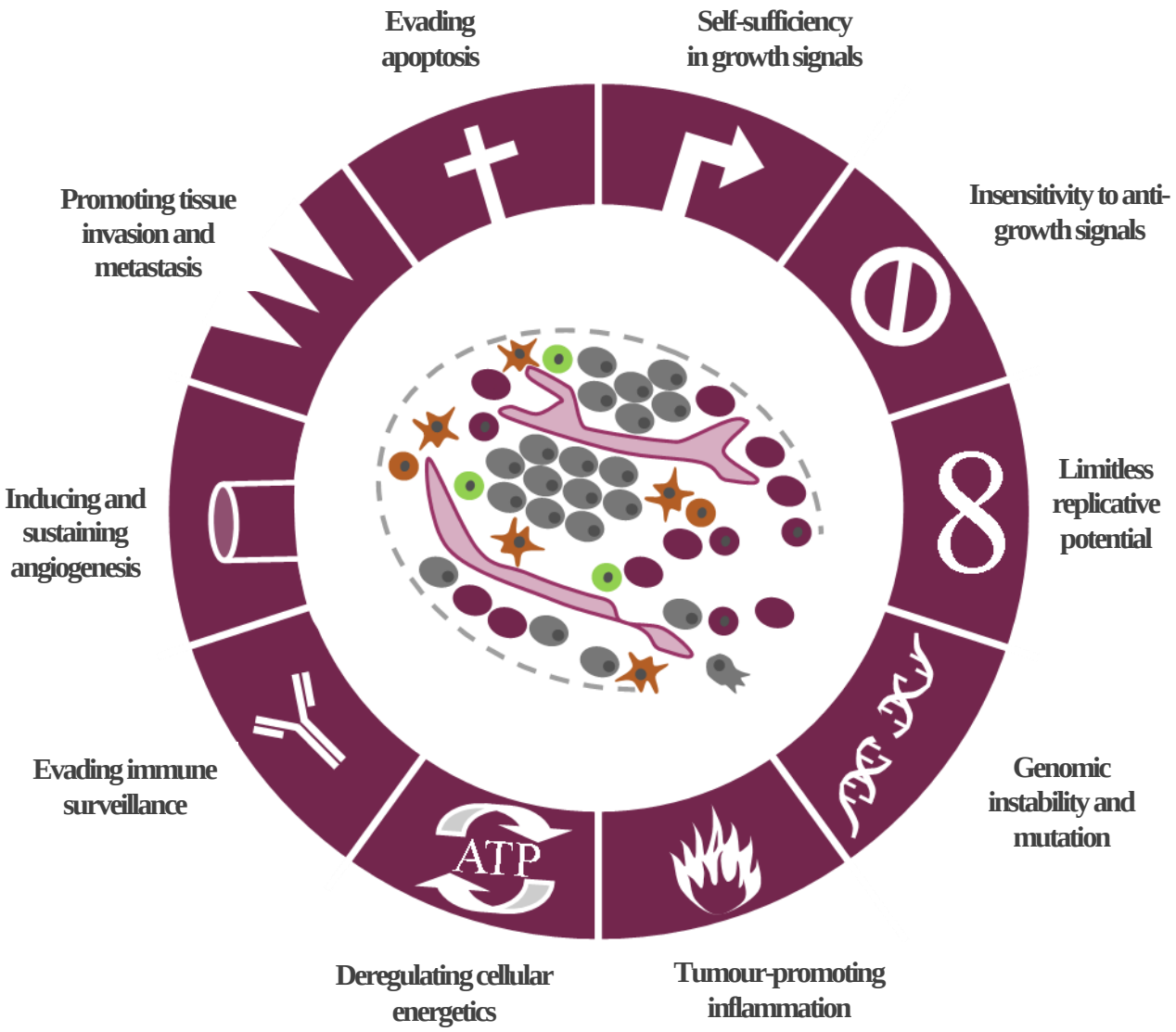


Figure 1.1 – The hallmarks of cancer. *(Adapted from Hanahan & Weinberg, 2011).*

It is thought that DNA could be exposed to tens of thousands events per day capable of causing damage as a consequence of normal metabolic processes and external environmental factors (carcinogens) (Fortini *et al.*, 2003; Lodish *et al.*, 2004). Continual or frequent exposure of DNA to carcinogens allow further mutations to accumulate, increasing the possibility of developing cancer, a notion supported by the observation between increasing incidences of most cancers and advancing age. The onset of tumour growth can be initiated by exposure to external causative agents that inflict damage to the cell genome which fall into the following categories of carcinogens:

Exposure to environmental carcinogens: Humans frequently encounter an abundance of initiating agents in their surrounding environment that are able to alter the physical

structure of genes, these agents originate from both chemical and physical sources. Chemical carcinogens encompass a wide range of agents including industrial chemicals associated with occupational cancers such as asbestos (lung cancer) and arsenic (skin and lung cancers), to more commonly known example, the by-products of tobacco smoke containing 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and benzo[a]pyrene (BaP), have been shown a causative agent to induce lung cancer (Hecht *et al.,* 1994). Prolonged exposure to physical carcinogens such as those that emit ionising radiation such as solar (UV) radiation and sources of radioactive decay (radon, radium and X-rays) have been shown to induce malignancy (Belpomme *et al.,* 2007).

Exposure to infectious pathogens: Oncogenic viruses are influencing factors in approximately 18% of neoplasia (Parkin, 2006). Two viral infections most frequently associated with cancer are human papillomavirus (HPV) which is strongly associated with cervical cancer and head and neck cancers and secondly, hepatitis B viruses (HBV) with hepatocellular carcinoma. Each of these viruses contributes differently to the initiation of the cell. HPV acts directly by inducing viral gene products E6 and E7 that inhibit the host cell signalling cascade of p53 and retinoblastoma (Rb) enhancing proliferation signals and inhibiting apoptotic signals (Song *et al.,* 1999). HBV on the other hand, indirectly induces chronic inflammation which consequently generates reactive oxygen species (ROS), a contributor to tumourigenesis (Jackson & Loeb, 2001). In addition to viruses, certain bacteria have been associated with cancer attributing to their induction of chronic inflammation, an example being *Helicobacter pylori* known to establish chronic ulceration in the stomach and has been linked to gastric cancer (Eslick *et al.,* 1999).

Epigenetic aberrations: Whilst mutations by factors previously mentioned are localised to the nucleotide sequence of genes, an aberration of processes involved in structural modifications that span whole chromatin can contribute to the transformation to cancer. Expression modifying events from which cancer arises include DNA methylation, covalent modification of histones and microRNA gene silencing often impacts on the activation of oncogenes and the silencing of tumour suppressor genes (discussed in sections 1.1.2.1 and 1.1.2.2). Unlike genetic changes, epigenetically altered gene expression can be restored and in some instances can be utilised for diagnostic and prognostic purposes. 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; Klimek *et al.,* 2008).

Pathways for DNA repair exist to counterbalance the onslaught of DNA damage in order to maintain the integrity of the genome. The inherent instability of DNA molecules themselves mean spontaneous DNA mutations can occur from within the cell by direct errors during replication, or indirectly following chemical damage most frequently due to ROS capable of inducing mutations in critical DNA stability genes (Jackson & Loeb, 2001). Cells are left highly vulnerable to further mutations evident in those carrying germline mutations in DNA repair genes having higher then normal genetic predisposition to cancer. The most well-known examples include defects in *BRCA* genes (Hall *et al.,* 1990) associated with an increased susceptibility to breast, ovarian, prostate, stomach and pancreas and colon cancer (Friedenson, 2005). Similarly, mutations in mismatch repair genes in hereditary non-polyposis colorectal cancer (Lui *et al.,* 1996).

Studies on common mechanisms between the processes involved in ageing and the initiation of cancer have implicated the decline in co-ordinated responses to oxidative stress and chronic inflammation as key elements toward cellular decline and with possible roles as mediators in tumourigenesis (Reuter *et al.,* 2010; Finkel *et al.,* 2007). Many of the carcinogens mentioned previously are termed as much due to their indirect induction of ROS creating an imbalance between oxidative stress and antioxidant defences in cells. Epidemiological studies correlating ethnic and geographical variations in the incidence and mortality across many cancers indicate toward the beneficial actions of certain dietary components. A number of dietary antioxidants, including β-carotene, lycopene and vitamins A, C and E have been shown to limit or prevent tumour growth in both *in vitro* and in animal studies; however large-scale epidemiology studies have proven far less conclusive. For example, several experimental studies demonstrated the individual anti-cancer actions of vitamin E and selenium in prostate cancer (Gunawardena *et al.,* 2000; Menter *et al.,* 2000). However, subsequent results in clinical trials such as the Selenium and Vitamin E Cancer Prevention Trial (SELECT) was stopped early due to the lack of efficacy for risk reduction (Lippman *et al.,* 2009) and the Physicians Health Study II (PHS II) also found no effect on the incidence of prostate cancer using combinations of vitamins E and C (Gaziano *et al.,* 2009) or vitamin E, selenium and soy protein on the progression

from high grade PIN (carcinoma *in situ*) to malignant disease (Fleshner *et al.*, 2011). Interestingly, data from a number of prospective studies supported the association of dietary supplements of vitamin E (Peters *et al.,* 2008) and intake of lycopene (Key *et al.*, 2007) reducing the risk of advanced prostate cancer among smokers. This difference could account for later trials designed to include screening for serum levels of prostate specific antigen (PSA), which would include biologically indolent cancers that would have gone undiagnosed in earlier investigation.

1.1.2 Genetic alteration: The underlying cause of cancer

Despite cells undergoing numerous divisions and under constant threat of potentially mutagenic events throughout life, the progression to cancer remains a relatively rare event. In normal cells, the process of cell division is tightly regulated by checkpoints during each of the four phases of the cell cycle (G_1, S, G_2 and M) ensuring that the genetic material is faithfully copied to each descendent cell. Following damage, these checkpoint controls either allow the cell passage through the cell cycle pending the repair of genetic damage or initiate the processes leading to programmed cell death (apoptosis) if the damage is too great. Central to maintaining tissue integrity is a turnover of viable cells byway of the retirement of those that have exhausted their replication limit (cellular senescence). This is a consequence of shortening of non-coding DNA sequences that cap chromosomes called telomeres which undergo a partial degradation following each round of genetic replication. This irreversible process serves to keep cellular proliferation in check. Tumour cells overcome these barriers to proliferation by disrupting control elements in the cell cycle leading to uncontrolled and unregulated cell division, jeopardising genomic integrity and inviting further mutations on each subsequent division. In this way, cancer is generally accepted to be clonal in nature expanded from a primary mutated cell which acquires an increasing number of mutations through an increasingly unstable genome (Stratton *et al.,* 2009). As illustrated in Figure 1.2, successful tumour cells balance subsequent proliferation and accumulative mutations to overcome barriers that restrict growth through gradual and increasing genomic instability without surpassing the threshold for cell viability (Cahill *et al.,* 1999).

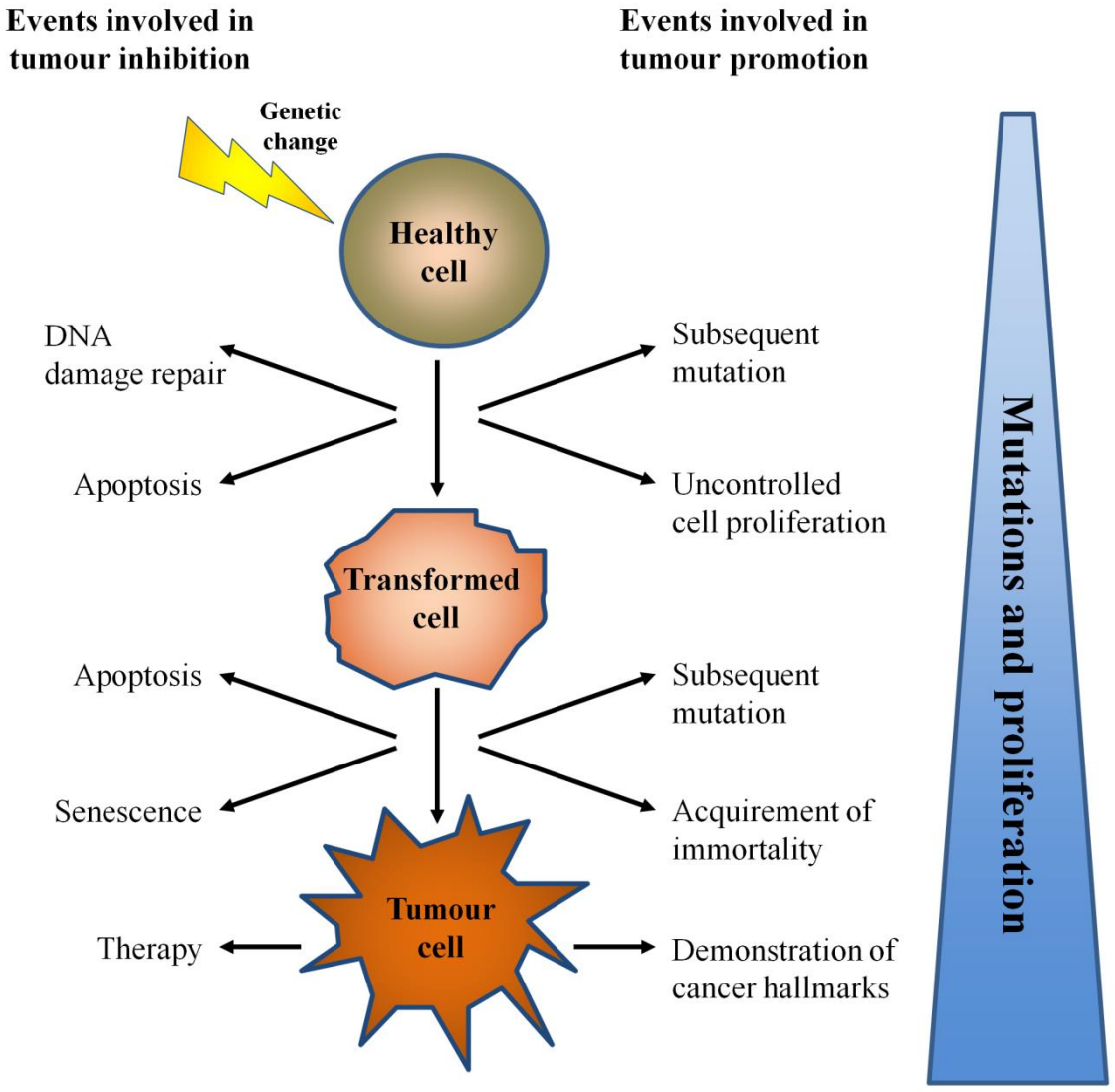


Figure 1.2 – The pathways to cancer. *An overview of the events involved during the progression of a cell to a malignant state. Cells accumulate mutations which must promote cell survival and overcome control mechanisms in place to eliminate the mutated cell. (Adapted from Bertram, 2000).*

For a cell to successfully reach the stage of exhibiting the classical hallmarks of cancer it must over time obtain mutations in at least five genes with each mutation enhancing the cells capability to adapt and grow in the host organism modelled in the development of colon carcinoma (Fearon & Vogelstein, 1990). Since this time, other solid-tumour cancers have been extensively scrutinised to identify early initiating molecular events involved in tumour progression. For example in prostate cancer, down regulation of *NKX3.1* homeobox gene through promoter methylation (Asatiani *et al.,* 2005) is an early initiation event that demonstrates roles in responses to oxidative stress and DNA damage (Ouyang

et al., 2005; Bowen & Gelmann, 2010). Somatic amplification of *MYC* oncogene has also been associated as a contributing factor to in the early onset of disease (Jenkins *et al.*, 1997) along with *TMPRSS2-ERG* fusions, a product of 21q chromosomal rearrangement resulting in a truncated ERG protein driven by androgen-responsive promotor elements of *TMPRSS2* (Tomlins *et al.*, 2005). The loss or reduction of cell cycle 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, up regulation of a histone-lysine N-methyltransferase encoded by *EZH2* gene due to loss of miR-101 has been frequently associated with late stage metastatic disease promoting cellular pathways such as Ras and NF-κB which are often deregulated in various cancers (Chiaradonna *et al.*, 2008; Sun & Xiao, 2003) (Figure 1.3). The development of prostate cancer is a predominantly slow and often asymptomatic process. Once symptoms are observed, the cancer usually signifies the onset of advanced stage disease and spread to distant organs (bone, lymph nodes, colorectal regions and brain).

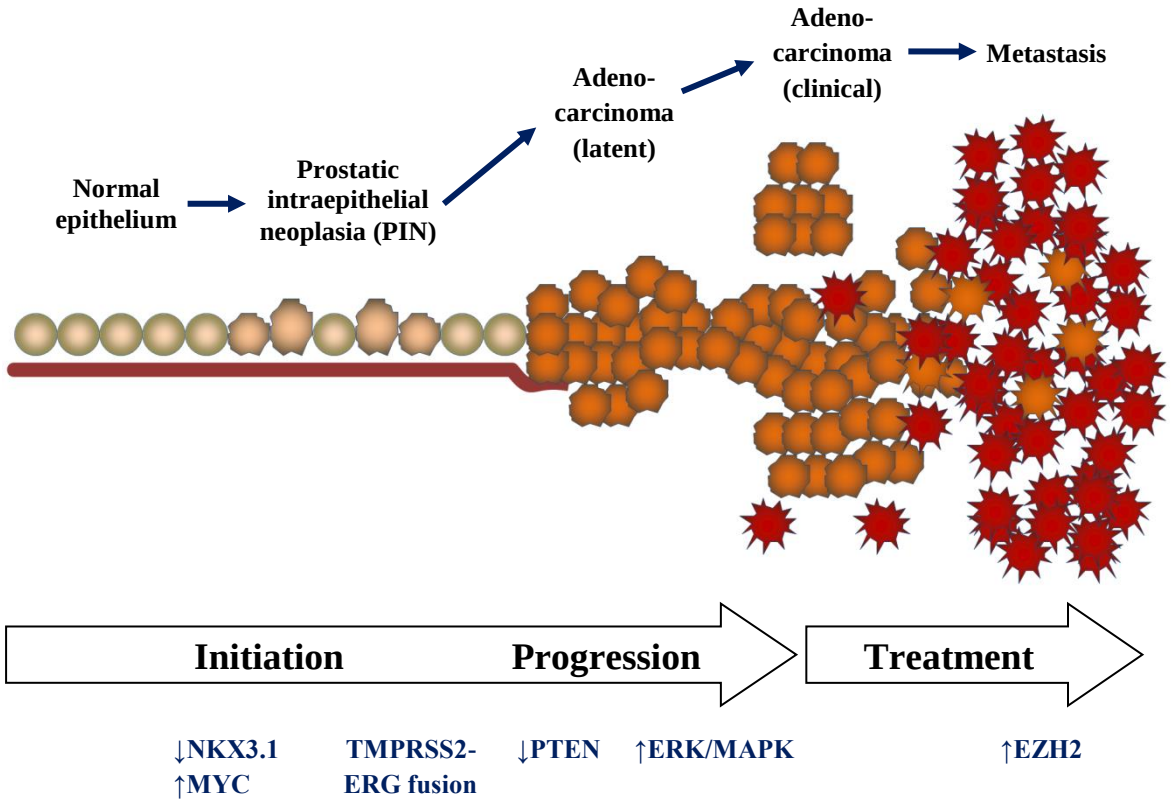


Figure 1.3 – The multistep progression of prostate cancer. *Commonly acquired gene mutations during the progression of disease from a high grade PIN to localised adenocarcinoma after breaching of the basal lamina, to progression into the lumen on the gland and the surrounding connective tissue. Finally, the progression to a malignant phenotype capable of distant metastases (Adapted from Abate-Shen & Shen, 2000).*

As alluded to previously, the progression of a healthy cell to a malignant phenotype exhibiting the cancerous hallmarks is essentially due to genetic mutations in cancer-related genes that aid initiation and stimulate growth (proto-oncogenes) and the silencing of those genes that inhibit cell proliferation and transformation (tumour suppressor genes).

1.1.2.1 The activation of proto-oncogenes

Oncogenes are derived from altered normal genes (proto-oncogenes) which encode for proteins that are involved in regulating growth and cellular differentiation in response from external signals. They are activated through interpretation of external growth signals resulting in changes in gene expression, and consequently, serve to promote cell proliferation, cytoskeletal remodelling, cell-to-cell adhesion, angiogenesis, cellular metabolism and inhibit differentiation and cell death. Mutations in locations where proto-oncogenes reside invariably lead to the deregulated activation of pathways that control cell growth, which under normal physiological conditions would remain silent. The excessive activity of oncogenic proteins can impact cell functions in a number of ways contributing significantly towards tumourigenesis. The activation of growth factors and their receptors, for example, the overproduction of platelet-derived growth factor B (PDGF-B) has been associated with tumour progression (Barnhill *et al.*, 1996). Erb-B family of growth receptor tyrosine kinases, in particular *ERBB2* gene (Her2/neu) have been implicated in the development several types of cancer due to genetic changes resulting in gene amplification leading to the overexpression of the receptor (Ross & Fletcher, 1999). Further downstream from growth receptors, the constitutive activation of intra-cellular signalling transduction kinases of the *RAS/RAF/ERK* families lead to self-sufficient cell growth and proliferation. *H-RAS* was the first oncogene to be discovered to induce neoplastic transformation of cells when present in its mutated form (Parada *et al.*, 1982). Since this time, mutations in *RAS* family oncogenes have appeared in various tumour types and have been extensively investigated in cancer (Bos, 1989). The direct activation of nuclear transcription factors which regulate gene expression of proteins involved in the cell cycle circumvent many of the control regulators further upstream in signalling pathways. Inappropriate activation of transcription factors through excessive expression or altered protein function drives continual cell proliferation. The most notable example being the overexpression *MYC* family oncogenes regulating in the accumulation of cyclin A and cyclin E proteins driving forward cell division (Dang, 1999).

1.1.2.2 The inhibition of tumour suppressor genes

In opposition to oncogenic signals, several safeguard mechanisms operate to prevent cells entering a state of uncontrolled cell division. These mechanisms contain within a group of cancer-related genes identified as tumour suppressor genes (TSG), which as the name suggests, are essential in averting cellular transformation and tumour expansion. The activities of their protein products are intrinsic to the cell cycle checkpoint responses, DNA damage and repair, promotion of apoptosis and the prevention of tumour progression (Sherr, 2004). Importantly, the realisation that most, if not all, pathways that promote cell proliferation also operate within an intrinsic growth inhibitory pathway networked to at least one TSG has placed further significance on their role in events leading to malignant transformation.

In contrast to proto-oncogenes, genetic mutations in both alleles of a tumour suppressor genes result in a loss of normal protein function through processes such as loss of hetrozygosity, methylation, cytogenic aberrations, and polymorphisms (Teh *et al.,* 1999). In 1971, the "two-hit" mutation theory proposed by Knudson stating that a germline mutation of one allele of a gene predisposes an individual to cancer following somatic mutation in the second allele still holds true for most TSGs (Knudson, 1971). Fifteen years later, the discovery of the first TSG isolated from retinoblastoma, and termed the *RB* gene (Friend *et al.,* 1986) is commonly found to be defective in multiple cancers through its role as a checkpoint control during transition to S phase of the cell cycle. In hereditary cancer syndromes, defective TSG expression predisposes individuals to further mutation and the development of cancer. For example patients with familial adenomatous polyposis have an increased risk of developing colorectal cancer due to mutations that reduce the activity of the adenomatous polyposis coli (*APC*) gene. This TSG operates within the Wnt signalling pathway, targeting free β-catenin for ubiquitination preventing the transcription of *cyclin D1* and *MYC* genes which drive progression of the cell cycle to S phase (Vogelstein & Kinzler, 2004). Germline mutations in stability genes *BRCA1* and *BRCA2* also predispose individuals to breast and ovarian cancer (King *et al.,* 2003).

Gene mutations that lead to the loss or functional impairment of p53 protein are perhaps the most frequent genetic alteration event observed in cancer. The *p53* gene acts as a transcription factor controlling the activation and inhibition of many genes and functions

and sits at the nexus of all cell cycle signalling pathways. It is activated in response to diverse cellular stress signals including nucleotide deprivation, oncogene activation and hypoxia following which *p53* orchestrates stress-specific pathways. Most significantly *p53* is instrumental in preserving cell function by activating proteins required for DNA repair by initiating cell cycle arrest in the event of DNA damage or triggering programmed cell death if the damage is too great (Bertram, 2000; Horn & Vousden, 2007). The importance of p53 and Rb and their network of associated proteins are further underscored by the fact that they are both are commonly defective across several types of cancer and virtually all oncoviruses directly or indirectly inactivate both *p53* and *RB* TSG pathways leading to tumour formation (Vogelstein & Kinzler, 2004).

1.1.3 Tumour associated antigens

Through successive divisions, tumour cells evolve to activate (proto-oncogenes) and inactivate (TSG) mutated genes providing selective advantages that support continual tumour growth and metastasis. The activation and inactivation of oncogene-TGS pathways expose the cell to further genome wide mutations as a combined effect of undue growth and the loss of genomic preservation. As a consequence, these events trigger signalling cascades affecting the expression of other gene products. The aberrant expression of these proteins seen to be restricted to tumour cells are called tumour associated antigens (TAAs). For this reason, identifying new TAAs have been the subject of intense investigation not only for their diagnostic utility but also for their application as tumour specific targets in treatment therapies. There has been particular focus on identifying short peptide regions (epitopes) derived from TAAs which are presented by major histocompatability complex (MHC) molecules that are recognised by T cells and are able to generate a tumour-specific T cell response. The first TAA identified from a melanoma patient (MAGE-1) using molecular cloning (van der Bruggen *et al.,* 1991) also provided the first tumour-specific T cell reactive MAGE-1 epitope by the same group the following year (Traversari *et al.,* 1992). Since then, techniques to identify TAA and their immunogenic epitopes have ranged from using genetic approaches (van der Bruggen *et al.,* 1991), modified serological analysis of recombinant cDNA libraries (SEREX) (Sahin *et al.,* 1995) and representational difference analysis (RDA) (Lisitsyn *et al.,* 1993) are discussed later.

Unsurprisingly, given the wide range of genomic mutations and epigenetic alterations that occur, a whole spectrum of altered TAAs have been identified which can be broadly classified into one of the following categories (Table 1.1).

Oncofetal antigens: Genes that are typically only activated during fetal development may also be re-expressed in tumour cells. Due to their restricted expression, oncofetal antigens have been investigated for their use as both serum biomarkers and for immunotherapy. For example, carcinoembryonic antigen (CEA), a glycoprotein produced in the gastrointestinal tissue during embryogenesis and is involved in cellular adhesion which may aid metastatic spread in colorectal carcinoma (Greiner *et al.,* 2002). Similar studies have been performed targeting of alpha-fetal protein (AFP) in patients with hepatocellular carcinoma (HCC) (Butterfield *et al.,* 2007).

Oncoviral antigens: Viruses associated with malignant transformation of infected cells are also a source of TAAs. Importantly, they invite considerable interest as targetable tumour antigens due to their involvement in tumour transformation and their expression being restricted to infected cells which encompass the tumour cell population. In addition, by virtue of being foreign (non-self antigen) to their infected host, T cell receptors specific to their viral molecules would not undergo deletion during early T cell selection processes and they would not induce autoimmunity. For example, antigens derived from human papillomavirus (HPV) namely E6 and E7 products act as oncogenes to inactivate TSGs p53 and pRb respectively (Münger & Howley, 2002) and have led to the development of two prophylactic vaccines against HPV (Cervarix and Gardasil) to prevent virally induced cervical carcinoma. Other targetable viral antigens originating from hepatitis B virus (HBV) in HCC and Epstein-Barr virus (EBV) in Burkitt's and Hodgkin's lymphomas have also demonstrated encouraging results (Gehring *et al.,* 2011; Kennedy-Nasser *et al.,* 2009).

Tumour-specific unique antigens: Tumour antigens arising from genes that undergo mutation can also provide a source of neo-antigens due to their newly exposed and possibly immunogenic epitopes that may potentially be recognised by T cells to engage in tumour rejection. So far, evidence of the immunogenic potential of some proteins involved in tumour progression has generated great interest. For example gene alterations in *BCR/ABL* in CML (Cai *et al.,* 2012) as a result of chromosomal translocation, point mutation in *CDK4* in melanoma (Wölfel *et al.,* 1995) and in immunoglobulin (Ig) idiotype

a gene fusion product in Non-Hodgkin's lymphoma (Redfern *et al.,* 2006). A notable feature of tumour cells is the deregulation of pre-mRNA splicing processes which may lead to the expression of protein variants that are specific to tumour cells. The advancement of genome-wide analysis technologies have allowed for the identification of cancer-specific variants as a result of deregulated alternative splicing and post-transitional modifications (Thorsen *et al.,* 2008; Eswaran *et al.,* 2013). Although questions remain as to whether these unique antigens accumulate as random by-products in tumour cells or whether they play an active role in tumourigenesis (Ghigna *et al.,* 2008). Examples of targeting variants assessed for their use in therapy include CD44v6 in head and neck cancer (Börjesson *et al.,* 2003) and Bcr/Abl in CML (Volpe *et al.,* 2007).

Cancer testis antigens: Similar to oncofetal antigens, the re-expression of normally silent cancer/testis (CT) antigens is restricted to tumour cells, male germline cells in the testis and placental tissue. As germline cells lack the expression of MHC molecules, they are considered to be immunoprivileged. The main appeal for therapy therefore lies in the ability to generate T cells that recognise CT antigens expressed on the surface of tumour cells in the context of their MHC allowing for immune responses elicited against tumour cells alone. These antigens are the best-characterised of the TAA classes and have been identified in multiple cancer types including the first CT antigen from melanoma, MAGE (van der Bruggen *et al.,* 1991) and later BAGE, GAGE (Boon & van der Bruggen, 1996) and NY-ESO-1 (Chen *et al.,* 1997) in multiple cancers.

Overexpressed antigens: These antigens are derived from a wide range of genes that are found to have higher expression in multiple malignant tissues compared to their generally lower expression in normal tissues. Examples include epidermal growth receptor 2 (Her-2) and mucin-1 (Muc-1) in breast and ovarian cancer and Wilm's tumour-1 (WT-1) in multiple cancers such as gastric, lung and HCC. Mutated and wild-type forms of p53 have been associated with multiple cancers as has survivin. Despite their low level ubiquitous expression in normal tissues, these antigens invite the opportunity to target tumours that overexpress these antigens via their HLA complex. Challenges still remain in identifying T cells that recognise these unaltered self-antigens which have avoided clonal deletion and are capable in generating a tumour-specific immune response.

Differentiation antigens: Differentially expressed antigen expression is characterised by their restriction to the tumour and the tissue from which it arose. To date, most of these antigens have been identified in melanoma sharing their expression in normal melanocytes and are involved in the production of melanin. Those that demonstrated immunogenicity and have advanced toward clinical assessments include Melan-A/Mart-1, gp100 and tyrosinase (Romero *et al.,* 2002; Schwartzentruber *et al.,* 2011; Valmori *et al.,* 2003). Other reported antigens include those from prostate tissue such as prostate acid phosphatase (PAP), prostate specific antigen (PSA) and prostate specific membrane antigen (PSMA) (Kantoff *et al.,* 2010; Corman *et al.,* 1998; Murphy *et al.,* 1996) respectively.

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	-
HPV(E6/E7)	Cervical 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 1.1 – Tumour associated antigens. *A list of the major classes of TAAs found in cancer with examples showing the types of malignancies in which they can be found and their expression in normal tissue. References to each TAA are detailed in the main text.*

1.1.4 The tumour microenvironment

Tumour cells engage in dynamic signalling interactions with their surrounding tumour microenvironment (TME) to further promote their growth and metastasis. The TME is a collective term referring to stromal tissues and local cells surrounding tumours, composed of tumour endothelial cells, pericytes, adipocytes, fibroblasts, dendritic cells, macrophages, T and B lymphocytes, natural killer (NK) cells and other inflammatory cells. Signalling components of the TME include chemokines and cytokines as well as danger signals from heat-shock proteins (HSPs), nucleic acids, stress induced proteins and other damage-associated molecular patterns (DAMPs) (Wilson & Balkwill 2002; Balkwill, 2012; Calderwood *et al.,* 2006; Lotze *et al.,* 2007). The crosstalk between tumour and the surrounding cells, particularly with macrophages and stromal fibroblasts, can be conditioned to aid growth, support angiogenesis and facilitate tumour invasion and distant metastasis through a number of mechanisms which are ordinarily intended to regulate cell behaviour and co-ordinate responses to danger or tissue damage. Malignancy occurs from tumour cells that have successfully achieved *i)* cell transformation and sustained accelerated growth *ii)* tumour survival *iii)* invasion of surrounding tissues *iv)* escape from detection and elimination *v)* dissemination and adaption to distant organs. It is becoming increasing apparent that the immune system plays a complex role in both the regression and progression of tumour (Hanahan & Coussens, 2012; de Visser *et al.,* 2006). Immunosuppressive components that further compound tumour progression are discussed later in section 1.3.2.2. The relationship between the TME, macrophages, fibroblast cells and their impact on events leading to the promotion of tumourigenesis, namely angiogenesis and metastasis are highlighted below.

1.1.4.1 Role of inflammation in tumourigenesis

Inflammation and the initiation of cancer: The causal relationship between inflammation and cancer was first theorised in 1863 by Dr Rudolf Virchow, in which he suggested that the presence of leukocytes in neoplastic tissue indicated that chronic inflammation contributes to tumour progression (Virchow 1863-67; reviewed in Balkwill & Mantovani, 2001). As exemplified in the increased incidence of colon cancer arising in individuals with autoimmune diseases such as inflammatory bowel diseases (chronic ulcerative colitis and Crohn's disease) (Triantafillidis *et al.,* 2009), and persistent infections and

inflammatory conditions such as *H. pylori* linked to stomach cancer (Eslick *et al.,* 1999) and in cases of prostatitis proceeding prostate carcinogenesis (Platz & De Marzo, 2004). Many environmental carcinogens are associated with chronic infections or inflammation such as tobacco smoke. In mice, obesity has been demonstrated to promote liver inflammation and hepatocellular carcinoma (HCC) through elevation of tumour promoting cytokines (interleukin 6 and tumour necrosis factors) activating oncogenic transcription factor *STAT3* (Park *et al.,* 2010). Leukocytes are essential during infection and injury as the first line of defence, however they may indirectly mediate cellular transformation. For example, during acute inflammation, phagocytic leukocytes infiltrating to the affected site engulf and destroy invading micro-organisms through the release cytotoxic mediators such as ROS, eradicating pathogens and neighbouring healthy cells alike. In chronic inflammation, persistent release of phagocyte-derived ROS may in fact contribute to the initiation of cancer (Azad *et al.,* 2008).

In response to tumour, infiltrating monocytes differentiate into immature dendritic cells (DC) under the influence of interferon gamma (IFNγ), tumour necrosis factor alpha (TNFα) and granulocyte macrophage colony factor (GM-CSF), which can then capture antigens and migrate to lymph nodes to prime T cells, termed the M_1 phenotype. However the production of interleukins; IL-4, IL-6, IL-10, IL-13 and colony-stimulating factor 1 (CSF-1) by tumour cells can cause precursors to assume a macrophage-like phenotype (M_2 phenotype) (Sica & Mantovani, 2012). The presence of macrophages with the M_2 phenotype in the TME have been shown to correlate with poor prognosis for many cancers such as in breast, prostate and bladder cancer (Leek *et al.,* 1999; Lissbrant *et al.,* 2000; Hanada *et al.,* 2000). Unlike T helper subtypes (discussed later), an important feature of macrophage M_1/M_2 differentiation is the high degree of plasticity in their phenotype enabling them to switch between polarities depending on the stage of tumour development. Thus, the predominantly M_2-like phenotype contributes to neoplastic formation in early stages of growth and later switches to tumour growth, angiogenesis and metastasis in established tumours (Biswas *et al.,* 2008).

Sustaining tumourigenesis: Once recruited to the TME, tumour-associated macrophages (TAMs) play a significant role in tumour progression producing a number of potent growth factors that accelerate angiogenesis primarily through their production of VEGF-A. Similarly, TAMs support other tumour related activities in the TME such as

lymphangiogenesis (basic fibroblast growth factor 2 (bFGF-2), TNFα), matrix remodelling (matrix metalloproteinases (MMPs)) and invasion/metastasis (plasminogen activators, macrophage colony stimulating factor (M-CSF), CSF-1, TNFα) (Ono, 2008). Furthermore, they can secrete mitogenic growth mediators (epidermal growth factor (EGF, TGFα) to stimulate tumour growth and also surrounding cancer associated fibroblasts (CAF). Like TAMs, CAFs can activate wound repair programmes which induce cell proliferation and neovascularisation (Hanahan & Coussens, 2012). Finally, TAMs produce growth factors and cytokines to promote anti-apoptosis (hepatocyte growth factor (HGF), IL-8), immunosupression (IL-10, TGFβ) and recruitment of other macrophages and neutrophils (monocyte chemoattractant protein 1 (MCP-1), chemokine ligand 5 (CCL5), M-CSF, VEGF-A) (Ono, 2008). Collaborative interactions between tumour cells, TAMs and CAFs produce a perpetual pro-inflammatory environment that fosters malignant growth in a scenario that represents tumours as "wounds that do not heal" (Dvorak, 1986).

Promotion of neovascularisation in established tumours: The delicate homeostasis between angiogenic inducers and regulators secreted by tumour cells and the cells of the TME is disturbed during tumourigenesis, which lead to the formation of new and often highly disorganised, networks of blood vessels. Increasing the activity of pro-angiogenic factors or a decrease of inhibitors, skew the "angiogenic switch" to favour vascular expansion, a critical step in tumour progression (Bergers & Benjamin, 2003). The VEGF family and in particular VEGF-A, are prominent stimulators of angiogenesis triggered through oncogenes and TSGs for example by *K-RAS*, *BCR/ABL*, *SRC*, *STAT3* and *p53* (Ikeda *et al.*, 2001; Ebos *et al.*, 2002; Ellis *et al.*, 1998; Niu *et al.*, 2002; Fujisawa *et al.*, 2003) as well as through cytokine-induced signalling during hypoxia (HIF1α and -β) and cell receptors and growth factors (EGFR, cyclooxgenase-2 (COX2) and platelet derived growth factor (PDGF)) (Bergers & Benjamin, 2003).

Tumours can successfully stimulate angiogenesis through the release of cellular stress signals. Growing tumours (>2.0 mm) require an increasing intra-tumoural blood supply to provide oxygen and nutrients whilst removing waste products. When this demand is not met, hypoxic tumour cells trigger the induction of hypoxia-inducible factor 1 alpha, transcriptional factor (*HIF-1α*) which activates a cascade of genes involved in the induction of angiogenesis such as *VEGF* and inducible nitric oxide synthase (*iNOS*) (Pugh & Ratcliffe, 2003). In addition, hypoxic tumours release a number of growth factors

including (CSF-1 and GM-CSF), transforming growth factor beta (TGF-β), cytokines and chemokines that recruit monocytes and macrophages. This can further cultivate an environment suited to tumour growth and vascularisation (Coussens & Werb, 2002). More recently, Lin and colleagues (2006) demonstrated that the presence of TAMs in pre-malignant mammary cells aided transition to a malignant phenotype through increased neoangiogenesis.

Many anti-angiogenic drugs (for example bevacizumab, sunitinib, and sorafenib) designed to target tumour-infrastructure have proven effective in halting tumour progression (Yang *et al.,* 2003, Motzer *et al.,* 2009, Llovet *et al.,* 2008). Although, a major consequence is that often only a transient affect is observed using these treatments resulting in the eventual selection of drug resistant tumour cells. Resistant cells are not dependent on angiogenic factors such as VEGR and circumvent hypoxia-induced apoptosis which restores tumour growth with an increasingly aggressive malignant phenotype (Bergers & Hanahan, 2008).

Promotion of metastasis: The production of various matrix metalloproteases (MMPs) released by infiltrating TAMs may also support escape and migration through ECM and further facilitate migration of tumours into blood and lymphatic circulation (intravasation). Firstly, the degradation of the endothelial basement membrane during inflammation is associated with TAMs which increase the mobility of metastatic cells (Kessenbrock *et al.,* 2010). Furthermore, the release of TAM-derived TNFα activates signalling cascades leading to the increased expression of pro-invasive factors such as extracellular matrix metalloprotease inducer (*EMMPRIN*). This activation is mediated by the activation of the nuclear factor kappa B (NFκB) pathway which is involved in the activation of other mechanisms that are favourable to tumour cells. These include the induction of pro-inflammatory cytokines and molecules which promote tumourigenesis (IL-6, TNFα, MMP's, COX-2 and iNOS and ROS). A consequence of NFκB activation is that it can inhibit TNFα-induced apoptosis through the activation of caspase-8 and BCL-2 family (Karin *et al.,* 2002). During metastasis, detached tumour cells may be eliminated through anoikis, apoptosis trigger by the loss of adhesion. A recent study demonstrated that α4-integrin expressing TAMs engage with breast cancer cells by binding to vascular cell adhesion molecule-1 (VCAM-1) on the tumour surface, which in turn lead to a cascade of signalling events leading to the suppression of apoptosis (Chen *et al.,* 2011).

1.1.4.2 Immunoediting and tumour escape

The policing of the microenvironment by immune cells can be effective in detecting and eliminating nascent transformed cells through a process described as immunosurveillance. The basis of this theory was first put forward by Burnet and Thomas (1957) and was later supported following observations that transplanted tumours could be repressed by the immune system in mice. Earlier observations demonstrating the rejection of tumours in athymic nude mice were later attributed to the actions of NK cells in response to tumour. Together with the observation that tumour-specific protection was generated in genetically identical mice suggested toward the existence of TAAs which formed the notion of immunosurveillance (Dunn *et al.,* 2002; Dunn *et al.,* 2004; Kim *et al.,* 2007). It is now broadly accepted that immunosurveillance forms part of a wider process in immune responses to cancer, in which some tumours are able to avoid elimination by immune cells through a process termed immunoediting (Dunn *et al.,* 2004). This concept proposes that certain tumour cells are successfully eliminated by both innate (NK cells and macrophages) and adaptive ($CD4^+$ and $CD8^+$ T lymphocyte cells) immune responses. As a consequence, this leads to either total eradication of tumour cells and restoration of normal tissue or the removal of those cells highly susceptible to immune attack, leaving a subgroup of immune resistant tumour cells. For the latter, continuous phases of cell elimination of the tumour leads to the development of tumour cells capable of avoiding immune destruction. This phase of immunoselection may occur over a long period of time in which the immense selective pressures maintain tumours whilst they acquire further mutation as a consequence of their increasingly unstable genomes. Those mutations that confer a selective advantage whilst clonally inheriting resistance from immune onslaught proceed to proliferate and metastasise unopposed by immune restraints (Dunn *et al.,* 2004). This process is illustrated in Figure 1.4.

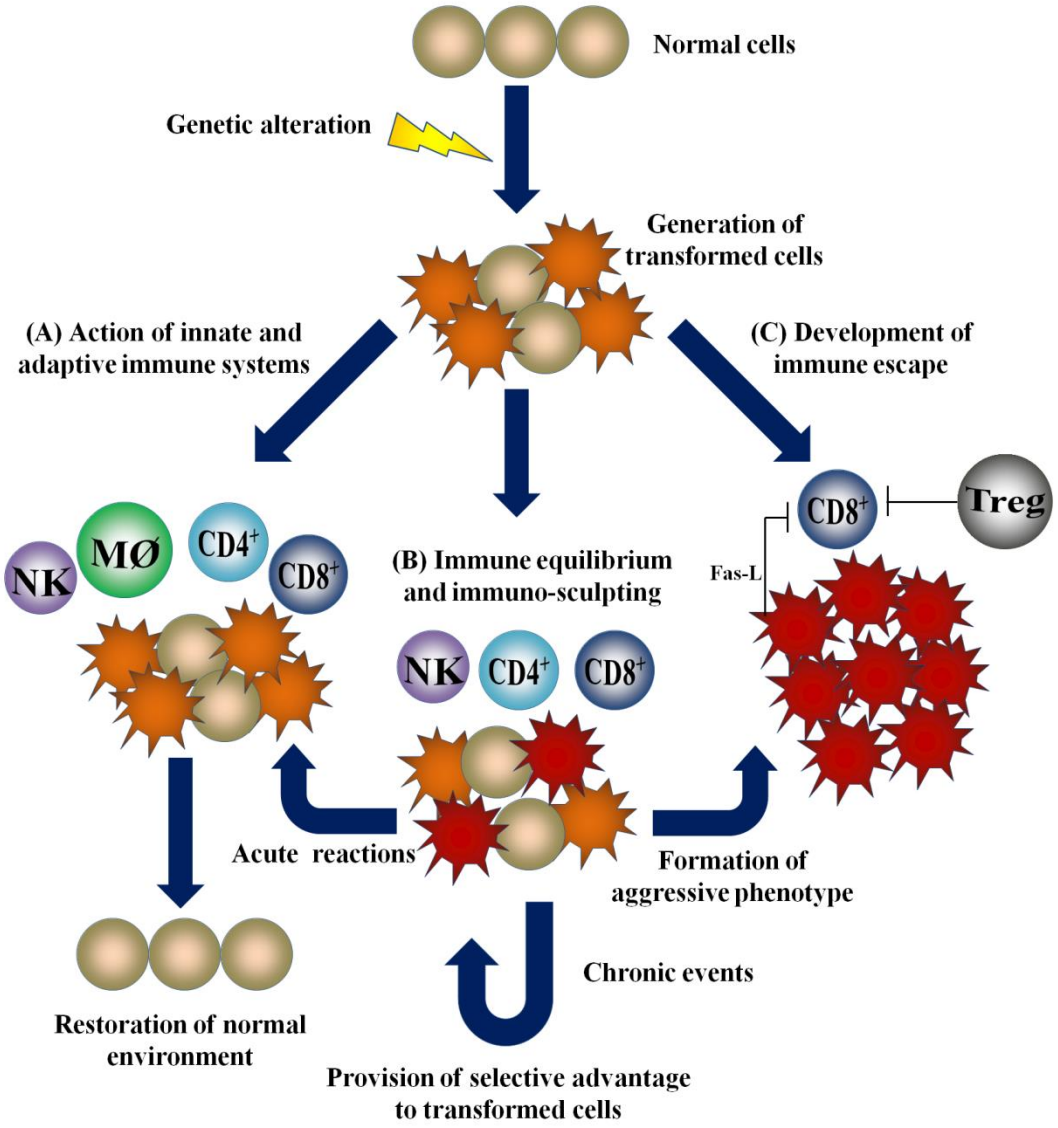


Figure 1.4 – Overview of the three phases of cancer immunoediting. *After transformation through genetic alterations, various immune events may occur. First, tumour cell elimination by cells of innate (NK=Natural killer cell, MØ=Macrophage) and adaptive immunity ($CD4^+$ and $CD8^+$ T lymphocyte populations) leading to the restoration of normal tissues (**A**). However, if unsuccessful, cells may enter a phase of equilibrium (**B**) in which they are maintained through selective pressures until acute immune reactions lead to tumour eradication or to a newly formed population of immunosculpted tumour cells which are resistant to immune elimination and displaying aggressive phenotype (**C**). This population may escape immune recognition through any number of tumour driven evasion mechanisms (e.g. up regulation of Fas-L) and immunosuppressive cell populations may also contribute to immune evasion (e.g regulatory T cells) (Adapted from Dunn et al., 2004).*

1.1.5 The cancer stem cell hypothesis

The origins of cancer have in the past, and continue to be, the source of intense debate. For many years the understanding that cancer arises from a single cell that is subject to mutational change leading to the gradual selection of clones remained the most widely accepted hypothesis, termed the clonal evolution theory. Accordingly, the majority of conventional therapies aim to target tumours based on this principle. The fact that current therapies often fall short of eliminating all tumour cells suggest that tumours contain a population of cells that are not only resistant to treatments, but are also responsible for reoccurrence and metastasis. The notion that only a rare subpopulation (cancer stem population) of cells holds the capacity to initiate and maintain tumour growth has formed the foundation for what is now referred to as the cancer stem cell model. In 1997, the first major breakthrough in providing evidence for the cancer stem cell model was the existence of leukaemic stem cell populations that were capable of generating tumours (Bonnet & Dick, 1997). The study demonstrated that injection of a rare stem cell population isolated from leukaemic cells with a specific surface expression ($CD34^+/CD38^-$) into non-obese diabetic mice with severe combined immunodeficiency disease (NOD/SCID) mice were capable of generating tumours that were phenotypically identical to that of the donor tumour. This finding prompted several investigations which identified tumour–initiating cancer stem cell populations in many tumour types including in breast, brain, prostate, colon and ovary (Al-Hajj *et al.*, 2003; Singh *et al.*, 2003; Collins *et al.*, 2005; O'Brien *et al.*, 2007; Zhang *et al.*, 2008).

Despite their discovery, many questions still remain as to the events that lead to their entry into tumourigenesis. The biological characteristics of these cells such as their ability to self-renew and also generate differentiated cell populations from CSC-derived progenitor-like cells, may possibly explain the unique features of some cancers such as teratocarcinomas (Andrews, 2002). Additionally, CSC possess more resistance to cell death then tumour cells when challenged with drug treatments, radiotherapy, apoptosis and their lack of surface MHC expression suggest that they may escape immune attention. The similarities shared between CSC and the stem cells from which they are named after give credibility to the proposal in which a self-replenishing population may indeed, drive tumourogenesis and could possibly initiate tissue invasion and cell dissemination through the generation of a metastatic CSC population (Brabletz *et al.*, 2005).

1.1.6 Current treatment strategies

There are more than 100 classified types of cancer that are distinguished according to their tissue and cell of origin. Genetic, environmental and lifestyle factor interactions can culminate to produce a given malignancy in a particular individual. It is therefore unsurprising that, given these layers of complexity, a varying degree of success is achieved across cancers and within subtypes with the current selection of treatments despite their refinement over time.

At present, first line treatments still largely depend on established traditional approaches which include surgery, radiotherapy, chemotherapy and hormone therapy. Surgical intervention is the mainstay in treating localised primary solid tumours and alone can be curative. However in the majority of cases, surgical removal of cancers that have metastasised beyond the primary site is ineffective and therefore require additional radiotherapy and/or chemotherapy treatments. Radiotherapy and chemotherapy can be used to target and destroy rapidly proliferating cancer cells. Due to their genomic instability, cancer cells are more susceptible to irreversible DNA damage as a consequence of their loss of damage repair mechanisms making them more vulnerable than normal cells to targeted radiation. Meanwhile chemotherapy using cytotoxic agents inhibits cell proliferation particularly in rapidly dividing cells leading to cell death. However, a major drawback to these treatments is that they also damage highly proliferative normal cells leading to the adverse and often severe toxicity. In addition, these two approaches are mostly effective in tumours which are well oxygenated making hypoxic areas within then resistant to radiation and poorly perfused by chemotherapeutic agents to be effective. Furthermore, chemotherapy and radiotherapy can, in some instances, also promote tumourigenesis through their impact on cells of the immune system. Finally, hormone therapy can be utilised block hormone-dependent tumour growth. Existing hormone therapies such as testosterone in prostate cancer are often only partially effective leading patients to relapse due to the generation of hormone-resistant tumours. In more recent times, approaches using monoclonal antibody technology and targeting cancer specific molecules using drugs have proven useful but are limited to those patients that sufficiently express the appropriate molecular target.

Often with these treatments, even when combined, fail to prevent cancer–related mortality. In the UK, for patients diagnosed with cancer in 2007, the predicted overall 5 year survival rates were 46% men and 56% for women (Cancer Research UK). These statistics, in part reflect on the limitations of the current treatment modalities, in particular their lack of specificity to cancer cells, damaging (and potentially initiating) surrounding normal cells and their severe toxic side-effects. Finally, current therapies that lead to the accumulation of treatment resistant tumour cells can arguably lead to a more invasive tumour phenotype and poorer survival outlooks. Thus, alternative curative approaches are required that limit their therapeutic action specifically toward tumours sparing normal cells. The concept of instructing the body's own immune system to specifically target and destroy tumour cells to avoid more invasive treatments which carry higher mortality risk and unwelcome side effects poses an attractive alternative approach. The current status of cancer immunotherapy is discussed further in section 1.4.

1.2 The Immune System and Cancer

The association between infection and the regression of cancer dates back to the late 18th century when Dr William Coley devised the first anti-cancer vaccine which involved triggering the immune system using bacterial toxins. Later in 1909, the concept of immunosurveillance was first formulated by Dr Paul Ehrlich who suggested that transformed cells are frequent occurrences in humans however they are recognised and eliminated by the immune system before they become clinically apparent. In more recent times, several investigations have added further weight to the notion that both the innate and adaptive immune system plays an influential role on tumours. In particular, the identification of natural tumour-specific circulating T lymphocytes as well as tumour reactive antibodies in the bloodstream of cancer patients (Valmori *et al.,* 2000; Schlichtholz *et al.,* 1992). Similarly, the release of cellular stress induced signals and the loss of surface MHC class I expression can activate NK cell mediated tumour cell lysis (Long & Rajagopalan, 2002). Over the years, as the knowledge of the intricate processes that orchestrate the anti-tumour response has expanded, it has also prompted virtually every facet of the immune system to be examined as a therapeutic strategy against cancer.

1.2.1 Innate immunity

The innate immune system is consider to be the "non-specific" arm of the immune system which includes defences that are constitutively present and prepared to mobilise immediately upon infection of a foreign pathogen. The innate system operates a rapid, inflexible first line response to quickly eliminate any invading pathogens that the body encounters. There are several effector cells that have been identified as important mediators of this response, including NK cells, NKT cells and γδ T cells originating from lymphoid progenitor cells, and phagocytic cells such as macrophages and DCs derived from myeloid lineage. These cells perform their function through cell-surface pattern recognition receptors (PRR) in order to identify pathogen-associated molecular patterns (PAMPs) which are associated with a wide range of conserved surface molecules found on microbial pathogens or are cellular stress signals. Similarly, damage-associated molecular patterns (DAMPs) have also been suggested to be recognised by PRRs during cellular damage (Seong & Matzinger, 2004). Following recognition of these molecules, phagocytic cells commence ingestion of pathogens in a process known as phagocytosis, in which phagocytes generate toxins (e.g. O_2^-, H_2O_2 and NOS) that assist in killing engulfed microorganisms. Likewise, NK cells release cytotoxic granules to induce either apoptotic or osmotic lysis of target cells. At this stage, activated cells are able to activate the adaptive immune response through cross-talk with antigen- presenting cells (APCs). A family of transmembrane protein structures referred to as toll-like receptors (TLR) are considered the most important type of PRR's. TLRs are common among vertebrates and invertebrate and to date in mammalian species, ten functional members and have been classified according to their known agonists. These include TLR recognition of peptidogylans (TLR2), double stranded RNA (TLR3), lipopolysaccharide and viral proteins (TLR4), flagellin (TLR5) and unmethylated CpG DNA motifs (TLR9) (Akira & Takeda, 2004). TLR mediated-activation of signalling pathways including NFκB and mitogen-activated protein kinases (MAPK) can induce pro-inflammatory cytokines and chemokines and the expression of co-stimulatory molecules which are essential for the induction of adaptive immunity via APCs (Medzhitov, 2001).

Although much attention to the role of the adaptive immune response has been given in tumour immunosurveillance, more recently it has been suggested that cells of the innate immune system are also capable of detecting tumour cells. It has been suggested that

endogenous danger signals released by damaged cells, principally uric acid (Shi *et al.*, 2003) or through exposure to certain inherent biological signals of the tumour itself (Seong & Matzinger., 2004) are sufficient to induce innate immune responses. The ensuing pro-inflammatory responses may be induced through the activation of TLRs by agonists such as heat-shock proteins (HSP's), high-mobility group protein B1 (HMGB1) and ECM derivatives (hyaluronic acid and heparin sulphates) (Dunn *et al.*, 2004). To date, a number of danger signals have been identified to be produced by tumours, which in turn allows for the recruitment of specific cells of the innate immune system. Taking the earlier example, tumours cells under hypoxic conditions induce molecules associated with stress called major histocompatability complex class I chain-related proteins A and B (MICA/B) and UL16-binding proteins. These molecules are cellular ligands of NK cell activating receptor (NKG2D) found on NK and NKT cells, cytotoxic T lymphocytes (CTL) and γδ T cells (Pende *et al.*, 2001). In NK cells, NKG2D acts as an activation receptor capable of triggering NK mediated tumour cell lysis and induce NK cells to secrete IFNγ, a potent pro-inflammatory stimulator, which among other activities is capable of direct cell killing through the inhibition of angiogenesis and metastasis and the recruitment of lymphocytes. In addition, IFNγ activates the adaptive response through the maturation of macrophages and DCs which in turn release IL-12 which enhances $CD8^+$ CTL and further promotes NK cytotoxic activity. In CTL, NKG2D serves as a co-stimulatory receptor of TCR-activated cells (González *et al.*, 2008). NK cells have also been found to recognise MHC class I molecules via specific surface receptors (Killer Ig-like receptors (KIRs), lectin-like receptor 1 (LIR-1) and CD94-NKG2A) that act to inhibit NK cytotoxic activity. The absence or insufficient concentration of MHC class I expression on the cell surface, a frequent immune evasion strategy used by tumour cells, fulfil the "the missing-self" criteria required for NK mediated cell lysis (Moretta & Moretta, 2004).

More recently, "unconventional" T cell populations have been studied for their role in bridging innate and adaptive immune responses. NKT cells represent a subset of $CD3^+$ T lymphocyte cells that express a CD1d-restricted TCR on their surface. These TCR differ from their conventional αβ T cells in their TCRs in that they are typically limited to recognising glycolipid antigens via PRR from bacterial microbes rather than peptide/MHC. Phenotypically, NKT cells more closely resemble cells of the innate immune system with NK-like features such as the rapid production of IFNγ, IL-4 and GM-CSF upon activation as well as exhibiting NK-like killing, however fail to generate immunological memory

(Berzofsky & Terabe, 2009; van Kaer *et al.,* 2011). Similarly, mature γδ T cells form functionally distinct subgroups that places them in both innate and adaptive divisions of the immune system. These cells are capable of recognising microbial invaders (Vδ2 T cells) and transformed cells (Vδ1 T cells) independently of antigen presenting and MHC molecules, can eliciting both NK cell and CTL anti-tumour effector responses, and can directly induce effector $CD4^+$ and $CD8^+$ T cell responses through APC-like activity and appear to have immunological memory (Holtmeier & Kabelitz, 2005; Kalyan & Kabelitz, 2013). These two populations provide evidence of the overlapping components that span over both the innate and adaptive arms of the immune system. Taken together the emerging evidence suggests that these populations have several influential roles from detecting "early danger" signals to immunosurveillance to switching from innate to regulating adaptive responses in cancer (Kim *et al.,* 2007).

The most effective cross-talk between the innate and adaptive immune systems occurs through three populations of cells that are responsible for antigen capture, processing and presentation via the MHC complexes termed professional antigen presenting cells (APCs). Through this process, B lymphocytes, macrophages and DCs are able to present effector cells of the adaptive immune response with specific antigens against which a targeted immune response can be initiated. Of these populations, DCs are considered the most potent and important of the APCs and sit centrally in the body's immune system, transmitting from innate to adaptive responses. DCs have been intensely researched to discover their various subsets and their role in order to determine how they could be harnessed for use in clinical therapy.

As previously mentioned DCs are derived from bone-marrow progenitor cells and are designated as sentinels of the immune system that are mostly positioned at the antigen entry points of the body. DCs reside in the periphery in an immature state, following an encounter with damaged or infected cells, DCs begin to engulf cellular debris through phagocytic mechanisms. Following antigen uptake, DCs begin to migrate into draining lymph nodes via the afferent lymphatics, during which time the cell undergoes various stages of maturation which culminate in the up regulation of surface MHC molecules and co-stimulatory molecules CD40 and B7. In addition, DCs process captured proteins into short peptides which are then displayed onto MHC molecules bound to the surface of the cell. Upon arrival at the lymph nodes, DCs activate naive $CD4^+$ and $CD8^+$ T cells that then

differentiate into antigen-specific effectors T cells. The maturation status of DCs is important in generating functional effector T cells, immature DCs that fail to present appropriate co-stimulation can lead to antigen-specific T cell anergy (Steinbrink *et al.*, 2009; Palucka & Banchereau, 2012).

1.2.2 Adaptive immunity

Unlike the innate immune response, the adaptive arm of the immune system consists of a sophisticated network of cellular components that co-ordinate measured responses through antigen-specific receptors. These responses can be classed under two headings, namely as humoral immunity consisting of B cell and antibody responses and/or cell mediated immunity relaying on T cell activity. The latter can be considered to be the chief instigator in the immune response against cancer through $CD4^+$ and $CD8^+$ T cell recognition of tumour associated antigens.

1.2.2.1 The humoral response

Humoral immunity is generated through antigen-dependent activation of B lymphocytes by DCs leading to either memory B cells or their clonal proliferation and terminal differentiation into antibody-secreting plasma cells. Circulating antibodies (Ab) can facilitate the ingestion of cells by phagocytic macrophages through antibody opsonisation, a process involving FAb mediated binding of antigen and activation of Fc receptors on the surface of macrophages. Similarly antigen bound antibodies can attract complement molecules to induce cell clearance via macrophages. Critically, B lymphocytes are themselves able to recognise and engulf soluble forms of their cognate antigen and display processed antigens bound to MHC class II molecules on their surface to T lymphocytes. Following B lymphocyte and $CD4^+$ T cell interactions, a further co-stimulation signal (via CD40/CD40L) activate B cell proliferation. Meanwhile $CD4^+$ T cell begin secreting cytokines to drive the development of the Th_2 response. Without the assistance of cognate $CD4^+$ T cells, plasma cells are unable to generate high-titre of antibodies. This has led to the identification of numerous TAA through SEREX suggesting that humoral responses are capable of inducing antibody mediated anti-tumour responses. During their development, B lymphocytes undergo negative selection to antigens in the bone marrow, a process that is less stringent than the selection mechanisms involved in T lymphocyte

development and therefore may be readily activated against tumour cells. Despite this, the role B lymphocytes play in anti-tumour immunity has remained debatable. Studies have demonstrated that an increase in B cell tumour infiltrates correlate with a poor prognosis in patients with metastatic disease (Dong *et al.,* 2006), contrary to other findings which associate them with improved survival (Nzula *et al.,* 2003; Kotlan *et al.,* 2005). The adoptive transfer of B lymphocytes into HPV16 mice lacking T and B cells restored the capacity of pre-malignant tissue to progress to full malignancy via the reinstatement of a chronically inflamed microenvironment (de Visser *et al.,* 2005). The down regulation of MHC class I molecules by tumour cells and their secretion of cytokines (IL-4 and IL-13) may preferentially skew the adaptive response to favour a Th_2 response in which case the use of therapeutic monoclonal antibodies may be beneficial either alone or in conjunction with Th_1 effectors to induce the rejection of tumours. Although it is evident that in isolation, the humoral response is detrimental to the effectiveness of the anti-tumour responses, it is becoming increasing clear that striking the correct Th_1/Th_2 balance between $CD4^+$, $CD8^+$ and B cells are critical to promote tumour rejection (Kao *et al.,* 2006; DiLillo *et al.,* 2010).

1.2.2.2 The cell mediated response

The cell mediated response is comprised for three functional classes of T lymphocytes which are capable orchestrating antigen-specific responses following their activation by APCs. Cytotoxic T lymphocytes or $CD8^+$ T cells recognise and eliminate infected or transformed cells. T helper lymphocytes (Th) or $CD4^+$ can differentiate into two further subtypes of effector cells namely, T helper 1 lymphocytes (Th_1 cells) which are involved in promoting CTL responses through APC signalling and drives the activation of macrophages. In contrast T helper 2 lymphocytes (Th_2 cells) promote the development of a humoral response via their B cell interaction.

Typically, DCs that are recruited to the tumour site and begin to internalise apoptotic and necrotic tumour cell debris and migrate to neighbouring lymph nodes where they interact with $CD4^+$ and $CD8^+$ T cells. During this migration period DC maturation occurs and as a consequence, they lose their ability to phagocytose further cellular material and instead, increase their ability to present processed antigens on their surface to effector T cell populations (Garrett *et al.,* 2000). Once receptor interactions with $CD4^+$ T cells occur,

DCs undergo further maturation which allow for exchanges between DCs and $CD8^+$ T cells to take place. This in turn leads to the differentiation and expansion of $CD8^+$ T cells into antigen specific CTL. This licences activated CTL and T helper cells to migrate to the site of the tumour and exercise antigen-specific killing of tumour cells bearing the same antigen through the release of cytolytic toxins or through the activation of death-inducing signal receptors on the cell-surface of target cells. This simplified overview describes the mobilisation of cellular components involved during the adaptive response. This response occurs as a consequence of the co-ordinated release of many cytokines that not only drive the induction of an adaptive immune response but are also central to the recruitment of immune cells that can either, depending on the cytokine profile, promote an anti-tumour offensive or dampen the response altogether.

(a) Major histocompatibility complex: structure and function

The major histocompatibility complex (MHC) molecules are transport complexes present on the surface membrane of cells. They provide the appropriate platform for the presentation of antigen fragments to T cells in order for them to initiate and regulate antigen-specific immune responses. The MHC molecules are encoded by a cluster of closely linked genes situated on chromosome 6 in humans and chromosome 17 in mice. Historically, human MHC molecules are known as human leukocyte antigen (*HLA*) and the mouse equivalent as *H2* (histocompatibility 2). The *HLA* genes are organised into three classes: the *HLA* class I, class II and class III genes. The former two classes encode for molecules that are important for the presentation of antigenic peptides to T cells. Both MHC class I and class II consist of a variation of an α and β chain which form unique peptide binding grooves in order to tether peptides at specified amino acid anchor residues on the peptide. MHC molecules are highly polymorphic and allow promiscuous binding of a diverse variety of peptides by a given MHC molecule (Reche & Reinherz, 2003). Once bound to membranous MHC molecules, peptides are recognised by T cell receptors (TCR) on the surface of T lymphocytes.

The TCR complex is heterodimer composed of two glycoprotein chains (α and β or γ and δ). The majority of T cells express the α and β subunits which each form a highly variable region (vα and vβ) and a constant region (cα and cβ). TCR share a similar structural composition to that of immunoglobulin (Ig) molecules. The variable region of TCR's are

encoded by "random" combinations of multiple variable (V), diversity (D), and joining (J) gene segments during early T cell development creating a vast and diverse pool of T cells which together are able to recognise a vast array of antigenic peptides. This pool is condensed down through the deletion of self-recognising T cells during thymic selection (discussed later in section 1.3.2.1). Unlike Ig, activation of T cells cannot be achieved through the recognition of free form antigens as they require presentation in the context of MHC molecules. TCR's are expressed alongside the CD3 protein complex consisting of γ δ and two ε chains and two intracellular ζ chains forming the immunoreceptor tyrosine-based activation motif (ITAM). Recognition of MHC-peptide complex by TCR, alongside binding of the respective MHC class I (CD8) or MHC class II (CD4) molecules and co-stimulation signals, triggers downstream signalling and T cell activation (Figure 1.5A). The interaction between MHC-peptide-TCR and the appropriate co-stimulatory signalling are crucial for T cells to become fully activated (Smith-Garvin *et al.*, 2009).

The human *HLA* genes encode for three class I variable α chain subunits (*HLA-A, -B* and *-C*) and three class II variable α and β chain subunits (*HLA-DR, -DP* and *-DQ*). The mouse *H2* equivalent consists of three class I α chain subunits (*H2-K, -L* and *-D*) and two class II α and β chain subunits (*IE* and *IA*). Genes associated with class II molecules namely β2-microglobulin (*B2M*) and the invariant chain (CD74) are situated away from the HLA locus on chromosomes 15 and 5 respectively. MHC class I and class II are the most polymorphic genes in the human genome. In addition, the expression of the HLA alleles is co-dominant, meaning that both sets of the principle 6 alleles are expressed simultaneously in heterozygous individuals. This introduces extensive diversity to the possible MHC binding groove; in fact each allele differs by up to 20 amino acids in this region of the MHC, allowing for a broad range of peptides that can be presented to TCR (Janeway, 2001).

In humans, the α chain region of the MHC class I molecule, also referred to as the heavy chain, consists of α1, α2 and α3 domains. This chain forms a non-covalent interaction between β2m and α3 domain to form the MHC class I complex. The α1 and α2 domains conform to create a deep pocketed binding groove which restricts the size of peptides suitable to bind to 8 to 10 amino acids in length. MHC class I molecules are constitutively expressed on nearly all somatic nucleated cells of the body with exceptions such as testis. Their biological function is to present intracellular proteins, to the $CD8^+$ T cells (Figure

1.5B). The presentation of "self-antigens" by healthy cells are surveyed by the immune system and are recognised as "normal" requiring no further exchange. However, in instances of virally infected cells, the profile of antigens presented on the cell surface change to include virally derived antigens leading to the recognition of "non-self" antigens. As a consequence, the immune system responds by destroying the infected cells. This mechanism is also crucial in monitoring "altered-self antigens" during cell transfection (Janeway, 2001). MHC class II complex is composed of two α and β chains (α1, α2 and β1, β2), which fold to create an open ended peptide binding groove between the α1 and β1domains capable of anchoring peptides between 10 – 16 amino acids in length (Figure 1.5C). These MHC molecules are constitutively expressed on the surface of professional APCs and present to $CD4^+$ T cells. As the peptides presented are predominantly of extracellular origin, this mechanism is plays a prominent role in evoking an adaptive immune response through the interactions with $CD4^+$ T cells (Janeway, 2001).

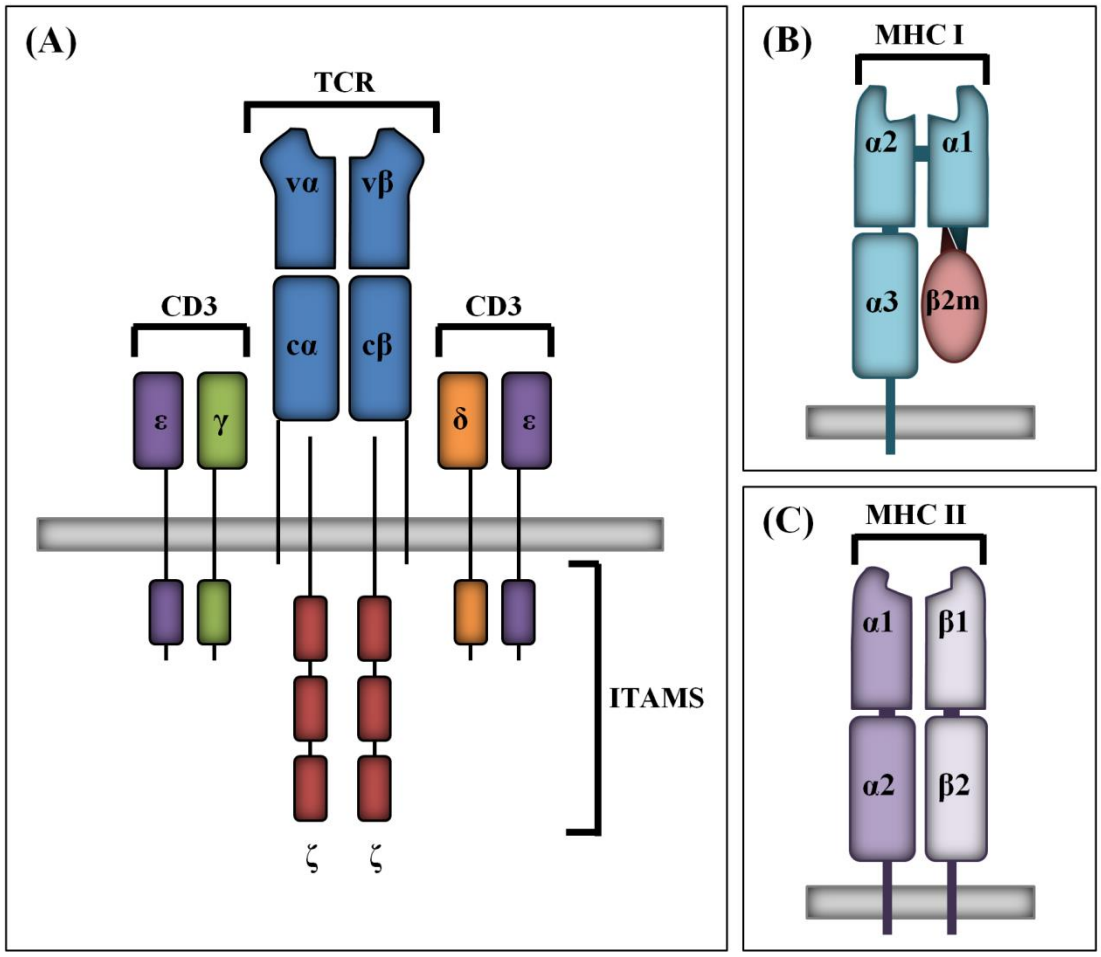


Figure 1.5 – Schematic representation of TCR and MHC class I and class II molecule structure. *The TCR consists of one α and one β chain which are themselves divided into one variable (v) and one constant (c) region. The variable regions form the binding domain for MHC bound peptides. The accompanying CD3 complex consisting of γ, δ, ε and ζ subunits and the ITAM mediate T cell activation and the ensuing intracellular signalling cascade (**A**). MHC class I consists of three α chain domains (α1, α2, α3) and β2m molecule. The α1 and α2 domains fold to form a cleft for peptides to bind (**B**). MHC class II consists of one α chain and one β chain domain (α1, α2 and β1, β2). Here the α1 and β1 domains fold to form the groove for peptides to binding (**C**) (Adapted from Wange & Samelson, 1996).*

(b) Antigen presentation pathways

As explained previously, the components of the immune system continuously assess the intra- and extracellular proteins for indications of infection or transformed cells. This process is mediated through MHC class I and class II molecules which present peptide fragments to T cells. These MHC-peptide complexes are generated with APCs and presented to T cells. The peptides and MHC molecules are generated in one of two

classical antigen presentation pathways. Typically, MHC class I molecules bind peptide fragments from intracellular sources (e.g. viral and altered self proteins) presented to $CD8^+$ T cell whereas MHC class II molecules present proteins arising from extracellular sources as a consequence of phagocytic activity by the APCs. The processing and presentation of antigens via MHC class I and class II pathways are illustrated in Figure 1.6.

Endogenous peptides destined for surface presentation by MHC class I molecules are derived from cytoplasmic proteins which undergo degradation by a multi-catalytic complex called the proteasome. Proteasomes are responsible for the disassembly of all miss-folded, damaged or foreign cytoplasmic proteins that have been covalently tagged with ubiquitin molecules regardless of whether they are transported to the endoplasmic reticulum (ER) (Michalek *et al.,* 1993). Proteasomes contain three IFNγ-inducible immunoproteasome subunits, LMP2, LMP7 and MECL-1 which induce proteasomal proteases that lead to a change in the nature of the peptides utilised for antigen presentation. Specifically, LMP2 and LMP7 enhance specific cleavage after hydrophobic and basic to create peptides with carboxyl ends which allow for easier transportation of peptide fragments and more suitable entry into peptide binding groove on the MHC molecule. The peptide fragments are transported to the ER via transporters associated with antigen processing (TAP) molecules, TAP 1 and TAP 2. MHC class I molecules are assembled within the ER lumen through the co-ordination of the peptide loading complex which consists of tapasin, ERp57, calreticulin and TAP molecules. The MHC heavy chain (α1, α2 and α3 components) and are stabilized by the glycoprotein binding chaperon protein called calnexin before binding with β2m. Tapasin also mediates the editing and loading of high affinity peptides onto the MHC class I molecule (Praveen *et al.,* 2010). TAP has also been shown to have a bias toward transporting amino acid sequences with hydrophobic side chains as this allows a stronger binding affinity between peptide and MHC (Momburg *et al.,* 1994). The binding of peptides enhance the structural stability of the MHC molecule and the complex is exported out of the ER to the Golgi complex and exocytosed to the cell surface via transporter vesicles for presentation to $CD8^+$ T cells (Figure 1.6).

Although MHC class I and II molecules share a similar structure and role in the cell, the assembly of the MHC class II molecules differs considerably to the mechanism described above. Exogenous proteins enter the cell via intracellular endosomal vesicles. As

endosomes advance further into the cell the pH environment within falls, which activates acid proteases (such as cathepsins) to degrade antigens into short peptide fragments. These fragments undergo lysosomal protease mediated degradation into smaller peptide fragments which remain within the endosomal vesicles. In the ER lumen, MHC class II α and β chain are synthesised and assembled in a dimer formation along the ER internal membrane. During this stage, an additional chaperon chain molecule associates to form a hetrodimer. This chain is termed the invariant chain (Ii) and is required to prevent binding of polypeptides to the peptide binding groove. The hetrodimer complexes are exported to the Golgi complex and trafficked through the cytoplasm via exocytic vesicles where proteases and cathepsins partially degrade the Ii so that only a small region inside the binding groove remains. This shorten region termed class II-associated Ii peptide (CLIP) ensures that no inappropriate binding to the MHC occurs. Endosomal and exocytic vesicles containing the peptide fragments and the MHC fuse to form late endosomes, CLIP then detaches from the MHC class II allowing dominant peptides with high affinity for the peptide binding groove on the MHC to load onto the complex. The vesicle containing the MHC class II-peptide complex shuttles towards the plasma membrane for presentation to $CD4^+$ T cells (Figure 1.6).

Although the two pathways mediate extracellular (exogenous route) and intracellular (endogenous route) these divisions are not strictly adhered to. Indeed, endogenous antigens internalised by DCs can generate peptide-MHC class I complexes that are cross-presented to $CD8^+$ T cells (Bevan, 1976; Shen & Rock, 2006). Similarly, endogenous proteins which are independent of the proteosome and TAP complexes may lead to peptide-MHC class II complexes recognised by $CD4^+$ T cells in a process known as cell autophagy (Li *et al.,* 2008). Mechanisms for cross-presentation are still to be determined although evidence suggests that exogenous antigens from phagosomes escape through channels or translocators into the cytostol or through phagosome-ER fusion leading to MHC class I presentation (Shen & Rock, 2006). Importantly, these overlapping mechanisms of cross-presentation can be exploited to induce antigen-specific immune responses. Whereby preparations of antigens are taken up by APCs and presented by MHC class I and class II molecules stimulating both $CD4^+$ and $CD8^+$ responses (discussed further in section 1.4).

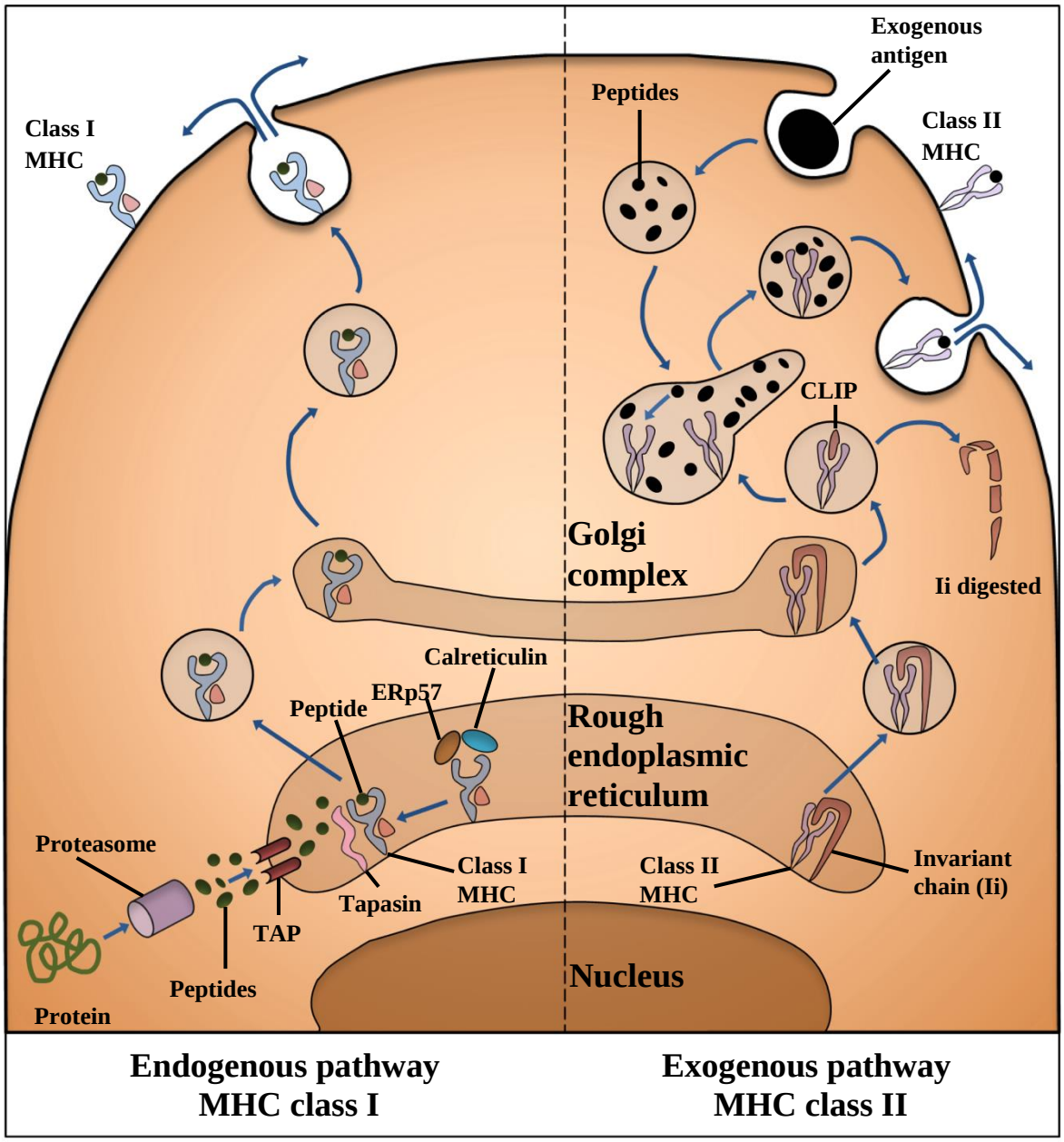


Figure 1.6 - Antigen processing and presentation on MHC class I and class II molecules. *(Left panel) Endogenous peptides generated in the cystol by the proteosome and cystolic proteases and are transported through the transporter associated with antigen (TAP) complex into the RER. Here MHC class I molecules are synthesised and are assembled along with the now processed 8-10 amino acid long peptides by the peptide loading complex (tapasin, ERp57, calreticulin and TAP molecules). The MHC-peptide complexes are trafficked via vesicles through the Golgi complex and to the cell surface to be presented to $CD8^+$ T cells. (Right panel) After MHC class II molecules are assembled in the RER with invariant chain they are transported via vesicles through the Golgi complex following which, the Ii undergoes proteolytic degradation leaving the class II-associated Ii peptide (CLIP). Exogenous antigens digested in endocytic compartments incorporate with vesicles transporting MHC class I molecules, CLIP is released from the peptide binding groove and peptides processed to the appropriate length (~10-16 amino acids) are loaded onto the MHC complex and shuttled to the cell surface to be presented to $CD4^+$ T cells and B cells.(Adapted from Kuby, 1997).*

(c) T cell effector functions

Naive lymphocytes cells require two specific signals to induce activation. The first is mediated through TCR recognition of cognate antigen bound to MHC molecules on the surface of DCs, in addition to the CD4 or CD8 co-receptor molecules binding to the MHC molecule. The second signal arises from the interactions of co-stimulatory receptor CD28 along with either CD80/CD86 surface proteins on APCs. The absence of either one of these signals during activation leads to the T cell undergoing apoptosis or left in a state of anergy (Harding *et al.,* 1992). This interaction between DCs and $CD4^+$ T cells promotes CD40/CD40L mediated cross talk which licences DCs to activate $CD8^+$ T cells and induce $CD4^+$ T cell differentiation or memory T cells. As a consequence, $CD4^+$ T cells are polarised towards either a Th_1 or Th_2 phenotype which produce cytokine profiles that orientate the immune response by either promoting CTL activation from $CD8^+$ T cells or promoting B lymphocyte production. Cytokine signalling from DCs constitute a third activation signal which determines the differentiation fate of T cells through the induction of T cell transcription factors. The secretion of IL-12 and IFNγ are key cytokines that are critical in the progression of Th_1 $CD4^+$ T cells leading to the production of their signature cytokines: IFNγ, IL-2 and IL-12 which promote $CD8^+$ mediated cytolytic activity. On the other hand, the secretion of IL-4 and IL-2 by DCs induce the development of Th_2 $CD4^+$ T cells leading to the release of cytokines (IL-4, IL-5, IL-10 and IL-13) which promote antibody responses towards the antigen. Therein, $CD4^+$ T cells sit centrally in the development and regulation of nearly all antigen-specific immune responses mediated through the function of their downstream subset of effector cell populations.

In regards to anti-tumour immunity, many *in vitro* and *in vivo* studies have now firmly established that $CD4^+$ T cell help is required to support the induction of strong CTL responses (Bennett *et al.,* 1997; Ossendorp *et al.,* 1998; Wang & Livingstone, 2003). The release of IFNγ and TNFα by $CD4^+$ T cells promotes cytotoxic effects towards tumour cells (Corthay *et al.,* 2005). In addition, IFNγ enhances tumour recognition and cell lysis by CTL by up regulating the cell surface expression of MHC class I and class II complexes on both immune cells and tumour cells (Dighe *et al.,* 1994). Activated tumour-specific $CD4^+$ T cells are also associated with the accumulation of increased frequency of activated $CD8^+$ T cells that infiltrate tumours (Wong *et al.,* 2008). Cognate memory $CD4^+$ T cells also assist in the expansion of cognate memory $CD8^+$ T cell population leading to

an increased elimination of tumour cells (Hwang *et al.,* 2007). The importance of $CD4^+$ T cell populations and their functions in the control of tumour are underlined by the use of adoptive therapies which engage tumour-reactive CTL. These early studies demonstrated that using CTL does not always promote effective tumour regression and that the lack of $CD4^+$ T cell help is an important factor owing towards the poor anti-tumour responses observed (Rosenberg & Dudley, 2004). Indeed in instances where $CD4^+$ T cells were either therapeutically depleted or were absent in knockout mice, the immune responses generated by $CD8^+$ T cells were reduced significantly (Hung *et al.*, 1998). It was proposed that in order to elicit strong and long lasting anti-tumour responses, DCs must engage both $CD4^+$ and $CD8^+$ T cells which recognise the same cognate antigen. Aside from promoting the expansion and maintenance of CTL for direct tumour killing, $CD4^+$ T cells have many diverse signalling functions which further contribute towards anti-tumour immunity. In fact, $CD4^+$ T cells have also demonstrated a capacity to recognise tumour antigens and induce direct tumour killing on their own (Daniel *et al.*, 2005). Several studies have demonstrated $CD4^+$ T cell direct tumour killing through a number of mechanisms including the use of perforins, Fas-FasL and TRAIL pathways (Porakishvili *et al.,* 2004; Stalder *et al.,* 1994; Thomas & Hersey, 1998). Moreover, $CD4^+$ T cell release of cytokines such as IFNγ recruit other immune effector cells to the tumour site which are able to engage in tumour killing (macrophages and eosinophils) (Hung *et al.*, 1998).

As a consequence, $CD4^+$ T cells can orchestrate anti-tumour immune activity through the release of IFNγ creating a pro-apoptotic and anti-proliferative tumour environment that encourages the immune cells present to activity seek and eliminate tumour cells (Ikeda *et al.*, 2002). Although $CD4^+$ T cells perform many roles in mediating indirect tumour killing, the induction of $CD4^+$ T cell specific direct tumour lysis is consisted to be a minor function of $CD4^+$ T cells as few tumours express MHC class II molecules on their cell surface. Therefore initial therapeutic approaches relied solely on priming $CD8^+$ T cells due to their primary function as cytotoxic effector T cells that directly destroy tumour cells.

$CD8^+$ T cells only recognise antigen in the context of presentation by MHC class I molecules and upon activation with secondary stimulatory signals result in their differentiation and proliferate to form a population of cytotoxic T cells (CTL). In the absence of the co-stimulatory signals, $CD8^+$ T cells generally produce a weakened, short-lived immune response causing these cells to be either be deleted or become anergic and

unresponsive to further stimulation. The extent of this response is variable from one T cell to another due largely to TCR affinity which dictates the antigen threshold required to stimulate a response in the absence of co-stimulation. In the presence of the appropriate co-stimulation signals and the presence of stimulatory cytokines (IL-2 and IFNγ) CTL proceed to elicit strong cytotoxic effect on tumour cells. The kinetics of TCR activation and sustained T cell functions are the focus of rigorous investigation with the intention of boosting anti-tumour immunity by isolating or engineering TCR's that demonstrate high avidity and affinity for their cognate antigen (Johnson *et al.,* 2009; Porter *et al.,* 2011; Curran *et al.,* 2012). Following tumour antigen recognition CTL mediated cell death occurs through one of two cell-cell lysis pathways. The first is through the targeted release of perforins and granzymes by CTL. Secretory lysosomes containing cytolytic toxins mobilise at a specific cleft that forms between CTL and target cell membranes releasing the contents into target cells causing apoptosis (Andersen *et al.,* 2006). The second mechanism is based on the activation of apoptosis via death receptors such as triggering Fas-FasL and TRAIL (TNF-related apoptosis inducing ligand) on the surface of target cells. The Fas ligand (FasL) is present on the surface of CTL and can trigger tumour cell apoptosis through its interaction with Fas receptor (CD95) on tumour cells. This interaction brings about the formation of a death inducing signalling complex (DISC) and subsequent initiation of downstream signalling of the caspase cascade resulting in programmed cell death. A similar response occurs following the engagement of death receptors on tumours via TRAIL. A third indirect pathway is mediated by the secretion of cytokines (TNFα and IFNγ) during CTL stimulation that bind to their respective receptors on target cells and lead to the direct triggering of caspase cascades (TNFα) and the induction of transcription factors that increase MHC class I presentation and FasL on the surface of tumours (IFNγ).

1.3 Tumour Immune Evasion

As discussed previously, the dynamic interactions between tumours and the immune system play an important role in tumourigenesis. The escape of tumours from the control of the immune system is a consequence of an amalgamation of multiple signalling mechanisms arising from the tumour and its influence on the surrounding TME and the immune response thereafter. Malignant cells expertly exploit the immune systems failsafe

mechanisms in place, which are intended to prevent autoimmunity and use those that promote wound healing following infection/injury to help facilitate tumour survival through the resistance of cell lysis, and promotion of cell proliferation and dissemination. A brief summary of these mechanisms have been described earlier in respect to immunosurveillance, "danger signals" and inflammation however for escape to occur, contributing factors from both the tumour cells and immune cells must be considered.

1.3.1 Tumour cells: Evading immune destruction

Several mechanisms employed by tumour cells have been identified which result in immunosuppression. As described earlier, the presentation of antigens in the context of MHC class I and class II molecules is essential in early activation phases during lymphocyte priming and during the effector phase of an immune response. Absence of antigen presentation through the loss or down regulation of genes that encode MHC complex associated proteins is a frequent occurrence in metastatic legions compared to primary tumour (Maeurer *et al.,* 1996; Hicklin *et al.,* 1999). The absence of MHC class I is often a consequence of mutations of the β2m subunit leading defective assembly which prevent immune recognition of tumours. Loss of MHC class I presentation may also be a consequence of defects in the tumour antigen processing machinery. More specifically, mutations leading to the loss or down regulation of immunoproteasomal subunits LMP2 and LMP7 (*PSMB9* and *PSMB8* genes) and peptide transporter genes *TAP1* and *TAP2* (Seliger *et al.,* 2003; Chen *et al.,* 1996; Agrawal *et al.,* 2004) have been associated with several tumour types such as melanoma, colorectal carcinoma, renal cell carcinoma pancreatic cancer and prostate cancer (Kageshita *et al.,* 1999; Cabrera *et al.,* 2003; Dovhey *et al.,* 2000; Pandha *et al.,* 2007; Sanda *et al.,* 1995). The loss of MHC class I leave tumours more susceptible to NK cell lysis via NKG2A/MICA/B interactions and suppression of the inhibitory KIR molecules, however one further strategy that the tumours may employ is the deletion or down regulation of surface expression of MICA/B (Khong & Restifo, 2002). The effective destruction of tumour cells presenting immunogenic antigens on the other hand, present the problem of epitope immunodominance leading to the loss of antigen variants to target. As a consequence this phenomenon it thought to have occurred in patients with recurrent metastasis following immunotherapy with a specific TAA epitope (Yee *et al.,* 2002; Jäger *et al.,* 1996). Thus, the adoption of simultaneous targeting of multiple TAAs, particularly those that are

essential for tumour growth may help avert the emergence of tumour variants that immune cells fail to recognise.

Many studies have drawn attention to the down-regulation of co-stimulatory molecules such as B7.1 and B7.2 by tumours as a factor leading to suboptimal immune cell activity and are seen as a contributor to T cell anergy (Koyama *et al.*, 1998; Harding *et al.*, 1992). In studies which transfect and restore the expression of B7 co-stimulatory molecules on tumours have demonstrated an increased induction of CTL mediated rejection in *in vitro* and in animal models (Hodge *et al.*, 1994; Townsend & Allison, 1993; Li *et al.*, 1994). Aside from mechanisms that impair surface MHC molecule expression, tumours may also alter the expression of surface receptors in order to avoid immune targeting. For example, the expression of non-classical *HLA* genes such as *HLA-G* and *HLA-E* protect MHC class I negative tumours from NK cell mediated lysis (Paul *et al.*, 1998; Marín *et al.*, 2003, de Kruijf *et al.*, 2010). To a similar end, tumours can down regulate or delete the surface expression of Fas and TRAIL receptors in order to prevent T cells activating cellular apoptosis through these death signalling pathways. The inhibition of these pathways are also a consequence of gene mutations of molecules involved in downstream signalling (Park *et al.*, 2001; Shin *et al.*, 2001). Besides avoiding death through apoptotic signalling, tumour can also prevent cytotoxin induced cell lysis by CTL and NK cells through their overexpression of a serine protease inhibitor, *PI-9/SPI-6* which inhibits granzyme B/perforins pathways (Medema *et al.*, 2001).

In addition to resisting T cell mediated destruction, tumours may also instigate a "counter-attack" through their own expression of FasL observed on the surface of several tumour types leading to T cell apoptosis. To date, the role of tumour FasL in mediating tumour escape has remained controversial due to it pro- and anti-inflammatory activities (Ryan *et al.*, 2005). Finally, tumours may also employ contact independent mechanisms as a means of immune escape. As discussed earlier, tumour cells can modulate the TME by secreting immunosuppressive cytokines and other factors such as VEGF, IL-10 and TGFβ which inhibit DC maturation, trafficking to lymph nodes and antigen presentation (Gabrilovich *et al.*, 2004). Other immunosuppressive molecules released by tumours such as ICAM-1, prostaglandin E_2, gangliosides, and indoleamine 2,3-dioxygenase (IDO) can also hinder anti-tumour responses (Liu *et al.*, 2005; Greenhough *et al.*, 2009; Birklé *et al.*, 2003; Munn & Mellor, 2007).

1.3.2 Tumour evasion mechanisms associated with the immune response

Without doubt the one crucial trait that tumours possess that gives them the advantage to succeed in populating the body is that they are derived from self. The immune system has in place a tightly regulated system of tolerance towards self to prevent T cells from reacting to self cells, meanwhile retaining the functions to survey and eliminate damaged or altered cells. The current knowledge of T cell development and tolerance to self and the more recent understanding of immune regulators and suppressive cell populations and their relevance in cancer must be considered as they present a major obstacle in generating anti-tumour responses through cancer immunotherapy.

1.3.2.1 Immunological tolerance and cancer

During their development, lymphoid progenitor cells emerging from the bone marrow are classed as double negative ($CD4^-/CD8^-$). Upon reaching the thymus, they undergo stages of development and selection and become single positive for either $CD8^+$ or $CD4^+$ depending on whether they recognise MHC class I or class II respectively. Following this, thymocytes enter the medulla where they undergo negative and positive selection processes through central tolerance mechanisms. During selection thymocytes are exposed to epithelial cells of the thymic cortex (cTECs) which present them with MHC molecules. Thymocytes that form any MHC-peptide complexes are maintained as this interaction also provides them with a survival signal; those that fail to form complexes are deprived of the survival signal and undergo death by neglect. Thymocytes that exhibit too high affinity TCR for self-antigen are eliminated by negative selection as a measure to prevent autoimmune reaction. Alternatively, these cells may avoid deletion by undergoing gene rearrangement at the TCRα loci, which consequently alters the specificity of the TCR in a process known as receptor editing (Santori *et al.,* 2002). Despite these processes, auto-reactive T cells may still escape into the periphery (Anderton *et al.,* 2002). The surviving population of thymocytes bear TCR which are capable of forming MHC-peptide complexes that are of medium affinities which are now of a committed linage. Next, thymocytes meet medullary thymic epithelial cells (mTECs) which express a diverse range of tissue specific self-antigens (TSA) promoted through the expression of a nuclear regulatory protein known as autoimmune regulator (AIRE). Once again high affinity auto-reactive thymocytes are deleted. Following rigorous selection, the selected pool of $CD4^+$

and $CD8^+$ T lymphocytes migrate to the periphery to begin to assess antigens presented by APCs in order detect pathogenic threats or infected and transformed cells. Following maturation, T lymphocytes pools are maintained through constant apoptosis which ensures homeostatic balance of the immune system. For example, following antigen recognition, reactive T cells trigger T cell expansion and engage and eliminate antigen bearing cells. Once the antigen is no longer detected, the presence of activated T cells reduces, returning back to a state of equilibrium. This winding down process relies on the elimination of surplus T cells leaving a small contingent to form a pool of memory T cells.

1.3.2.2 Immune regulators and suppressor cells

As alluded to earlier, macrophages are classed in accordance to their response to tumour in a similar manner as is designation for $CD4^+$ Th cells (Th_1 and Th_2). Macrophages that are "classically activated" in response to TME stimuli such as IFNγ and microbial molecules (such as LPS) are polarised towards the M_1 phenotype. These cells are characterised by their production of IL-12 and IL-23 and subsequent activation of Th_1, anti-tumour cytotoxicity, production of iNOS and proficiency as APCs for T lymphocytes. In contrast, macrophages that are "alternatively activated" in response to IL-4 and IL-13 revert to a M_2 phenotype. The distinguishing features of these cells are their promotion of ECM remodelling, secretion of growth factors which support tumourigenesis and high levels of IL-10 production promoting Treg cells (Sica & Mantovani, 2012). Like Th_1/Th_2 balance, the loss of homeostasis between M_1/M_2 populations could induce chronic inflammatory environments (excess M_1) or induction of immune suppression (excess of M_2). Tumour associated macrophages (TAM) recruited to the site of tumour share resemblance to M_2 macrophages and as a consequence their cytokine profile helps to shape a Th_2 immune response whilst down regulating a Th_1-mediated adaptive response (Martinez *et al.*, 2009).

Myeloid derived suppressor cells (MDSCs) are inflammatory monocytes composed of incomplete inactivated granulocytes, macrophages and dendritic cells and are defined with a $CD11b^+/CD33^+/CD14^-$ phenotype (Peranzoni *et al.*, 2010). High levels of MDSCs have been detected during chronic inflammation and tumourigenesis (Almand *et al.*, 2001; Ostrand-Rosenberg & Sinha, 2009). MDSCs are capable of direct suppression of both $CD4^+$ and $CD8^+$ T cell activity through a number of mechanisms. Firstly, through their surface expression of CD28L (B7.1) which bind with high affinity to T cell surface

molecule cytotoxicity T lymphocyte antigen 4 (CTLA-4) which brings about negative control and inhibits activity of T cells. It has also been shown that MDSCs can also induce Treg cells by their release of IL-10, increase production of arinase and depletion of cysteine reduce the activation and function of T cells and up regulate COX/PGE_2 (Ostrand-Rosenberg & Sinha, 2009).

To date, the most well studied population of immunosuppressive cells are $CD4^+/CD25^+/FoxP3^+$ regulatory T (Treg cells). These cells can be broadly divided into two distinct classes; the first are naturally occurring Treg cells selected on their high avidity interactions in the thymus and the others are antigen-induced Treg cells generated the peripheral tissues in order to enforce peripheral tolerance to self-antigens (Curotto *et al.,* 2009). The latter originate from $CD4^+/CD25^-$ T lymphocytes which acquire $CD25^+$ expression induced by suppressive cytokines such as IL-10 and TGF-β (Chen *et al.,* 2003). Treg cells are able to suppress anti-tumour activity by inhibiting T cells via their surface expression of CTLA-4, a major inhibitory signal for activated T cells which subsequently dampens the adaptive immune response. Treg cells may present the most formidable immunosuppressive huddle to be overcome in order to generate strong, long lasting anti-tumour responses using immunotherapy. In 2004, Chakraborty and colleagues monitored antigen-specific CTL following immunisation of melanoma patients with APC-loaded with peptide or autologous tumour lysates. They observed a peak in CTL response by day 7 post-vaccine, followed by a return to pre-vaccine levels by day 28. The decline showed an inverted correlation to the expansion of $CD4^+/CD25^+$ Treg cells and increased level of IL-10-secretion Treg cells in post-vaccine peripheral blood lymphocytes (Chakraborty *et al.,* 2004). Many studies have since demonstrated the therapeutic benefit of depleting or inhibiting Treg cells prior to vaccination can lead to enhanced and prolonged CTL-mediated responses (Dannull *et al.,* 2005; Quezada *et al.,* 2008). The more recently identified Th_{17} are generated under the influence of TGF-β and IL-6 released from activated $CD4^+$ T cells. Th_{17} cells are credited with the augmentation of anti-tumour effects through the secretion of IL-17 (Martin-Orozco *et al.,* 2009) however some studies suggest that their inflammatory activities may stimulate Treg cells and promote tumourigenesis (Charles *et al.,* 2009; Wu *et al.,* 2009).

1.4 Cancer Immunotherapy

Although forms of immunisation therapies against cancer have been attempted for well over a hundred years, it is only during the last fifty years that have seen the identification of antigens, TCRs and molecular modulators that are responsible for immune recognition of tumour cells. The emergence of new technologies, bring further insight into the inner workings of the immune system and how it could be harnessed to destroy tumours without targeting healthy self tissue. Immunotherapy can be broadly divided into three major categories; passive immunotherapy which utilise immune-effector molecules mostly through the administration of immunoglobulins active immunotherapy which attempt to activate immune responses directly within the patient, whereas the final approach, adoptive immunotherapy is built on *ex vivo* T cells which are stimulated prior to reinfusion back into the patient.

1.4.1 Passive cancer immunotherapy

One of the most effective therapeutic strategies to date can be found in the form of exogenously produced monoclonal antibodies (mAb) which are immunoglobulins designed to target TAAs and cell receptor molecules that are important for tumour growth. Treatment with mAbs attempts to mimic the existing mechanism of immunoglobulin mediated immune responses within the body. Immunoglobulins that have been generated to bind previously identified TAAs or other molecules expressed by tumour cells can trigger antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). In effect, this action allows tumours to become more visible to NK cells which secrete Th_1 promoting cytokines such as IFNγ, helping to potentiate an adaptive immune response (Weiner, 2010). One of the most widely used mAb therapies is an anti-CD20 mAb (Rituximab) for B cell non-Hodgkin's lymphoma (Scott, 1998; Dotan *et al.,* 2010). Alternatively, mAbs may act as receptor blockade mechanisms which can be used to prevent access of growth factors or inhibit the transduction of signals. Perhaps the most well-known drug trastuzumab (Herceptin), specifically targets a tyrosinase kinase receptor molecule HER2 found to be over-expressed on the cell surface in certain breast carcinomas. The mAb inhibits the signalling cascade that normally activates following receptor interaction with HER and the tumour cells undergo apoptosis (Hudis *et al.*, 2007).

In addition to targeting antigens involved in tumourigenesis, mAbs can be utilised against immunological targets that are critical to the anti-tumour response. For example, a mAb known as Ipilimumab acts as a CTLA-4 blockade mAb which potentiates T cell activation by preventing inhibitory receptor signalling (CTLA-4) from occurring allowing antigen-specific activated CTL to destroy metastatic melanoma when used in combination with peptide vaccines (Hodi *et al.,* 2010). The therapeutic success of Ipilimumab has drawn attention on other immunological targets such as PD-1 blockade (PD-L1/B7.1) which has demonstrated durable anti-tumour activity in colorectal cancer, castrate resistant prostate cancer and renal cell carcinoma (RCC) (Brahmer *et al.,* 2010). Further mAb therapies have been developed to utilise mAbs as vehicles to deliver potent cytocidal payloads to the site of tumours. This has included engineering of Fc regions of mAbs using cytocidal agents such as toxin (Mylotarg) or radionuclide (Zevalin) or pro-drug conjugates which home to tumour cells and exert their effects (Ducry & Stump, 2010; Scott *et al.,* 2012).

1.4.2 Active cancer immunotherapy

Whole cell vaccines: Whole cell vaccines consist of genetically modified autologous or allogeneic tumour cells that are irradiated before injection into the patient. These *in vitro* cell modifications can facilitate the cells to secrete immunostimulatory cytokines (GM-CSF, IL-7 IFNγ and IL-2) and express co-stimulatory molecules (ODN, TLR ligands, OX40) in order to present antigen to T cells via APC (Parmiani *et al.,* 2000; Ali *et al.,* 2000). The use of autologous material ensures that patients are vaccinated with antigen that are relevant to their tumour and the approach does not require elucidation of individual antigen. Alternatively the use of allogeneic tumour cells can bypass time consuming therapy with patient tumour cells. Moreover, cross priming of $CD8^+$ T cells by APCs does not require HLA-matching between the tumour cell vaccine and the recipient (Nemunaitis *et al.,* 2006). The results from clinical trials have proven to be only partially effective in generating clinically relevant responses. Clinical trials have reported complete or partial responses in only a small proportion of patients treated with autologous tumour cells expressing cytokines in melanoma and kidney cancer (Parmiani *et al.,* 2000). More recent efforts to further stimulate immune responses of whole cell vaccines are begin pursued by combining other treatment modalities such as administering mAbs that target cognate antigen found on injected tumour cells and using immune blockade (CTLA-4) (Kim *et al.,* 2008; Schoenfeld *et al.,* 2010). Despite their limited successes to date, whole

cells vaccines have provided an important stepping stone towards antigen specific vaccines.

Heat shock proteins: Heat shock proteins (HSPs) consist as part of a large family of ubiquitously expressed inducible proteins that are responsible for transportation of intracellular peptides to APCs. These molecular chaperons are also responsible for the induction of both cellular (CTL and antibody generated) and innate (activation of DC and NK cell activity) immune response via HSP receptors such as Lox-1, SR-A, TLR2 and TLR4 present on APCs (Srivastava, 2002; Tsan & Gao, 2009). Most HSP clinical trial vaccines followed the removal and purification of HSP-peptide complexes (HSP-PC) from the primary site of the tumour and were reinfused back into the patient. In a phase I trial conducted using autologous HSP96-PC derived from various malignancies, it was shown that 6/12 patients developed CTL and 8/13 patients showed increased NK cell populations after immunisation (Janetzki *et al.,* 2000). Several clinical trials have been conducted using an autologous tumour gp96-PC preparation commercially known as Vitespen and have so far demonstrated safety and feasibility colorectal cancer, melanoma, non-Hodgkin's lymphoma, pancreatic adenocarcinoma and RCC (Randazzo *et al.,* 2012). Approaches have been taken to enhance the potency of HSP-PC in therapeutic settings such as extracting HSP-PC from DCs fused to tumour cells which would contain more immunogenic antigens compared to with HSP-PC derived from tumour (Gong *et al.,* 2010). Studies to induce tumour killing using *ex vivo* preparations of IL-2 activated NK cells pulsed with an HSP70-derived peptide (TKD) demonstrated NK cell mediated cytolytic responses in a phase I trial of colon and lung cancer patients (Krause *et al.,* 2004). Moreover, the 14mer TKD peptide was used to generate a mAb (cmHsp70.1) which was shown to recognise membrane bound HSP in human tumours and is capable of significantly inhibiting tumour growth in mice bearing CT26 tumours through ADCC, an effect that could be further enhanced with the addition of IL-2 (Stangl *et al.,* 2011). The major advantage of this approach in cancer immunotherapy is that it relies primarily on NK cell activity and other macrophages to mediate tumour killing and therefore not restrained to targeting only MHC-positive tumour cells or face the challenges of overcoming tolerance to self-antigens. Although, increased NK cell activity and consequently their secretion of IFNγ may in turn promote CTL mediated tumour destruction and help skew $CD4^+$ T cells to perpetuate Th_1 responses (Cheng et *al.,* 2013).

Peptide vaccines: The identification of many proteins that are associated with cancer has subsequently led to the identification of short epitopes that are presented and recognised by CTL in the context of MHC class I molecules. Peptide vaccines were formed on the basic principle that antigens specific to tumours could be made targets of the immune system. The discovery that some patients expressed T cells specific for CT and differential antigens in their tumours instigated the development of peptide vaccines. To date, hundreds of TAA peptide sequences have been reported (http://cancerimmunity.org/peptide/) leading to the design of peptide vaccines (Table 1.1). First generation peptide vaccines consisted of one or multiple HLA class I restricted peptide epitopes which were emulsified with Freund's incomplete adjuvant or Montanide or transfected into DCs prior to vaccination. Later, findings that indicated towards the pivotal role of $CD4^+$ T helper cells in the induction of CTL and memory responses lead to their inclusion alongside MHC class I epitopes (Toes *et al.,* 1999; Overwijk *et al.,* 1999). These earlier trials highlighted many issues need to be overcome in order to reap therapeutic benefits, not least the need to enhance the immunogenicity of peptides. The binding affinities of peptides to the cognate MHC molecules vary from one peptide to another leading to competition during peptide editing when loading onto MHC molecules during processing. One novel method of increasing the potential for MHC presentation is using TAA derived peptides linked to (LRMK) Ii-Key, which offers the associated peptide preferential binding to MHC class II molecules. Studies using HER2-derived peptide vaccine (Ii-Key/Her2/neu), reported that the linkage helped increase the potency immunological responses (Voutsas *et al.,* 2007; Sotiriadou *et al.,* 2007) and have demonstrated safety and high immunogenicity in two phase I trials on $HER2^+$ breast and prostate cancer patients (Sears *et al.,* 2011; Perez *et al.,* 2010b). The combined use of IL-2 and a gp100 derived peptide was recently shown to enhance anti-tumour response and prolong progression-free survival compared with IL-2 therapy alone in melanoma patients demonstrated the need to stimulate the immune system in order to generate more durable responses (Schwartzentruber *et al.,* 2011). Some of the more recent promising agents to partner peptide vaccines include blockade of immune checkpoints using anti-CTLA-4 and anti-PD-1 Ab which are expected to improve tumour reactivity in T cells and enhance anti-tumour immunity (Pardoll, 2012). Programmed cell death 1 (PD-1) is an inhibitory surface membrane molecule expressed on T and B cells, its ligand is expressed in some peripheral tissues and in tumours and has been assessed in cancer patients with $PD\text{-}1^+$ tumours demonstrating objective responses in 9/25 (36%) cases (Topalian *et al.,* 2012).

There are several advantages of synthetic peptide vaccines have over other immunotherapy approaches. In addition to their ease of manufacture and modification, stability, and safety demonstrated for clinical use they have the benefit of personalising vaccines to individual patients based on their tumour antigen fingerprint in feasible time and financial scale. Although, short peptide vaccines have the limitation of potentially binding to non-professional APCs (e.g. fibroblasts) on administration and thus inducing tolerance to the antigen. Moreover, prior knowledge of immunogenic epitopes that have been assessed for HLA-compatibility before use reduces the number of patients that can be treated with the same peptide. Finally successful elimination of tumours bearing cognate antigens may give raise to tumours capable of immune evasion due to their lack of antigen expression. Therefore current peptide vaccines have shifted to multi-epitope (HLA class I and class II) and multi-valent approaches. Longer peptide sequences (ranging from 12 – 50 amino acids in length) circumvent many of the issues raised above. Long peptides are also more likely to contain multiple HLA class I and HLA class II epitopes, which increases their application to all patients irrespective of HLA type. A number of recent studies in clinical trials have demonstrated their ability to induce cytokine responses attributed to the activation of immune responses (Speetjens *et al.,* 2009; Welters *et al.,* 2008; Leffers *et al.,* 2009; Bijker *et al.,* 2008). Peptide cocktail vaccines administered to induce responses using multiple antigen targets can help combat the generation of antigen-loss variant tumours. Two phase II clinical studies consisting of multiple TAA (IMA901) plus pre-treatment with cyclophosphamide to reduced Treg cell populations have demonstrated immune responses and longer overall survival in HLA.A2^{+} renal cell cancer (Walter *et al.,* 2012) and colorectal cell cancer patients (Walter, PIVAC conference 2011) with further phase III studies currently underway (Walter *et al.,* 2012).

Dendritic cell vaccines: As reiterated throughout, the actions of DCs are essential in determining the fate of the immune response to cancer. Therefore it is unsurprising that DCs are the topic of extensive investigation for immunotherapeutic intervention due to their unique function in orchestrating both innate and adaptive immune responses. This approach has also lead to the first active cancer immunotherapy meeting FDA approval in 2010 for the vaccine Provenge (Sipuleucel-T) for use in castration resistant prostate cancer (Kantoff *et al.,* 2010a). Sipuleucel-T consists of *ex vivo* generated DCs cultured with a recombinant fusion protein composed of TAA prostatic acid phosphatase (PAP) linked to GM-CSF with results demonstrating a four month-prolonged median survival in phase III

trials (Higano *et al.,* 2009; Kantoff *et al.,* 2010b). The using of *ex vivo* generated DCs hold some key advantages in that they are capable of priming both $CD4^+$ and $CD8^+$ T cells. Studies have demonstrated that anti-tumour responses can be improved by targeting specific sub populations of DCs that favour the induction of Th_1 cell mediated immune responses (Bonifaz *et al.,* 2004). DCs that express CD205(Ly-75) on their cell surface present antigens to both MHC class I and class II molecules whereas, DCs that are positive for 33D1 antigens present only on MHC class II (Dudziak *et al.,* 2007). Several DC vaccine trials have been conducted using *ex vivo* cultured DCs and loaded with GM-CSF and/or IL-4 and tumour specific targets including HLA-restricted peptides in metastatic prostate cancer and melanoma (Murphy *et al.,* 1999; Thurner *et al.,* 1999), tumour eluted peptides or lysates in melanoma, RCC, and glioma (Nestle *et al.,* 1998; Höltl *et al.,* 1999; Yu *et al.,* 2001), autologous or allogeneic RNA in colon and prostate cancer (Nair *et al.,* 2002; Mu *et al.,* 2005) and using DC co-stimulation molecules such as NKT cell ligand α-Galactoslceramide (αGalCer) in advanced cancers (Chang *et al.,* 2005). Taken together, these studies all demonstrated that DC-based vaccines are safe, are capable of inducing antigen-specific T cell expansion with clinically significant responses in some patients. These studies also highlighted that clinical responses are gradual however patients can undergo long lasting remission.

DNA vaccines: DNA constructs consist of DNA coding sequences derived from a TAA inserted into a bacterial plasmid which can be delivered to patients via liposomes, nano-particles, bacteria and viruses. An advantage of using such an approach is that CTL's the presentation of antigens to APCs may be generated against multiple epitopes of both MHC classes and can be designed to encode co-stimulatory molecules, cytokines, CpG oligonucleotide sequences to enhance the immunological effectiveness of targeted APCs. One vaccine strategy uses a DNA vector encoding an antibody and T cell epitopes (termed ImmunoBody) can directly present or cross present epitopes via the high affinity Fcγ (CD64) receptor in APCs that can consequently activate high avidity T cells capable of recognising and destroying tumours (Pudney *et al.,* 2010). A phase I/II using a DNA vaccine (SCIB1) using the ImmunoBody approach is currently underway in patients with melanoma (ClinicalTrial.gov Identifier: NCT01138410/completion 2017). DNA vaccines are administered through a number of routes including intramuscular, intranasal, intramucosal or intradermally by coating DNA constructs onto gold micro-particles and administering them using gene gun immunisation (Stoitzner *et al.,* 2006). The constructs

are taken up by surrounding cells or residing langerhans cells which allow APCs to cross present and initiation of the immune response. Previous studies using animal models have demonstrated that the mode of administration seem to be a determining factor in the type of response generated. For example, intramuscular immunisation predominantly favours a Th_1 response whereas gene gun assisted delivery favours a Th_2 response (Weiss *et al.*, 2002). A recent study has challenged this notion suggesting that gene gun immunisations can polarise T cell to Th_1 responses demonstrated the induction of antigen-specific CTL capable of *in vivo* and *in vitro* tumour cell killing (Nguyen-Hoai *et al.*, 2012). To date, studies conducted in numerous phase I clinical setting confirm DNA vaccines and their various routes of administration mentioned above to be safe and capable of eliciting clinically relevant antigen-specific immune responses (Senovilla *et al.*, 2013).

Viral vector vaccines: Vectors encoding TAAs attempt to exploit the potent immune responses directed towards viral components to potentiate immune reactivity against the TAA. Retrovirus, poxvirus, adenovirus and HPV have all been investigated in pre-clinical models with human trials currently underway. In a recent phase II trial, 125 castrate-resistant prostate cancer patients receiving PSA-targeted poxviral vaccine (PROSTVAC-VF) which included co-stimulatory molecules (B7.1, ICAM-1 and LFA-3) demonstrated an overall survival benefit of 25.1 months over the control cohort (16.6 months) immunised with empty vector (Kantoff *et al.*, 2010b). Many recombinant bacterial vaccines derived from strains of *Salmonella choleraesuis, Bacillus Calmette-Guerin* (BCG) and *Listeria monocytogenes* are currently under evaluation for their potential use in immunotherapy (Avogadri *et al.*, 2005; Yuk *et al.*, 2010; Wood *et al.*, 2009).

1.4.3 Adoptive cancer immunotherapy

In contrast to active immunotherapy, adoptive therapies achieve tumour reactive T lymphocyte *ex vivo* through the activation and expansion of autologous tumour specific T cell populations which are then transferred back into the patient. The principle behind adoptive T cell therapy (ACT) is on the observation that certain lymphocytes migrate to tumour sites and may promote some form of anti-tumour response. This cells referred to as tumour infiltrating lymphocytes (TILs) are extraction from tumour lesions and clonally expanded *in vitro* under the influence of a cocktail of cytokine modulators including IL-2. Prior to ACT therapy, patients may undergo pre-conditional lymphodepletion using non-

myeloablative chemotherapy, total body irradiation (TBI) and cytokine therapy to remove the patients host immune system. Although this may seem counter-intuitive, studies have demonstrated that the depletion of immune cells prior to transfer of reactive TILs augment anti-tumour efficacy (Rosenberg *et al.,* 2011). This may be a consequence of the elimination of immunosuppressive immune components such as Treg cells and MDSCs (using cyclophosphamide and fludarabine), the depletion of endogenous immune cells that compete for activating cytokines and the increased activation and availability of APCs (Gattinoni *et al.,* 2006). ACT therapy has been used in several clinical trials conducted by Rosenberg and colleagues in the treatment of late-stage melanoma where it has demonstrated impressive results in achieving significant regression of tumour lesions (Dudley *et al.,* 2005; Rosenberg *et al.,* 2008). This approach does highlight the over-represented role of regulatory cells and other negative immune modulators (TGF-β) that exist in tumours. Indeed, selective depletion of Treg cells through CTLA-4 and PD-1 blockade or TGF-β prior to ACT therapy many enhance tumour regression (Phan *et al.,* 2003). In mice, T cells pulsed with a NKT cell ligand known as α-Galactoslceramide (αGalCer) prior to infusion potential enhanced anti-tumour responses through the maturation DCs and activation of NK, T and B cells (Choi *et al.,* 2011). Further investigation of the differentiation features of effector T cells concluded that the later effector T cells differentiate in long term culture, the less effective they were in generating *in vivo* responses. This challenged the clonal expansion principle that the methodology using long term culture relied upon (Gattinoni *et al.,* 2005). Recently, the same group identified a stem cell-like memory T cell population which have phenotypic characteristics similar to that of naive T cells, however also expressed (CD95, IL-2Rβ, CXCR3 and LFA-1) and compared to naive T cells, they had increased proliferative capacity and elicited stronger anti-tumour responses in animal models (Gattinoni *et al.,* 2011). New findings such as these which appear to single out T cell subset populations could significantly improve the efficacy of ACT therapy in the future. A limitation of this strategy is often deriving large populations of reactive T cells specific to each individual is problematic.

ACT therapy has been further refined to activate autologous T cells that recognise identified TAAs such as gp100 and MART-1 which are prevalent in melanoma (Powell *et al.,* 2006; Duval *et al.,* 2006). Genes that encode for α/β TCR's isolated from T lymphocytes that demonstrate high avidity for a selected TAA can be delivered to lymphocytes using viral vectors in order to generate a whole population of T cells with the

same high avidity specificity for a particular antigen. Despite initially achieving successful regression of metastatic melanoma, there have been reports of adverse toxicity in which patients experienced including severe transient colitis and evidence of autoimmunity against normal tissues expressing rare cognate antigen (Parkhurst *et al.*, 2011; Johnson *et al.*, 2009). This autoimmune response may be attributed to a TCR cross-pairing phenomenon occurring, in which an endogenous α/β TCR may recombine with the respective α/β chains of a transgenic TCR forming a third hybrid TCR. The new hybrid TCR may in turn lose specificity for the antigen of interest in favour for another, self-antigen expressed on normal cells (Brenner, 2010). As described earlier, tumour may evade immune recognition by down regulating their expression of MHC class I molecules which renders the application of engineered α/β TCRs redundant in some cases.

One approach that may help target MHC-negative tumours is using engineered T cells to express chimeric antigen receptors (CARs) designed to induce high avidity antigen-specific immune responses against surface antigens found on tumour cells, bypassing the need of MHC-peptide presentation (Bridgeman *et al.*, 2010). The CARs are constructed as a hybrid between a TCR and an immunoglobulin in that the light and heavy chain of variable region of Ig's, or single chain Fc (scFv), with a flexible hinge or spacer, transmembrane domain and endodomain (for intracellular signalling) upon scFv recognition of antigen. The current generation of CARs also include one or more T cell co-stimulatory molecules in the endodomain to circumvent T cell activation through APC interaction (Jena *et al.*, 2010).

Studies that combine immunotherapies with conventional modalities such as chemotherapy suggest valuable synergistic effects that could be utilised to circumvent tumour immune evasion and tolerance issues (Andersen *et al.*, 2008). The future of cancer treatment may indeed lie in the use of multiple approaches such as debulking tumour masses (chemotherapy, radiotherapy, surgery), in combination with immunotherapeutic treatments that enhance tumour specific T cell responses (peptide vaccines plus agonistic mAbs cytokines, TLR ligand adjuvants) whilst counteracting immunosupressive components (depletion of Treg cells and MDSCs and immune blockade of CTLA-4/PD-1).

1.5 T21: A Potential Tumour Associated Antigen and Target for Cancer

1.5.1 Serological identification of T21

To date, among the multitude of identified and characterised human tumour-associated antigens, research continues towards determining the most effective candidate antigens to incorporate into antigen-specific immunotherapeutic vaccines. Several tumour associated antigens identified by T cell responses such as Her2/neu and MAGE-1 have demonstrated that $CD4^+$ and $CD8^+$ T cells and B cells form an integrate immune response against tumour, having also been defined using serological analysis of cDNA expression (SEREX) (Sahin *et al.,* 1997). Since the specificity of B cell responses are regulated by negative selection in the bone marrow, the induction of high-titre antibody responses require cognate $CD4^+$ T cell help indicating towards the capability of both the adaptive and humoral systems to elicit an orchestrated response against tumours. SEREX technology was first devised in 1995 (Sahin *et al.,* 1995), and combines serological analysis with antigen expression and cloning approaches to identify, in this instance, human tumour antigens which elicit antibody responses in cancer patients. This method involves extraction of mRNA from tumour derived tissue which is then expressed in λ-phage display vectors to create cDNA expression libraries, these libraries are screened with patient sera, the positive colonies are isolated and cloned and sequenced and are finally put forward for DNA databank search.

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 (Figure 1.7). 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).

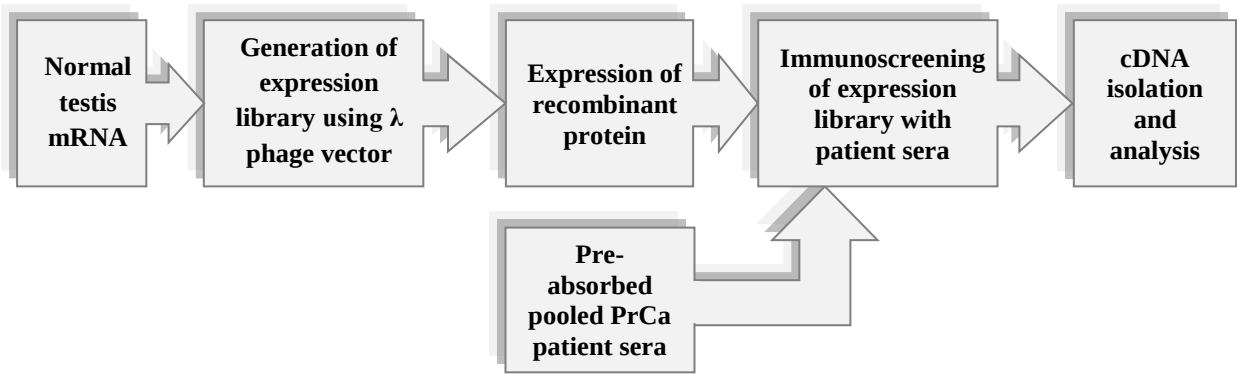


Figure 1.7 – Modified SEREX approach used to identify T21. *A normal testicular cDNA library was screened with allogeneic sera from prostate cancer patient, from which positive clones were isolated and analysed.*

1.5.2 Proposed study rationale

The identification of T21 and initial molecular characterisation has warranted further study to determine if it has the potential to be a tumour biomarker and/or be utilised as a target antigen for immunotherapy. This project proposed to achieve this within the scope of prostate cancer by advancing the current understanding of T21 through analysis of gene sequence similarities observed with *CEP290* and begin to extrapolate possible functional attributes of T21 and the role it may play in cancer. In order to validate the use of T21 as a target for immunotherapy, this study aims to complete a comprehensive mRNA and protein expression profile on various human prostate cancer cell lines (and other malignancies) and normal tissues. Evaluation of T21 as a potential prognostic indicator of prostate cancer using and both prostate cancer and benign tumours will be sought. Finally, this study aimed to evaluate the immunological potential of T21 derived overlapping 15mer peptides to generate immune responses in a pre-clinical transgenic mouse model as a pre-requisite for the development of a T21 directed cancer vaccine. In addition, positively identified peptides will be taken forward to assess their utility for immunotherapy and immunomonitoring using human prostate cancer patient PBMCs.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Reagents and a list of suppliers

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

Culture media	**Supplier**
C.T.L-Test media	Cellular Technology Ltd
KSFM, Opti-MEM media (w/o phenol)	Invitrogen
DMEM, HAMS-F12, McCoy's, RPMI media	Lonza

Culture media supplements	**Supplier**
2-mercaptoethanol (2-ME)	Sigma
Blasticidin	Invitrogen
Fetal calf serum (FCS)	Perbio Thermo Fisher
Fungizone	Lonza
HEPES buffer	Lonza
Human AB serum	Sigma
L-glutamine, Pyruvate	Lonza
Non-essential amino acids (NEAAs)	Lonza
Penicillin/Streptomycin (PenStrep)	Lonza

Additional culture reagents	**Supplier**
Acetic Acid	Fisher Scientific
Dimehyl Sulphoxide Hybri-Max (DMSO)	Sigma
IL-2, GM-CSF (murine)	R&D Systems

Incomplete Freund's Adjuvant (IFA)	Sigma
INTERFERin transfection reagent	Polyplus Transfection
Lipofectamine-2000	Invitrogen
Lipopolysaccharide (LPS)	Sigma
Phosphate buffer saline (dPBS)	Lonza
Polyinosinic Polycytideylic acid (Poly I.C)	Sigma
Staphylococcal exterotoxin B (SEB)	Sigma
Trypan blue	Sigma
Trypsin/Versene	Lonza
Vitamin E	Calbiochem

Chemical reagents	**Supplier**
2-methylbutane (Isopentane) and Acetone	Acros Organics
Acetonitrile	Sigma
Acrylamide (30%)	National Diagnostics
Agarose	Bioline
Ammonium sulphate	National Diagnostics
Boric acid and Calcium chloride ($CaCl_2$)	Sigma
Bovine Serum Albumin (BSA)	Calbiochem
Chloroform	Sigma
Coomassie Brilliant Blue	PhiBio Systems
Decon 90	Decon Laboratory Ltd
Dextran Sulphate	Sigma
Dithiothreitol (DTT)	Apollo Scientific
dNTPs	Promega
DNA ladders (1kb and 100bp)	Promega
Dried marvel skimmed milk	Premier Brands
Dry Anhydrous Ethanol	Sigma
Ethanol	BDH Merck
Ethylenediaminetetraacetic acid (EDTA)	Sigma
GelRed Nucleic Acid Gel Stain	Biotium
Glycerol	Sigma
Gold microcarriers (1.0µm)	Bio-Rad
Hydrochloric acid (HCl)	Fisher Scientific
Isopropanol	Fisher
iQ SYBR Green SuperMix	Bio-Rad
Magnesium chloride ($MgCl_2$)	Promega
Microscint-O Scintillation media	Perkin Elmer
M-MLV RT and 5X Reaction Buffer	Promega
Nuclease-Free water	Ambion
Octyl β-D-glucopyranoside (OGP)	Apollo Scientific
Oligo$(dT)_{15}$ Primer	Promega
Orange G powder	Sigma
Paraformaldehyde	Sigma
Polyinosinic-polycytidylic acid (Poly-IC)	Sigma
Polyvinylpolypyrolidone (PVP)	Sigma
Presept	Johnson and Johnson
RapidStep ECL Reagent	Calbiochem
RNasin Ribonuclease Inhibitor	Promega
RNA STAT-60	AMS Biotechnology

Spermidine	Sigma
Sodium dodecyl sulphate (SDS)	Sigma
Sodium chloride (NaCl)	Fisher Scientific
Sulphuric Acid	Fisher Scientific
TEMED	National Diagnostics
Tepol	Johnson and Johnson
[6-^{3}H]-Thymidine (^{3}Thy), 1 mCi (37 Mbq)	Amersham
Tris and Tris HCl	Fisher Scientific
Trifluroacetic acid (TFA)	Fisher Scientific
Tris glycine transfer and SDS running solutions	National Diagnostics
Trizma base and Tween-20	Sigma
Trypsin gold MS grade	Promega
Ultima Gold	Packard Instrument Co.
Urea	Sigma
VectorShield with DAPI	VectorLabs
Xylene	Acros Organics

2.1.2 Equipment and a list of suppliers

Equipment	**Supplier**
4°C Refrigerators	Lec
4°C Refrigerated centrifuges	Eppendorf/Sanyo
-20°C Freezers	Lec
-80°C Freezers	Revco/Sanyo
96-well plate reader	Tecan
Autoclave	Rodwell
Autostainer XL	Leica
Bench top vortex mixers	Scientific Industries
Cell Harvester, Filtermate Harvester	Packard
Centrifuges (MSE Mistral 2000R)	Sanyo
Class II safety cabinets, Microflow biological safety cabinet	Walker
Cryostore, Cryo 200	Forma Scientific
Drying cabinet	SLS
Electrophoresis gel tanks and apparatus	Bio-Rad/Geneflow
EliSpot Reader, Immunospot analyzer	Cellular Technology Ltd
Fluorescence microscope	Olympus BX51
Fujifilm intelligent dark box	Fujifilm Life Sciences Products
Gallios Flow Cytometer	Beckman Coulter
Gamma counter – Topcount scintillation counter	Packard
Gel documentation system InGenius	Syngene
Haemocytometers	Weber
Hairdryer	Fransen
Helios Gene-Gun System	Bio-Rad
Helium gas and cylinder	BOC
Incubators CO_2 water jacked incubators	Forma Scientific/Galaxy
Light microscope	Nikon/Olympus
Light microscope camera	Nikon

Liquid Nitrogen and canister	British Oxygen Company
MACSMix Tube Rotator	Miltenyi Biotech
Microcentrifuge, Microcentraur	MSE
Microwave	Matsui
MoFlo XDP High-Speed Cell Sorter	Beckman Coulter
Nanodrop 8000 Spectrophotometer	Thermo Scientific
Nitrogen gas and cylinder	BOC
Orbital plate shaker	VWR
PCR workstation cabinet	Grant Instruments
pH meters	Metler Toledo
Pipettes and multichannel pipettes	Gilson, Star labs, Eppendorf
Pipette guns	SLS, Eppendorf, Star labs
Plate rocker	VWR
Powerpacks	Bio-Rad
Real Time qPCR Thermal Cycler	Qiagen
Tecan ULTRA	Tecan Group Ltd.
Thermo cycler TC-5000	Techne
Transfer apparatus, (wet transfer)	Bio-Rad
Transfer apparatus, Trans-blot cell (semi-dry)	Bio-Rad
Tube prep station	Bio-Rad
Ultra sonicator	VWR
Ultrapure water dispenser	Barnstead
Ultraflextreme MALDI-TOFTOF MS	Bruker Daltonics
UNO-Thermoblock	Biometra
UV transilluminator	Ultra Violet Products
Water baths	Grant Instruments
Weighing scale	Fisherbrand
Whirlmixers	Scientific Industries

2.1.3 Consumables and a list of suppliers

Disposable equipment and plastic-ware	**Supplier**
0.2 µm filters	Sartorius
8-well chamber culture slide	BD
10 ml syringes	Becton Dickenson
24-well plate round coverslips	SLS
25 cm^2, 75 cm^2, 175 cm^2 tissue culture flasks	Sarstedt
90 mm petri dishes	Phillip Harris Scientific
96-well plate covers	Star Labs
96-well plate harvester filters	Perkin Elmer
96 Uni/Filter scintillin-coated plates	Perkin Elmer
96-well ELISA plates	NUNC
Bijou tubes (5 ml)	Sterilin
Blotting paper	SLS
Cell scrapers 12 mm blade	TTP
Coverslips	Fisher
Cryovials	TTP
Dry ice pellets	BOC
EliSpot plate 96-well, multiscreen HA filter	Millipore

Eppendorf tubes (0.5 ml, 1.5 ml)	Sarstedt
Filter tips (10 µl, 100 µl, 200 µl, 1000 µl)	Sarstedt
Flat bottom culture dishes (6-, 12-, 24-, 96-well)	Sarstedt
Flow cytometry tubes	Tyco Healthcare Group
Gel cutting tips	Alpha Laboratories
Microscope glass slides	SLS/Menzel-Glaser
Needles and syringes (0.5 ml, 1 ml)	Becton Dickinson
Nitrocellulose membrane	Amersham
Nylon Membranes, positively charged	Roache
Pasteur pipettes	Sarstedt
PCR tubes	Qiagen/Micronic Systems
Polypropylene screw top tubes (15 ml, 50 ml)	Sarstedt
Round bottom culture dishes (96-well)	Sarstedt
Scalpels	Swan Morton
Serological pipettes (2 ml, 5 ml, 10 ml, 25 ml)	Sarstedt
Sequenza slide racks and coverslips	Thermo Fisher
Sterile hypodermic needles 25 G	Becton Dickinson
Syringes (5 ml)	Stem Cell Technology
Pipette tips (20 µl, 200 µl, 1000 µl)	Sarstedt
Tefzel tubing	Bio-Rad
Universal tubes (25 ml)	Sterilin
Weigh boats	SLS
Whatman 3 MM filter paper	Whatman
ZipTipC_{18} pipette tips	Millipore

2.1.4 Cell lines and media compositions

Cell culture media was prepared and used within the expiry date and maintained at 4°C when not in use. Complete BM-DC media was prepared fresh immediately prior to use. All serum was heat inactivated at 56°C for 30 minutes prior to use.

T cell media
RPMI
10% (v/v) FCS
2 mM L-glutamine
20 mM HEPES buffer
50 µM 2-ME
50 U/ml Penicillin/Steptomycin
0.25 µg/ml Fungizone

BM-DC media
RPMI
5% (v/v) FCS
2 mM L-glutamine
20 mM HEPES buffer
50 µM 2-ME
50 U/ml Penicillin/Steptomycin
0.25 µg/ml Fungizone

Complete BM-DC media
BM-DC media + 1 ng/ml mGM-CSF

Complete PBMC media
RPMI
5% (v/v) AB serum
2 mM L-glutamine

Complete C.T.L media
C.T.L media + 2 mM L-glutamine

Human prostate derived cell lines: All grow as a monolayer

***Cell Line** / Source*	***Tumour origin and cell line features***	***Tumour-igenicity***	***Androgen receptor***	***Prostate Specific Antigen (PSA)***	***Prostatic acid phos-phatase (PAP)***	***Culture media***
DU145 / *ATCC*	Prostate carcinoma derived from brain	moderate	+ (low)	no	no	DMEM + 10% FCS, 1% pyruvate
LNCaP / *ATCC*	Androgen dependent prostate carcinoma derived from lymph node	low	+	yes	yes	RPMI 1640 + 10% FCS + 1% L-glutamine
PC3 / *ATCC*	Prostate adenocarcinoma derived from bone	high	+ (low)	no	no	HAM-F-12K + 10% FCS
OPCT-1 / *Onyvax*	Derived from prostate tumour epithelium	moderate /low	-	+ (low)	-	KSFM + 2% FCS
PNT1a / *ECACC*	Benign prostatic epithelial	non	-	-	-	RPMI 1640 + 10% FCS + 1% L-glutamine
PNT2 / *ECACC*	Benign prostatic epithelial	non	-	-	-	RPMI 1640 + 10% FCS + 1% L-glutamine

Human breast derived cell lines: All grow as a monolayer

***Cell Line** / Source*	***Tumour origin and cell line features***	***Estrogen/ Progesterone receptor***	***Luminal / Basal Type***	***Her2 over-expression***	***Culture media***
T47D / *ATCC*	Breast invasive ductal carcinoma	+/+	Luminal A	-	RPMI1640 + 10% FCS + 1% L-glutamine
MCF7 / ATCC	Breast invasive ductal carcinoma	+/+	Luminal A	-	DMEM + 10% FCS
MDA468 / ATCC	Breast adenocarcinoma	-/-	Basal B	-	RPMI1640 + 10% FCS + 1% L-glutamine
MDA231 / *Dr de Gremoux, Institut Paris, (gift)*	Breast adenocarcinoma	-/-	Basal B	+	RPMI1640 + 10% FCS + 1% L-glutamine
SkBr3 / *Sheffield Medical School (gift)*	Breast adenocarcinoma	-/-	-	Her2/c-erb-2	McCoys + 10% FCS

Table 2.1 - Cell lines used for in vitro studies, descriptions and propagation conditions

2.1.5 Antibodies and kits

All antibodies and kits were prepared and stored in accordance to manufacturer's instructions and used within the expiry date. Optimum antibody dilutions and assay conditions were determined for ELISA, Immunofluorescence (IF), Western blotting (WB), Immunohistochemistry (IHC) and Northern blotting (NB).

Custom Primary Antibodies	**Dilution/application**	**Supplier**
Anti T21/CEP290 pAb (Rb) (3 mg/ml) Seq: (VELERQLRKENEKQKNEL)	1:300 (IF) 1:500 (WB)	Pacific Immunology
Anti T21u pAb (Rb) (0.4 mg/ml) Seq: (ADIELEHHRSQAEQ)	1:50 (IF) 1:50 (IHC) 1:100 (WB) 1:1000 (ELISA)	Pacific Immunology

Primary Antibodies	**Dilution/application**	**Supplier**
Anti CEP290 pAb (Gt) Seq: (RNSKHLKQQQYRAEN)	1:100 (IF) 1:200 (WB)	Everest Biotech
Anti Pericentrin (Mu)	1:100 (IF)	Abcam
Anti Ki67 (Mu)	1:50 (IF)	Serotec
IgG Rabbit Isotype control	As applicable	Serotec
IgG Mouse Isotype control	As applicable	Invitrogen
Anti b-actin (Rb)	1:1000 (WB)	Cell Signalling
Precision Plus Protein Ladder	5 µl (WB)	Bio-Rad
RiboReady™ Color RNA Ladder	5 or 6 µl (NB)	Amresco

Secondary Antibodies	**Dilution/application**	**Supplier**
Alexafluor 488 – goat anti-rabbit IgG	1:1500 (IF)	Invitrogen
Alexafluor 488 – chick anti-rabbit IgG	1:1200 (IF)	Invitrogen
Alexafluor 488 – goat anti-mouse IgG	1:1500 (IF)	Invitrogen
Alexafluor 568 – goat anti-rabbit IgG	1:1000 (IF)	Invitrogen
Alexafluor 568 – goat anti-mouse IgG	1:1000 (IF)	Invitrogen
Anti-rabbit IgG HRP – conjugate	1:1000 (WB)	Cell Signalling
Anti-mouse IgG HRP – conjugate	1:1000 (WB)	Cell Signalling
Precision StrepTactin HRP – conjugate	1:5000 (WB)	Bio-Rad
Biotinylated – rabbit anti-goat	1:1000 (WB)	Dako
Biotinylated – goat anti-rabbit	1:500 (ELISA)	Dako

Kits	**Supplier**
Bio-Rad protein assay	Bio-Rad
Chemiluminescent nucleic acid detection module	Thermo Scientific
Click-iT EdU Alexafluor 647 Flow cytometry kit	Invitrogen

Colour blue developing reagent kit	R and D Systems
ELISA Substrate Reagent Pack & ELISA Streptavidin-HRP	R and D Systems
GeneRacer® Kit with SuperScript® III RT and TOPO	Invitrogen
GoTaq	Promega
Human Total RNA Master Panel II	Clontech
Immunoprecipitation Kit - Dynabeads® Protein G	Invitrogen
LIVE/DEAD Cell Stain Kit, 405 nm excitation	Invitrogen
hIFNγ and mIFNγ EliSpot development modules	R and D Systems
Novolink Polymer Detection System	Leica
Pierce 3' end biotinylation kit	Thermo Scientific
Phusion High-Fidelity DNA Polymerase	NEB
QIAquick PCR purification kit & RNAeasy Mini Prep kit	Qiagen
Wizard SV gel & PCR clean up system	Promega

Oligonucleotides and plasmids and peptides	**Supplier**
DNA oligonucleotide primers for PCR	Eurofins
siRNA oligonucleotides	Eurogentec
RNA molecular probes	Sigma
T21 shRNA and negative control shRNA plasmids	Origene Technologies
Recombinant T21 protein	GTP Technology
Overlapping long peptide library	ProImmune

2.1.6 Buffers and solutions

All buffers and solutions were prepared in sterile duran glass bottles or glass vials and maintained at recommended temperature when not in use with some solutions prepared fresh immediately prior to use.

Cell counting solutions:

Trypan blue solution:
0.1% (v/v) trypan blue solution in dPBS

White cell counting solution:
0.6% (v/v) acetic acid in dPBS

Buffers used for DNA or protein analysis and Polymerase Chain Reaction (PCR):

50X TAE:
242 g Tris
57.1 ml glacial acetic acid
100 ml 0.5 M EDTA (pH 8.0)
Completed to 1 L ddH_2O

1% agarose gel electrophoresis:
1 g agarose powder
100 ml 1X TAE
8 µl GelRed

Orange G DNA loading buffer:
0.25% (w/v) orange G powder
30% (v/v) glycerol
Complete to final volume with ddH_2O

Cell lysis buffer:
9.5% (w/v) urea
2% (w/v) DTT
1% (w/v) OGP
Complete to final volume with ddH_2O

Protein assay working dye reagent:
1 parts Dye reagent concentrate
4 part ddH_2O

Sample reducing buffer:
0.5 M tris-HCl, pH 6.8
2% (w/v) SDS
10% (w/v) DTT
Complete to final volume with ddH_2O

Tris buffer saline (TBS):
50 mM tris
150 mM NaCl
pH adjusted to 7.5 with HCl
Completed to 1L dH_2O

TBS-Tween-20 (Marvel):
0.05% Tween-20
5% (w/v) marvel milk
Complete to final volume with TBS

Resolving gel:
30% acrylamide
1.5 M tris-HCl (pH 8.8)
dH_2O
10% (w/v) SDS
10% (w/v) APS
TEMED

Stacking gel:
30% acrylamide
0.5 M tris-HCl (pH 6.8)
dH_2O
10% (w/v) SDS
10% (w/v) APS
TEMED

Running buffer:
0.25 M trizma base
2 M glycine
1% (w/v) SDS
Complete to 1 L with dH_2O

Transfer buffer:
48 M tris
39 mM glycine
20% (v/v) methanol
Complete to 1 L with dH_2O

De-staining solution:
10% (v/v) acetic acid
30% (v/v) methanol
Complete to 1 L with dH_2O

Buffers and solutions for Northern blotting:

5X TBE buffer:
0.44 M tris base
0.44 M boric acid
10m M EDTA
Complete to 1 L with DEPC treated dH_2O

Resolving gel:
30% acrylamide
5X TBE buffer
dH_2O
10% (w/v) APS
TEMED

Buffers and solutions for immunohistochemistry and for immunofluorescence:

Novolink Polymer Detection System:

Citrate solution:
10 mM citric acid (anhydrous)
0.05% (v/v) Tween-20
pH adjusted to 6.0 with NaOH
Complete to 1 L with dH_2O

TBS:
50 mM tris
150 mM NaCl
pH adjusted to 7.5 with HCl
Completed to 1 L with dH_2O

Fixing solution:
4% (w/v) paraformaldehyde
Dissolved in 1X dPBS
pH adjusted to 7.2 with HCl

PBST wash buffer:
0.1% (v/v) Tween-20
Complete to final
volume with 1X PBS

Blocking solution:
10% (w/v) BSA
Complete to final volume using
1X PBST wash buffer

Buffers and solutions for ELISA and EliSpot assay:

Wash buffer (TTBS):
50 mM tris HCL (pH7.4)
150 mM NaCl
0.05% (v/v) tween-20 in ddH_20

Coating solution:
15 mM Na_2CO_3
30 mM $NaHCO_3$
0.02% (w/v) azide
pH adjusted to 9.6 with HCl

Blocking buffer:
4% (w/v) BSA in TTBS

Dilution buffer:
2% (w/v) BSA in TTBS

Substrate solution:
1 volume colour reagent A
1 volume colour reagent B

Stop solution:
2 N H_2SO_4

Wash buffer:
0.05% (v/v) Tween-20 in dPBS

Reagent diluent solution:
1% (w/v) BSA
Complete to final volume with dPBS

Buffer for flow cytometry:

Wash buffer:
1% (w/v) BSA in 1X dPBS

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Tissue culture cell line propagation

Cell line storage conditions: Cell lines and culture conditions are described in Table 2.1. All cell culture work was performed in class II safety cabinets. All cell lines were frozen in 1 ml 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. Cell lines were maintained in a humidified incubator at $37^{o}C$, 5% CO_2 culture flasks or plates.

Culture of adherent cells: Confluent monolayer cells were washed 3 times with an appropriate volume of dPBS to remove residual media, and dislodged at 37°C using 1X TrypsinVersene stopped by the addition of complete growth media, centrifuged at 400 g, $4^{o}C$ for 3 minutes, counted using trypan blue exclusion method and seeded at the appropriate density into cell culture flasks or plates. Seeded DCs were harvested from culture plates by scraping using a pipette tip. Seeded PBMCs and cells for lysate or RNA extraction were removed by gentle mechanical scrapping.

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

2.2.2 Genomic expression analysis

Studies involving investigation of genomic expression were performed by analysing RNA expression in samples. For these studies, RNA was routinely extracted from cell lines and reverse transcribed into complementary DNA (cDNA) which was then used in quantitative real time PCR (RT-Q-PCR) and semi-quantitative PCR reactions. Semi-quantitative PCR products underwent size separation using agarose gels, DNA purification and clean up and finally DNA sequencing.

2.2.2.1 RNA extraction and cDNA synthesis

RNA extraction by RNA spin columns: RNA was extracted using RNAeasy mini kit (Qiagen) according to the manufacturer's instructions. In brief, adherent cells (up to $x10^7$ cells) were collected (as described in section 2.2.1.1) into universal tubes and washed once using dPBS by centrifugation at 86 g, 21°C for 3 minutes and the supernatant pipetted off. To the tubes, 350 µl of RTL lysis buffer followed by 350 µl of 70% ethanol were added and the pellet dispersed through pipetting up and down several times. The cells were then transferred onto an RNAeasy spin column and centrifuged at 8600 g for 15 seconds and the flow through discarded. To remove DNA and other contaminants 500 µl of RW1 wash buffer was applied to each column, which was then centrifuged and the flow through discarded as before. Following this, 500 µl of Buffer RPE was added to the column and centrifuged as before. After discarding the flow through, 500 µl of Buffer RPE was again added to the column and centrifuged 8600 g for 2 minutes to wash the membrane and ensure removal of ethanol prior to the elution steps. The column was then transferred to a fresh collection tube and between 10 and 25 µl of RNAase free water was added directly to the column membrane and centrifuged 8600 g for 60 seconds to elute the purified RNA into the collection tube. RNA concentration was measured using a Nanodrop spectrophotometer and resuspended to a concentration of 1 µg/µl in ddH_2O ready for reverse transcription or stored at $^-$80°C for up to 6 months for future use.

RNA extraction by isopropanol precipitation method: RNA was extracted using RNA STAT-60 according to manufacturer's instruction. Briefly, tissues were ground to a powder in liquid nitrogen. The powder was collected into 1.5 ml eppendorfs and 1 ml of RNA STAT-60 added. Cell lines were homogenised using 500 µl per $2x10^6$ cells of RNA STAT-60 in 1.5 ml eppendorf tubes and left at room temperature (RT) for 3 minutes. To this, 100 µl of chloroform per 500 µl of RNA STAT-60 was added and mixed by shaking vigorously for 60 seconds and again tubes were left for 3 minutes at RT. Tubes were then centrifuged at 17000 g, 4°C for 15 minutes and the colourless upper aqueous phase containing RNA was carefully removed into a fresh 1.5 ml eppendorf and the phenol red phase discarded. To this, 250 µl of isopropanol per 500 µl of RNA STAT-60 was added and the solution left at RT for 8 minutes in order to precipitate out RNA before again centrifuging at 12000 g, 4°C for 10 minutes. The supernatant was removed and discarded leaving a white pellet, and the RNA washed using 1 ml of 75% ethanol followed by

centrifugation at 4800 g, 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 10-30 µl) 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 1 µg/µl in ddH_2O ready for reverse transcription.

Reverse transcription: First-strand cDNA synthesis from RNA was prepared following the manufacturers protocol described below per sample. Into a 0.5 ml eppendorf, 2 µg of RNA, 0.5 µg of the $Oligo(dT)_{15}$ primer and ddH_2O were added to give a final volume of 15 µl. 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: 5 µl of M-MLV 5X reaction buffer, 1 µl of 40 mM dNTP's, 0.7 µl of RNasin ribonuclease inhibitor, 1 µl M-MLV RT and 2.3 µl ddH_2O. Samples were mixed gently and incubated for 60 minutes at 42°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 PCR.

2.2.2.2 Semi-quantitative PCR amplification

Primer design and optimisation: PCR was performed on a 96 well automated DNA thermal cycler using oligo-primers designed using online primer designing tools (http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/), (http://frodo.wi.mit.edu/) and (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) purchased from MWG Eurofins. Primers were optimised in accordance to protocols described elsewhere (Roux, 2009; Bustin *et al.*, 2009). PCR tubes were prepared following manufacturer's protocols specific to the Taq DNA polymerase used.

PCR set-up conditions: For product amplification using GoTaq DNA polymerase, each tube was prepared using 5 µl of 5X Reaction Buffer, 13.3 µl of ddH_20, 1.5 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP mix, 1 µl of each primer and 0.2 µl of 5 U/µl GoTaq DNA polymerase and 2 µl of template cDNA. Gradient PCR was performed under the following program conditions; Initial denaturation at 95°C for 2 minutes (1 cycle), denaturation 95°C

for 60 seconds, annealing 60°C at 12 gradient for 60 seconds, extension 75°C for 60 seconds/kb (35 cycles), final extension 75°C for 5 minutes (1 cycle) and final hold at 4°C.

2.2.2.3 DNA isolation and purification for sequencing analysis

Gel electrophoresis and visualisation: PCR products were separated using agarose gel electrophoresis and visualised with 1:10,000 GelRed stain using a UV transilluminator. To achieve good separation and resolution, percentage agarose gels were prepared in accordance to expected product size. Gels trays were loaded into the electrophoresis tank and samples were loaded with 3 µl orange G onto the gel wells and ran at 70-110 V for 30-60 minutes. Gels were then removed from trays and visualised using a UV transilluminator and images captured using GeneSnap (Syngene) software.

DNA clean-up of post PCR product: Following PCR, DNA clean-up was performed using QIAquick PCR purification kit (Qiagen) following manufacturer's instructions. The PCR product was transferred to a 1.5 ml eppendorf tube and diluted in 5 volumes of buffer PB before adding to the QIAquick column assembly. Tubes were centrifuged at 14500 g for 40 seconds and the flow through discarded from the collection tube. A further 750 µl of buffer PE was applied to the column and tubes were centrifuged as before, discarding the flow though. The column was further centrifuged as before for 60 seconds to remove residual wash buffer before being transferred to fresh 1.5 ml eppendorf tube and 50 µl of buffer EB was applied directly to the column membrane to elute DNA. After 60 seconds incubation at room temperature (RT), the column assembly was centrifuged for 60 seconds at 14500 g and the eluted DNA collected and quantified using a nanodrop and stored at between 4°C and $^{-}$20°C for future use.

Gel band extraction and DNA purification: Following electrophoresis gel bands were excised under ultraviolet (UV) light using GelX tips and placed in 1.5 ml eppendorf tubes. Purification of DNA was performed using a Wizard SV gel and PCR clean up system (Promega) following the manufacturer's instructions. Briefly, gel slices were weighed and 10 µl of membrane binding solution was added per 10 mg of gel. Tubes were then vortex mixed and incubated at 50°C until the gel slices were completely dissolved. Following incubation, an equal volume of membrane binding solution was added to the tubes before the dissolved gel mixture was transferred to the column assembly and incubated at RT for

60 seconds. Tubes were centrifuged at 17000 g for 60 seconds and the flow through discarded from the collection tube. A further 700 µl of membrane wash solution was applied to the column and tubes were centrifuged as before, discarding the flow though. Columns were washed and centrifuged at 17000 g for 5 minutes with 500 µl of membrane wash solution with the flow through discarded. The column was further centrifuged as before for 60 seconds to remove residual ethanol before being transferred to fresh 1.5 ml eppendorf tube and 50 µl of nuclease-free water added to elute DNA. After 60 seconds incubation at RT, the column assembly was centrifuged for 60 seconds at 17000 g and the eluted DNA collected and quantified using a nanodrop and stored at between 4°C and -20°C for future use.

2.2.2.4 Quantitative real time PCR (RT-Q-PCR)

PCR set-up conditions: A panel of normal tissue mRNA's were purchased from Clontech and subjected to reverse transcription (as described in section 2.2.2.1). All oligo-primers were designed to be between 15 – 30 bases in length and would generate products no greater then 250bp for RT-Q-PCR. All oligo-primers were purchased from Eurofins MWG Operon and diluted to a working concentration of 10 pg/µl using ddH_2O and stored at -20°C. Real time quantitative PCR was performed on a Real Time qPCR Thermal Cycler using iQ SYBR Green fluorescence dye. PCR tubes prepared for each reaction were prepared in 12.5 µl volume containing 6.25 µl of iQ SYBR Green SuperMix, 4.75 µl of ddH_2O, 0.5 µl of each primer and 0.5 µl of template cDNA. In cases of Non-Template Control (NTC), cDNA template was substituted with ddH_2O. PCR was performed under the following program conditions; Initial melt step at 95°C for 5 minutes, followed by 40 cycles of denaturation 95°C for 30 seconds, primer specific annealing (detailed in Table 2.2) for 30 seconds, extension 72°C for 30 seconds and a final extension at 98°C for 60 seconds. Samples were run in duplicate and repeated at least 3 times to ensure validity of results which were then analysed using the ΔCt calculation (Bustin *et al.*, 2009).

Amplicon	Primer	Sequence (5' – 3')	Product size	Tm.	Ext.
CEP290	CEP290 F4	**GCTTCGATTGCCTGCCACTGC**	214bp	62°C	30sec
	CEP290 R4	**GCAGTGGCAGGCAATCGAAGC**			
T21 unique region	T21 qPCR F	**CAAAGAATGAAATCATAGCACAGG**	100bp	55°C	30sec
	T21 qPCR R	**TTCTGTTCTGCCTGGCTTCT**			
HKG: Transferase	hHPRT1 F	**TGACACTGGCAAAACAATGCA**	94bp	57°C	30sec
	hHPRT1 R	**GGTCCTTTTCACCAGCAAGCT**			
HKG: TATA box binding	hTBP F	**TGCACAGGAGCCAAGAGTGAA**	132bp	57°C	30sec
	hTBP R	**CACATCACAGCTCCCCACCA**			
OAS1: induced by IFN	OAS1 F	**CAAGCTCAAGAGCCTCATCC**	150bp	59°C	30sec
	OAS1 R	**TGGGCTGTGTTGAAATGTGT**			
STAT1: activated by IFN	STAT1 F	**AAATTCCTGGAGCAGGTTCA**	166bp	59°C	30sec
	STAT1 R	**TGGCCCCAAGTCACTTAATC**			
HKG: β-actin	β-actin F	**CTCTTCCAGCCTTCCTTCCT**	116bp	57°C	30sec
	β-actin R	**AGCACTGTGTTGGCGTACAG**			
CEP290/T21	T21 – F1	**ATGCCACCTAATATAAACTGGAAAG**	1829bp	62.5°C	2min
	T21 – R1	**TTTCATTCTTTGATTGTGCTTCA**			
CEP290/T21	T21 – F3	**AGAAGCCAGGCAGAACAGAA**	1508bp	59.4°C	2min
	T21 – R3	**CAAACCTCAGCAAATTTGGTTTC**			
CEP290/T21	CEP290/T21 F	**ATGAGTGAAGCACAATCAAAG**	194/ 287bp	60°C	30sec
	CEP290/T21 R	**TCAGGATCTTTCTGCATTTCC**			

Table 2.2 – Primers used for PCR, descriptions and amplification conditions. *Abbreviations: bp – base pairs; Tm. - annealing temperature; Ext. – extension time; HKG - House Keeping Gene.*

2.2.3 Rapid Amplification of 5' and 3' cDNA Ends (RACE)

Rapid Amplification of cDNA Ends (RACE) technique allows for the identification of the 5' and 3' untranslated regions of a gene using known partial sequences obtained by library screening methodologies such as SEREX. Preparation of RNA was performed using GeneRacer kit (Invitrogen) which is based on RNA ligase-mediated and oligo-capping RACE methods resulting in selective ligation of primers to the 5' ends of decapped mRNA using T4 RNA ligase.

2.2.3.1 Preparation of total RNA to full length mRNA

To commence preparation of 5' RACE, total RNA was required to be processed for full length capping which was performed by dephosphorylation of non mRNA and truncated mRNA, removal of the mRNA cap structure and ligation with GeneRacer RNA Oligo to full length mRNA.

Dephosphorylation: First, dephosphorylation reaction was performed in a 0.5 ml eppendorf tube using 3 µg of prostate total RNA (Clontech) to which 1 µl of 10X CIP buffer, 1 µl RNaseOut (40 U/µl), 1 µl CIP (10 U/µl) and 4 µl DEPC water was added. Tubes were gently mixed and briefly centrifuged before being incubated at 50^{o}C for 60 minutes. Following incubation, tubes were again briefly centrifuged and placed on ice. Next, RNA was precipitated out by adding 90 µl of DEPC water and 100 µl phenol:chloroform with the mixture then vortex mixed for 30 seconds before centrifugation for 5 minutes at 14500 g in order to separate the phases. The top layer aqueous phase was carefully removed and transferred into a fresh eppendorf before proceeding with RNA precipitation. Next, 2 µl of mussel glycogen (10 mg/ml) and 10 µl of 3 M NaOAc (pH 5.2) was added followed by 220 µl of 95% ethanol taking care to vortex mix the tubes well after each addition. Tubes were then super-chilled on dry ice for 10 minutes. Following incubation, tubes were spun at 4^{o}C for 20 minutes at 17000 g and the supernatant carefully removed and discarded so not to disturb the pellet. The remaining pellet was washed with 500 µl of 70% ethanol and centrifuged at 17000 g for 2 minutes at 4^{o}C. After discarding the supernatant, the pellet was allowed to air dry for 2 minutes at RT before being resuspended in 7 µl of DEPC water.

Decapping of 5' structure: Upon dephosphorylation and precipitation of the RNA, decapping of the 5' structure was performed in a 0.5 ml eppendorf tube on ice by gently mixing 1 µl 10X TAP buffer, 1 µl RNaseOut (40 U/µl), 1 µl TAP (0.5 U/µl), followed by briefly centrifuging the tubes to collect liquid and incubated at 37^{o}C of 60 minutes. Following incubation tubes were again briefly centrifuged and placed on ice. Subsequent mRNA precipitation was performed as described previously. Following the decapping procedure, the precipitated mRNA was ready for ligation with the Generacer RNA Oligo, specifically to the 5' end of the decapped mRNA. First, 7 µl of dephosphorolated, decapped mRNA was added to the lyophilised pre-aliquoted GeneRacer RNA Oligo

(0.25 µg), mixed well and briefly centrifuged to collect the liquid at the bottom of the tube prior to incubation at 65°C for 5 minutes followed by incubation on ice for 2 minutes. Tubes were briefly centrifuged and the following components added to each tube: 1 µl of 10 Ligase buffer, 1 µl ATP (10mM), 1 µl RNaseOut (40 U/µl), 1 µl T4 RNA ligase (5 U/µl) before being incubated at 37°C for 60 minutes. After incubation, tubes were briefly centrifuged and placed on ice and the RNA was precipitated as described previously and the resulting pellet resuspended in 10 µl of DEPC treated water.

2.2.3.2 Reverse transcription of mRNA for 5' and 3' ends

Reverse transcription: Reverse transcription was performed on the unprocessed RNA for 3' RACE and processed RNA for 5'RACE described in the previous section. Briefly, 3 µl of unprocessed total RNA from prostate and 7 µl of DEPC for 3'RACE or 10 µl of processed RNA processed for 5'RACE were prepared in 0.5 ml eppendorfs to which 1 µl of GeneRacer Oligo dT primer (50 µM) and 1 µl of dNTP mix 25 mM) was added. Next, RNA secondary structures were removed by incubating for 5 minutes at 65°C followed by incubation on ice for a further 2 minutes. Tubes where then briefly centrifuged and the following components were added to the primer mixture and ligated RNA: 4 µl of 5X RT buffer, 1 µl of cloned AMV RT (15 U/µl), 2 µl sterile water and 1 µl RNaseOut (40 U/µl). Tubes were mixed well and incubated at 45°C for 60 minutes followed by incubation at 85°C for 15 minutes to inactivate Cloned AMV RT. Tubes were once again briefly centrifuged and the cDNA stored at $^{-}20^{o}$C for future use.

2.2.3.3 Rapid Amplification of 5' and 3' ends of T21

Semi quantitative RT PCR together with Phusion High-Fidelity DNA Polymerase was used to amplify the previously untranslated region 5' and 3' ends of T21 from prostate tissue cDNA. For amplification of 5' and 3' ends, T21 unique region specific sequence reverse and forward primers were used respectively along with the GeneRacer primers provided.

T21 specific primer	T21 specific primer sequence	Tm.
T21 specific 5' RACE primer reverse	**GAATTCCTGTGCTATGATTT**	65.2°C
T21 specific 3' RACE primer forward	**AAATCATAGCACAGGAATTC**	61.5°C

Table 2.3 – Primers used for RACE PCR

PCR set-up conditions: For amplification using Phusion High-Fidelity DNA Polymerase, each PCR tube was prepared to a total reaction volume of 50 µl using 10 µl of 5X Hi-Fidelity Buffer, 32.5 µl of nuclease-free water, 1 µl of dNTPS (10 mM), 3 µl of GeneRacer 5' Primer (10 µmole/µl) for 5'end amplification or 1 µl of T21 sequence specific forward primer (10 µmole/µl) for 3' end amplification, 1 µl of T21 sequence specific reverse primer (10 µmole/µl) for 5'end amplification or 3 µl of GeneRacer 3' Primer (10 µmole/µl) for 3' end amplification, 0.5 µl of Phusion Hi-Fidelity Taq polymerase and 2 µl of template cDNA.

PCR was performed under the following program conditions; hotstart at 98°C, initial denaturation at 98°C for 30 seconds (1 cycle), denaturation 98°C for 10 seconds, annealing (as indicated in the table above) for 30 seconds, extension 72°C for 30 seconds (35 cycles), final extension 72°C for 10 minutes (1 cycle) and final hold at 4°C. Upon completion, PCR products were loaded onto 1.2% agarose gel and underwent visualisation, band extraction and purification using methods described in section 2.2.2.3.

2.2.4 Northern Blotting

Northern blotting allows for the detection of specific RNA transcripts using complementary sequence probes that are labelled using either radioactive isotopes such as (^{32}P) or with chemiluminescence using enzyme substrates directly or indirectly attached to probes labelled with chemiluminescent molecules such as biotin. This technique allows for identification of novel gene transcripts and determining transcription variants in relation to specific binding and transcript size as well as observations of gene expression patterns and transcript abundance from tissue to tissue.

2.2.4.1 RNA 3' end biotinylation of probes and RNA/probe hybridisation

RNA 3' end biotinylation of probes: RNA probes purchased from Sigma were resuspended at a concentration of 100 µM using dH_20 to prepare a working stock of 10 µM. For the RNA ligation reaction, 5 µl of RNA probe were transferred into a 0.5 ml eppendorf tube and heated for 5 minutes at 85°C before being placed on ice. Next, the labelling reaction mix was prepared and the components added as follows: 3 µl nuclease-free water, 3 µl 10X RNA ligase reaction buffer, 1 µl RNase inhibitor (40 U), 5 µl RNA,

1 µl of biotinylated cytidine (Bis)phosphate (1 nmol), 2 µl T4 RNA ligase (40 U) and 15 µl of 30% PEG. The labelling reaction mix was pipette mixed and incubated at 16°C overnight for approximately 18 hours. Following ligation, 70 µl of nuclease-free water was added to the reaction followed by 100 µl of chloroform:isoamyl alcohol used to extract the RNA ligase. The mixture was vortex mixed before centrifugation for 3 minutes at 17000 g in order to separate the phases. The top layer aqueous phase was carefully removed and transferred into a fresh eppendorf before proceeding with RNA precipitation. Next, 10 µl of 5 M NaCl, 1 µl of glycogen and 300 µl of cold 100% ethanol was added to each tube and centrifuged for 15 minutes at 17000 g at 4°C. The supernatant was discarded and the pellet washed with a further 300 µl of cold 70% ethanol before being discarded and the pellet left to air dry for 5 minutes. The dried pellet was then resuspended in 20 µl of nuclease-free water ready for hybridisation with RNA.

Probe No.	Target gene	RNA sequence
1	T21	**AUAGCACAGGAAUUCUUGAUCA**
2	CEP290	**UCCCUGACCCUAGUUUGCCCCU**
3	CEP290/T21	**GAAAACGAUCAUUAUCAACUUCAAG**
4	T21	**CAAACCUCAGCAAAUUUGGUUUC**

Table 2.4 - RNA probes used for northern blotting

RNA/probe hybridisation: Following labelling, 1 µl of RNA probe was mixed with 4 µl of a 250 ng/µl stock of total RNA derived from various tissue in a 0.5 ml eppendorf tube and incubated at 95°C for 5 minutes. Tubes were then place immediately on to ice for 30 minutes to avoid formation of secondary structures. Tubes were then incubated at RT for 30 minutes before commencing to polyacrylamide gel electrophoresis (PAGE).

2.2.4.2 Northern blotting

PAGE, membrane transfer and cross-linking of RNA/probe: Polyacrylamide gels were made with the constituents listed below and cast between two glass plate gel casters and with a spacer comb inserted between the glass plates. After a minimum of 30 minutes following gel polymerisation, the glass plates were removed from caster apparatus and transferred to a running tank and covered with 0.5X TBE buffer and the spacer comb

carefully removed and the gel was pre-run for 30 minutes a 130 V to equilibrate the gel and remove any traces of ammonium persulphate and any other trace contaminants that may interfere with the running of RNA.

Polyacrylamide Gel	**12%**
30% Acrylamide	3.300
5X TBE	2.000
dH_2O	4.700
10% Ammonium persulphate	0.100
TEMED	0.010

Table 2.5 - Polyacrylamide gel formulation used for northern blotting. *Values given in (ml)*

Next, 15 µl of RNA/probe was mixed with 5 µl Orange G (used for sample tracking) and was carefully loaded into the wells and the tank was then run for 30 minutes at 130 V. Gels were then removed from the apparatus and placed in 0.5X TBE buffer to equilibrate before transfer onto positively charged nylon membranes. Following electrophoresis, a stack was constructed were 3 gel sized pieces of blotting paper, the nylon membrane, the gel and again 3 pieces blotting paper all soaked in 0.5X TBE buffer were placed onto the semi-dry transfer unit and run at 200 mA for 30 minutes. After transfer, nylon membranes were removed from the transfer apparatus and placed onto an A5-sized sheet of blotting paper soaked with 0.5X TBE buffer before being completely covered by a sheet of Whatman 3MM paper also soaked in 0.5X TBE buffer to prevent the nylon membrane from drying out. This stack containing the membrane was incubated at 80°C for 15 minutes in a pre-heated drying cabinet in order to cross-link RNA to the membrane, which was then placed in a container ready for detection.

Detection of immobilised RNA/probe: The detection of RNA/probes was performed using a chemiluminescent nucleic acid detection module (Thermo Scientific) as per the recommended instructions. Briefly, for a 10 x 10 cm membrane 16 ml of pre-warmed blocking buffer was added to the membrane and left to incubate at RT for 15 minutes with gently rocking before being removed. Next, 50 µl of streptavidin-horseradish peroxidase conjugate diluted in 16 mls of blocking buffer was added to the membrane and was left to

incubate as described above. Meanwhile, a working concentration of 1X wash solution was prepared using 40 mls of pre-warmed X4 wash buffer and 120 ml ddH20. Membranes were transferred into a fresh container and rinsed with 20 mls of 1X wash buffer prior to washing 4 times for 5 minutes whilst gently rocking. Following the final wash, membranes were transferred to a fresh container and incubated with 30 mls of substrate equilibrium buffer for 5 minutes under gentle rocking. Meanwhile, the chemiluminescent substrate working solution was prepared by mixing 6 mls of luminol/enhancer solution to 6 ml of stable peroxide solution in a tube concealed from light. Following incubation, membrane edges were carefully blotted onto absorbent paper to remove excess buffer before being transferred into a polythene bag containing 12 mls of chemiluminescent substrate solution and was left to incubate for 5 minutes on a flat surface without shaking. Membranes were then removed from the bag and the excess liquid removed by blotting using absorbent paper and membranes subjected to luminescence detection.

2.2.5 Protein expression analysis

Protein expression and localisation studies were performed by employing methods involving immunoblotting and immunosorbent assays, immunohistochemistry and immunocytochemistry. For each assay, antibody concentrations were used were from manufacturers suggested recommendations. Optimal assay conditions and antibody concentrations were determined prior to the use of customised antibodies, all of which are detailed in section 2.1.5.

2.2.5.1 Indirect immunofluorescence

Cell preparation: A day prior to immunofluorescent staining, cells were seeded into 24 well culture plates at a concentration of between 2-10x10^4 cells/ml at 1 ml per well each containing one sterile round glass cover slip. For LNCaP cells (low adhesive properties), fluorescence was performed in 8 well chamber slides and all solution volumes were scaled down 5-fold. Cells were then incubated for at least 24 hours at 37^{o}C, 5% CO_2 so that cells would be between 85-95% confluent at the time of staining.

Immunofluorescence staining: The following day, media was removed from the wells and 200 µl of cold 4% paraformaldehyde solution was added to each well for 15 minutes at 4^{o}C

in order to fix cells. Wells were then washed once for 10 minutes using dPBS whilst on a plate rocker followed by blocking for 60 minutes using blocking buffer. After blocking, primary antibodies and isotype controls were prepared in blocking buffer at optimal dilutions optimised in house or suggested by manufacturers and added to appropriate wells and left to incubate overnight at 4°C on a plate rocker. Following incubation, wells were washed using wash buffer x3 times for 10 minutes and the secondary Alexafluor antibodies prepared using blocking buffer and added to appropriate wells. Plates were then wrapped in foil and incubated at RT 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^{o}C until ready to view using a fluorescence microscope.

2.2.5.2 Immunohistochemistry staining of paraffin-embedded sections

Pre-staining procedure: Slides purchased from US Biomax were stained using the following procedure. For paraffin embedded tissue microarray sections (TMA) slides were initially heated on a hotplate for 10 minutes at 60°C to melt the paraffin wax before beginning automated deparaffinisation/hydration process using an Autostainer XL (Leica) programmed automation as follows: emersion of slides in xylene bath for 5 minutes, second xylene bath 5 minutes, three consecutive graded alcohol baths (100%, 100% and 70% ethanol) each for 2 minutes and a final dH_20 bath for 5 minutes. Slides were then placed in antigen retrieval buffer (0.01 M citrate solution pH 6) and microwaved for 20 minutes from boiling after which, slides were then allowed to cool under cool tap water for 5 minutes. Whilst underwater, slides were loaded onto sequenza coverslips and carefully inserted into sequenza racks. Slides were washed using TBS which was applied between the slide and to the coverslip creating a reservoir allowing liquids to run down the slide and out of the bottom via capillary action.

Tissue staining: Staining was performed using a Novolink kit following the manufacturer's instructions (all kit solutions were applied in 100 µl volume each time followed by x2 wash stages using TBS for 5 minutes). Initial peroxidase block was applied to the slide and following washes, protein block was added and again left for 5 minutes followed by washing as before. Next primary antibody prepared in antibody diluent was

applied to the slide and incubated overnight at 4°C followed by washing. Next, post primary block was added and washed after 30 minutes followed by addition of polymer for 30 minutes and subsequent wash. Finally, DAB working solution was added for 5 minutes followed by wash and counterstained using haematoxylin for 6 minutes after which dH_20 was added to rinse of excess haematoxylin and slides were removed from the sequenza apparatus and placed into racks to begin automated dehydration process using Autostainer XL programmed as follows: immersion of slides in three consecutive graded alcohol baths (70%, 100% and 100% ethanol) each for 2 minutes, xylene bath for 5 minutes, second xylene bath 5 minutes, and a final dH_20 bath for 5 minutes. Coverslips were then mounted on to slides using DPX mounting medium.

2.2.5.3 Cell protein preparation and quantification for SDS-PAGE

Cell protein preparation: Protein lysates were prepared from adherent cultures grown to 70 – 80% confluency in culture flasks. Cells were then washed twice with dPBS to remove residual calf serum, and homogenised with a cell scraper and transferred into a 15 ml universal tube. Lysed cells were then centrifuged at 8600 g for 5 minutes at 4°C following which the supernatant discarded and the pellet resuspended in dPBS and centrifuged once again. Supernatant was then removed leaving a dry pellet which was suspended in $2x10^6$ cells/100µl of cell lysis buffer. Cell pellets were sonicated to disrupt the pellet for 5 minutes followed by 5 minutes of cooling on ice. Following this, $2x10^6$ cells/100µl of cell lysis buffer containing 0.1% TFA at ratio of 1:3 was added and the solution was then subjected to a further 2 cycles of 5 minutes under sonication followed by 5 minutes on ice. The samples were centrifuged at 17000 g for 60 seconds at 4°C to pellet cell debris and stored at $^-$20°C for one month or at $^-$80°C for long term storage.

Protein quantification: All protein concentrations were determined using Bradford assay. This assay utilises coomassie brilliant blue G-250, an acidic detection dye used to bind to aromatic amino acid residues present on proteins. This results in differential colour change forming a blue complex measurable at wavelength of 590 nm.

First a standard calibration curve was determined following the manufacturers protocol by preparing serial dilutions of BSA using lysate buffer and 40 µl of duplicates of BSA standard or protein lysate sample added to a 96 well round bottom plate. Next, 200 µl of

working dye reagent was added to each well and the plate was sealed with film and incubated at 37°C for 10 minutes with shaking. The absorbance was then measured at 590nm wavelength using a Tecan plate reader, enabling a standard curve to be constructed from the BSA serial dilutions following which the sample protein concentration could be derived based on the absorbance values obtained.

2.2.5.4 SDS-PAGE and transfer

Gel preparation and electrophoresis: The western blotting method was utilised to assess protein expression derived from cell lines. Gels were cast between two glass plate gel casters. Firstly the resolving gel made with the constituents listed on Table 2.6 depending on the protein molecular weight was added using a 1 ml pipette into the gel casters up to 3cm from the top of the glass plates. A thin layer of distilled water was added to the top of the gel for gel levelling and to stop evaporation. After a minimum of 45 minutes following complete gel polymerisation, the water was removed from the gel using blotting paper and the stacking gel added to the top of the gel followed by insertion of the spacer comb. The stacking gel allows for packing of the protein bands after loading and the resolving gel then allows for good protein band separation.

Following polymerisation of the stacking gel, glass plates were removed from the caster apparatus and transferred to a running tank and covered with running buffer and the spacer combs carefully removed. Next, 30-60 µg of protein lysate were heated to 95°C for 5 minutes with x3 sample reducing buffer (3:1 sample:buffer) in order to break disulphide bonds, after which samples and a biotinylated protein ladder were carefully loaded into the wells. The tank was then run for 15 minutes at 70 V allowing protein to travel through the stacking gel followed by another 60 – 90 minutes at 140 V until bands were approximately 1cm from the bottom of the gel. Gels were then removed from the apparatus, the stacking gel cut off and discarded and the gel placed in cold transfer buffer to equilibrate for up to 5 minutes before staining or nitrocellulose transfer. Alternatively, gels could be stained for protein using coomassie staining.

Resolving Gel	**6%**	**10%**
30% acrylamide	1.000	3.300
1.5M tris HCl (pH 8.8)	1.300	2.500
dH_2O	5.300	4.000
10% SDS	0.050	0.100
10% ammonium persulphate	0.050	0.100
TEMED	0.004	0.004
Stacking Gel	**5%**	
30% acrylamide	0.330	
1M tris HCl (pH 6.8)	1.250	
dH_2O	1.400	
10% SDS	0.020	
10% ammonium persulphate	0.020	
TEMED	0.002	

Table 2.6 – Polyacrylamide gel formulations used for western blotting. *Formulations are given for 2 gels with volume in ml, lower molecular weight proteins require high percentage gels whereas lower percentage gels can give better resolution for larger proteins.*

Protein transfer from SDS-PAGE to nitrocellulose membrane: After band separation using SDS-PAGE, a stack was constructed were two gel sized pieces of blotting paper, the nitrocellulose membrane, the gel and again two pieces blotting paper all soaked in transfer buffer were placed onto the transfer unit and run at 30 V overnight at 4°C.

Ponceau red staining: To assess for successful transfer of proteins, membranes were rinsed in TBST and placed in Ponceau Red solution and agitated for 5–10 minutes. Membranes were washed extensively using dH_2O to reveal defined red/pink stained protein bands. Membranes were then destained completely using TBST before proceeding to western blotting.

Coomassie staining: Gels were stained to determine if proteins have migrated uniformly and show equal protein loading or to extract bands using a coomassie blue staining method. To allow band extraction, the proteins are fixed in the gel in 50% methanol, 40% dH_2O and 10% glacial acetic acid for 60 minutes with shaking, the solution discarded and replaced with fresh solution and left overnight with gentle agitation. Gels were then stained with coomassie blue solution overnight with gentle shaking followed by several destaining

washes using 50% dH_2O, 40% methanol and 10% glacial acetic acid until the gel was fully destained leaving well-defined blue protein bands.

2.2.5.5 Western blotting

Membrane probing: Membranes were blocked to prevent non-specific binding of antibodies using TBST-5% marvel milk solution for 60 minutes followed by 5 minutes x2 washes using TBST and the addition of the primary antibody diluted at a working concentration in TBST-3% marvel milk solution and incubated at 4°C overnight under gentle rocking.

Chemioluminescent detection: In order to remove excess and non-specifically bound primary antibody, membranes were washed for 10 minutes x5 washes with TBST before the addition of the secondary HRP-conjugated antibodies diluted using TBST-3% marvel milk solution for 2 hours at RT under agitation. The membranes were washed as before and developed using 3 – 4 sprays of RapidStep ECL to wet the membrane with chemioluminescence solution and left to stand without agitation for 60 seconds and the excess blotted off using absorbent paper and membranes subjected to luminescence detection using Fujifilm intelligent dark box.

2.2.5.6 Protein immunoprecipitation using Dynal magnetic beads

Preparation of antigen sample and Dynabeads: Before commencing immunoprecipitation, protein lysates were obtained from individual cell lines, quantified and frozen at $^{-}80°C$ (refer to section 2.2.5.3). For each sample, 50 µl of Dynabeads protein G was transferred into a 1.5 ml eppendorf tube and magnetically separated into beads and supernatant.

Binding of antibody: Following separation, the now clear supernatant was removed from the tube and replaced with 1-10 µg of appropriate antibody diluted in 200 µl of antibody binding and washing buffer (anti-T21u antibody at 10 µg and IgG controls 5 µg). Tubes were incubated with rotation for 10 minutes at RT before the unbound antibody was removed by placing tubes in a magnetic separator and the supernatants carefully removed. The tube containing the Dynabead-antibody complexes were removed from the magnet

and gently washed using 200 µl of antibody binding and washing buffer before being placed on the magnet to remove the clear supernatant.

Immunoprecipitation of target antigen: Protein lysates containing antigen were added to the Dynabeads-antibody complexes and thoroughly mixed by flicking the tube. Tubes were then incubated with rotation for 30 minutes at RT. Following incubation tubes were again transferred to the magnet and the supernatants removed. Dynabead-antibody-antigen complexes were then removed from the magnet and washed using 200 µl of washing buffer before being replaced onto the magnet to remove the supernatants. This wash step was repeated a further three times. After the removal of the final supernatant wash, Dynabead-antibody-antigen were resuspended with 100 µl of washing buffer and transferred to a fresh eppendorf tube.

Elution of target antigen: Tubes were placed on the magnet and the supernatants were completely removed, before the addition of 30 µl the elution mix (20 µl elution buffer containing 10 µl of sample reducing buffer used for western blotting). Tubes were heated to 70°C for 10 minutes and replaced on the magnet and the eluted antibody and antigen collected in the resulting supernatant. Samples were then loaded onto SDS-PAGE gels for western blotting.

2.2.5.7 In-gel band digestion using trypsin for MALDI MS analysis

Gel preparation - Protein staining and de-staining: Following electrophoresis of immunoprecipitated samples, SDS-PAGE gels were stained overnight with coomassie blue and then washed using de-staining solution for 4 hours under agitation changing the de-staining solution every 30 minutes. The protein bands observed at the desired mass were carefully excised and placed into 1.5 ml eppendorf tubes were gel pieces were homogenised into roughly 1 x 1 mm pieces using a scalpel. Tubes were then stored at the 4°C until further use.

Protein in-gel digestion with trypsin: The gel pieces were washed using ddH_2O ensuring all liquid was carefully removed prior to re-hydration with an equal volume of 50 nM $(NH_4)HCO_3$ added along with 100% acetonitrile (ACN) and left to partially dissolve in an oven at 37°C. Following incubation approximately 50 µl of $(NH_4)HCO_3$/ACN solution, at

a ratio of 1:1, was added to the tubes and left to incubate at RT under agitation for 15 minutes. Following incubation, the liquid was carefully discarded and gel pieces underwent dehydration by addition of enough 100% ACN to fully immerse the gel pieces which were again left to incubate under agitation until the pieces were observed to have reduced in size. The ACN was removed and 200 µl of 50 nM $(NH_4)HCO_3$ was added to hydrate the gel pieces for 5 minutes. Next, 200 µl of 100% ACN was added to the tubes and left under agitation for 15 minutes. The liquid was then fully removed and 100% ACN was once again added to dehydrate the gel pieces before being removed and the gel pieces left to dry for 5 minutes. Finally dH_2O was added to hydrate the gel pieces and then immediately removed. Proteins underwent typsination by addition of 7.6 µl of ddH_2O, 16.6 µl of $(NH_4)HCO_3$ and 1 µl of mass spectrometry grade trypsin to tubes and were left to incubate at 37°C in an oven overnight.

Sample preparation using ZIP-TIP and analysis using LC-MALDI: Following digestion, protein fragments were purified and concentrated using ZipTipC_{18} pipette tips. Briefly, 80% ACN was applied to pre-wet the ZipTip before equilibrating using 0.1% trifluroacetic acid (TFA). Next, 1 µl of 1% TFA was added to the overnight sample in order to halt the trypsin reaction and the sample was taken up into the ZipTip by pipetting up and down five times followed by immersion of the ZipTip with bound peptide fragments into 80% ACN and the pippetting action repeated three times. Next the ZipTip was immersed in 0.1% TFA and the pippetting action repeated once more. The same ZipTip was again immersed in the sample and pipetted up and down 15 times followed by two cycles of pippeting using 10 µl of 0.1% TFA which were discarded from the ZipTip. Finally bound peptide fragments were eluted from the ZipTip by pipetting up and down 15 times using 4 µl of 80% ACN into a fresh tube containing 18 µl of 0.1% TFA. Samples were then spotted onto an MS target plate which was loaded in the ultrafleXtreme MALDI-TOFTOF Mass Spectrometer (Bruker Daltonics). Results were observed as spectral peaks of the masses of the trypsin-digested peptides. The data was searched against a database made against CEP290 and the T21 hypothetical protein and using MASCOT 2.2 (Perkins *et al.*, 1999).

2.2.5.8 Enzyme Linked Immunosorbent Assay (ELISA)

Plate preparation: Indirect ELISA was utilised to detect binding of specific peptide or protein in samples and was performed as follows. One day prior to assay, samples were

diluted in coating buffer and applied at 50 µl/well to 96 well NUNC plates and sealed with an adhesive plate cover. In this instance, diluted samples consisted of either, cell line or protein lysates isolated from patient tumour sections at 1mg/ml, recombinant T21 protein at 0.3 µg/ml or T21 antibody specific peptide serially diluted from 10 ng/ml to 0.01 pg/ml.

ELISA and detection: Following overnight incubation at 4°C, wells were aspirated, blotted and washed with wash buffer x3 and wells were then incubated with 100µl/well of chilled blocking buffer at RT for 2 hours. Wells were washed as previously described and primary antibodies were prepared in chilled dilution buffer and seeded at 50µl/well into the appropriate wells. Plates were incubated at RT for 60 minutes followed by aspiration and washing as described above. Wells were then incubated for 2 hours with biotinylated secondary antibody prepared as described previously. Following incubation, wells were again washed as previously described and wells were incubated 100µl/well of HRP Strepavidin diluted to a working stock of 1:200 in dilution buffer. Plates were again incubated at RT for 20 minutes before a final wash as described previously. To each well, substrate reagent solution was added at 100µl/well comprising of 1 part colour reagent-A (stabilised hydrogen peroxide) and 1 part colour reagent-B (stabilised tetramethylbenzidine) and allowed to develop concealed from light at RT for 15 minutes. Finally 50 µl of acid stop solution (H_2SO_4) was added to each well and the optical density was measured at 450 nm wavelength using a Tecan microplate reader.

2.2.5.9 Cell cycle analysis

Cell preparation and staining: Cell cycle analysis was performed using the Click-iT EdU ALEXAfluor 647 flow cytometry kit following the manufacturers guidelines. Briefly, 5-ethynyl-2´-deoxyuridine (EdU) was added to 24 well plate cell cultures at a concentration of 10 µM/well 2 hours prior to harvesting into flow cytometry tubes. Following this, cells were centrifuged at 400 g for 3 minutes at 4°C, supernatants were discarded and the cell pellet resuspended in dPBS and centrifuged as before. The supernatants were discarded and the pellet resuspended in 500 µl of 4% paraformaldehyde fixative solution (PFA) for 15 minutes at RT. Cells were then centrifuged as before and washed with 1% BSA in dPBS wash buffer to remove any residue PFA (In the cases of time-point studies, cell pellets were stored at 4°C for up to 5 days before continuing the procedure). Following washing, 500 µl of the following mixture was prepared from the kit components provided

and added to each tube and incubated in the dark at RT for 30 minutes, 438 µl of Click-iT 1X reaction buffer, 10 µl of $CuSO_4$, 2.5 µl of fluorescent azide (CellCycle 647-red 7-AAD) and 50 µl 1X reaction buffer additive. Following incubation, cells were washed using 1:10 dilution of saponin buffer and centrifuged as before. The supernatants were discarded and the cell pellet resuspended in 500 µl of 1:10 dilution of saponin buffer. To measure the DNA content of the cells 1 µl of cell cycle dye 405-violet along with 2 µl of RNAse was added to the resuspended cells and incubated in dark for 15 minutes. DNA/cell cycle analysis was performed using a Gallios flow cytometer and data obtained analysed using the Kaluza Flow Analysis Software (version 1.0) from Beckman Coulter. Cell analysis was performed by plotting DNA content (x-axis) against EdU (y-axis) measured in linear mode (LIN).

2.2.6 Cell transfection for gene silencing studies

Targeted suppression of gene expression using interfering RNA (iRNA) is a widely utilised tool for the investigation of gene function. Cell transfection of small interfering RNA (siRNAs) specifically designed to target the RNA sequence of a gene enable initiation of gene silencing via the degradation of the mRNA sequence. To induce long-lived stable gene suppression, an alternative short hairpin RNA (shRNAs), employing the use of an expression vector can be used.

2.2.6.1 Small interfering RNA (siRNA) transfection

Transfection of cell lines using siRNA was done using INTERFERin transfection reagent according to the manufacturers protocol.

Cell preparation: Briefly a day prior to transfection, cells were seeded into a 24 well flat bottom plate at a concentration of $5x10^4$ cells/ml and left to incubated overnight at 37^{o}C, 5% CO_2 so that cells would be between 30-50% confluent at the time of transfection.

Cell transfection: The following day, siRNA stocks were prepared at 40 µM/well in 100µl/well of serum free Opti-MEM media and mixed. To these mixtures 2 µl/well of INTERFERin reagent was added and vortexed for 10 seconds. Further wells containing commercially available control siRNA (Eurogentec), INTERFERin only and Opti-MEM

media alone were prepared and all mixtures were incubated at RT for 10 minutes to allow complexes to form. These complexes were then added dropwise whilst on a plate rocker 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 37ºC, 5% CO_2 for 6 hours after which 500 µl of pre-warmed complete media was added to each well and incubated (time dependent on the assay performed).

Target gene	siRNA duplexes	Sequence (5' – 3')
T21	1	**GUGUAGAAAUGCAGAUAUA** **UAUAUCUGCAUUUCUACAC**
T21	2	**AGAUAUAGAGCUUGAACAU** **AUGUUCAAGCUCUAUAUCU**
T21	3	**GCACAGGAAUUCUUGAUCA** **UGAUCAAGAAUUCCUGUGC**

Table 2.7 – RNA duplexes designed for siRNA transfection.

2.2.6.2 Short hairpin RNA (shRNA) transfection

Stable transfection of cell lines using a pGFP-B-RS vector with blasticidin resistance gene (see appendix Figure Ia for vector map) 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.

Cell preparation: Briefly a day prior to transfection, cells were seeded into a 24 well flat bottom plate at a concentration of $5x10^5$ cells/ml and left to incubated overnight at 37ºC, 5% CO_2 so that cells would be between 85-95% confluent at the time of transfection.

Cell transfection: The following day, shRNA stock was prepared in HAMS-F12 media at a concentration of 1.2 µg per 50 µl per well and mixed gently. Alongside to this, 2 µg per 50 µl per well of Lipofectamine-2000 was also prepared in HAMS-F12 and both preparations were left to incubate at RT for 5 minutes before combining the diluted DNA and Lipofectamine-2000 allowing complexes to form at RT for 30 minutes. During which,

media from the wells of the overnight cultures were removed and replaced with 100 µl of the complexes containing the DNA/Lipofectamine-2000 solutions which were added dropwise to the wells whilst on a rocking platform and then incubated at 37^{o}C, 5% CO_2 for 6 hours, after which a further 900 µl of pre-warmed complete media was added and left to incubate. Following 48 hours of transfection, media from the wells was removed and fresh media containing 25 µg/ml of blasticidin selective antibiotic was added. The cells were then observed over the following days to monitor cell viability.

Clonal selection: 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 RT-Q-PCR and immunofluorescent staining over several passages to ensure stable transfection.

Cell Sorting: 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.

2.2.7 Animals and peptides

2.2.7.1 Animals

C57B/6 HLA-A2.1/DR1 transgenic mice (HLA-A2/DR1) used were murine class I knockout, but express chimeric HLA-A2.1 molecule with human α1, 2α and β2-microglobulin and 3α domain murine and were received as a gift from Dr. Lemonnier, Institut Pasteur, Paris. Mice were bred and all work was carried out under Home Office approved project licence number 40/3563 and in accordance to Home Office regulations at the Nottingham University Animal facility and the Nottingham Trent University Animal facility.

2.2.7.2 Peptides

Overlapping 15mer peptides were either purchased (ProImmune Ltd) or obtained through collaboration (Dr Cave and Dr Mundell, Nottingham Trent University) and used for immunisation experiments. All peptides for injection were resolubilised in 100% DMSO at a final concentration of 10 mg/ml and stored at -80°C. Peptide received from Pacific Immunology was resolubilised following manufacturer's instruction in 50% ACN and 50% ddH_20.

2.2.8 Immunisations and procedures

2.2.8.1 Peptide immunisations

Double transgenic HLA-A2/DR1 mice used between 6 – 12 weeks were given a bolus injection of 100 µl of test peptide(s) (100 or 110 µg) in PBS emulsified in Incomplete Freund's Adjuvant (IFA) in a ratio of 1:1 subcutaneously at the base of the tail. In accordance with experimental requirements, mice could be given a further 2 rounds of weekly immunisations 7 days following initial immunisation. Between 7-10 days post final immunisation, spleens were harvested for *in vitro* assays.

2.2.8.2 DNA bullet preparation and DNA immunisation

DNA bullet preparation - Gold microcarrier solution preparation: Gold "bullets" containing the gold labelled DNA were produced according to kit manufacturers protocol with the following adjustments. DNA (see appendix Figure Ib for vector map) to be labelled was made up to a working concentration of 1 µg/µl in ddH_2O in a final volume of 36 µl. This gave a dose of 1 µg of DNA/bullet/animal. To the measured gold, 200 µl of 0.05 M spermidine was added and vortex mixed, then sonicated to break up gold clumps. The DNA was then added to the gold and spermidine mixture and briefly vortexed, then whilst sonicating 200 µl of 1 M $CaCl_2$ was added dropwise to the mixture. The mixture was left precipitate at RT for 10 minutes. The microcarrier solution was centrifuged at 14500 g for 30 seconds and the supernatant discarded. Following this, the pellet was washed three times using 1 ml of 100% anhydrous ethanol briefly vortexing between each wash. Following the final wash step the supernatant was discarded and the pellet once

again vortexed briefly. To the microcarrier solution, 1 ml of 0.025 mg/ml of PVP (Polyvinylpolypyrrolidone) was added and the solution was transferred to a 15 ml polypropylene centrifuge tube. The tube previously containing the microcarrier solution was then used to rinse using again 1 ml of 0.025 mg/ml of PVP and transferred into the same polypropylene tube giving a final volume of 2 ml.

Loading onto the Prep Station: Prior to loading the microcarrier solution, the gold-coating tubing was completely dried by purging with nitrogen gas for 15 minutes at flow set to 0.3-0.4 LMP on the Tubing Prep Station. The dried tubing was then removed, the adaptor tubing fitted with a 10 ml syringe was fixed at one end. The other end of the tubing was placed into the gold suspension, then whilst sonicating, the suspension was quickly drawn into the gold-coating tubing. The tubing was then loaded onto the Tubing Prep Station with the gas turned off. The microcarriers were left to settle for 3-5 minutes before the ethanol was removed using the 10 ml syringe. The tubing was then turned 180° for 5 minutes to allow the gold to begin coating the inside surface of the tubing. Following this the tubing was set to rotate for a further 30 minutes before the nitrogen gas was switched on for 5 minutes at flow set to 0.3-0.4 LMP to completely dry the gold microcarriers. The Tubing Prep Station was then switched off and the gold-coated tubing removed and placed into the tubing cutter to cut the gold coated areas of the tubing into 1.0 cm pieces. These "bullets" were stored in a tube at 4°C for DNA immunisations.

DNA immunisation: Mice aged between 6 – 12 weeks were administered with DNA encoding whole T21 protein coated onto 1.0 µm gold particles which were then administered intradermally by the Helios Gene Gun as per manufacturer's instructions, with each mouse receiving 1 µg of DNA per immunisation. This was repeated at weekly intervals for a total of three immunisations. Between 7 – 10 days post final immunisation, spleens were harvested for *in vitro* assays.

2.2.9 *In vitro* generation of CTL and murine IFNγ EliSpot assay

2.2.9.1 LPS blast cell generation

Preparation of LPS blast cells: Splenocytes from naïve animals were used as APCs during *in vitro* restimulation of immunised splenocytes and were therefore harvested 2 days prior

to spleens of immunised animals were collected. Spleens were excised post-mortem and transported in dPBS, then using a Petri dish and sterile scissors and forceps the spleens were prepared firstly by carefully cut away and removing fatty tissue deposits followed by gentle massage of the spleen to break down internal compartments. Next, a 10 ml syringe containing 10 ml of T cell media was used to inject media into the ends of the spleen and the forceps used to gently flush the contents until the splenic sack became translucent. The splenic sack was then homogenised using scissors and both spleen and the flush were collected and placed in a 15 ml polypropylene tube and further broken down using a pasteur pipette. After allowing the debris to settle the supernatant was collected and centrifuged at 400 g, 4ºC for 3 minutes. The cellular pellet was resuspended in T cell media and cells were counted using 0.06% acetic acid, and then trypan blue, to exclude red blood cells and dead cells which appear blue under the microscope. The cell suspension was then seeded into a T75 flask at a concentration of $1.5x10^6$ cells/ml with 25 µg/ml of lipopolysaccharide (LPS) and 7 µg/ml of dextran sulphate, used to mature cells (Firat *et al*., 1999), and 1.6 µg/ml of Vitamin E, used to minimise cell death. These cells were then incubated at 37ºC, 5% CO_2 for 48 hours. Following incubation, cells were then harvested and irradiated with 21.35 Gy for 15 minutes, washed twice and resuspended using T cell media at $20x10^6$ cells/ml in universal tubes with 10 µg/ml of relevant peptide(s) and incubated with a slightly loosened lid to allow sufficient ventilation at 37ºC, 5% CO_2 for 60 minutes. Following incubation, cells were once again washed, counted and the concentration adjusted to $0.5x10^6$ cells per 500 µl per well and were seeded into flat bottom 24 well plates alongside splenocytes from immunised animals.

2.2.9.2 *In vitro* restimulation with peptide

Spleens from immunised animals were harvested 7 days after final immunisation from which splenocytes were flushed and processed from the spleen as mentioned above. The cellular pellet was resuspended at $2.5x10^6$ cells per 500 µl per well alongside peptide-pulsed LPS blast splenocytes into flat bottom 24 well plates and placed at 37°C, 5% CO_2 for 6 days.

2.2.9.3 Bone marrow-derived dendritic cell generation

Processing and culture of BM-DC: Bone marrow-derived dendritic cells (BM-DCs) were generated by taking bone marrow from mouse hind limbs 8 days prior to carrying out the proliferation assay. Limbs were placed in a petri dish and surrounding muscle and tissue was carefully removed using sterile scissors and forceps to expose the hind leg bones. The bones were then separated from the knee joint and placed in a petri dish containing BM-DC media. The ends of the femur, and tibia were cut and using a 10 ml syringe containing 10 ml of BM-DC media, bones were injected with media, in turn, from each end flushing the marrow into the petri dish. The marrow cells were further broken down using a pasteur pipette and passed through a cell strainer into a universal tube. The suspension was centrifuged at 400 g, 4^{o}C for 3 minutes. The cellular pellet was resuspended in BM-DC media and cells were counted using 0.06% acetic acid and trypan blue. The cell concentration was adjusted to 1x10^{6} cells/ml with 1 pg/ml of GM-CSF and seeded into 24 well flat bottom plates at 1ml per well. These cells were then incubated at 37^{o}C, 5% CO_2.

Washing DC cultures: After 2 and 4 days, non-adherent cells were removed by gentle wash with 700 µl of BM-DC media contained in the well. Wells were then replenished with 750 µl of fresh media containing 1 pg/ml of GM-CSF.

2.2.9.4 Dendritic cell maturation for antigen presentation

DC maturation: After 7 days of culture, DCs were harvested from wells and centrifuged at 400 g, 4^{o}C for 3 minutes and the cellular pellet resuspended in BM-DC media and cells were counted using trypan blue. Cell concentration was adjusted to 0.5x10^{6} per ml containing 1 pg/ml of GM-CSF and 1 µg/ml peptide(s). Plates were then left for incubation at 37^{o}C, 5% CO_2 for 4 hours after which, 2.5 µg/well of LPS in 100 µl per well of BM-DC media was added to induce maturation and left to incubate overnight. The following day, the mature DCs were harvested from wells and centrifuged at 400 g, 4^{o}C for 3 minutes and the cellular pellet, resuspended in 1 ml of BM-DC media into universal tubes and pulsed with 1 µg/ml of relevant peptide(s). An additional 12.5 µg/ml of Poly-IC, used to induce full maturation of cells, was added and incubated at 37^{o}C, 5% CO_2 for 2 hours with the lids screwed loosely on to allow cells sufficient ventilation.

After incubation, centrifuged as before and the cellular pellet resuspended in 1 ml of T cell media and counted using trypan blue. The cell concentration was adjusted to $5x10^3$ cells per 50 µl in T cell media and used as antigen presenting cells in the proliferation assay.

2.2.9.5 Murine interferon gamma EliSpot assay

Plate preparation: EliSpot assays were performed using manufacturer's procedure guidelines. Microplates were pre-wet using 100 µl of 75% ethanol for 5 minutes. Meanwhile, capture antibody was used at a 1:60 dilution from the stock using dPBS allowing for 10 mls per plate. The ethanol from the wells was aspirated off and the plate blotted onto tissue paper to remove residual liquid. Immediately 100 µl of the diluted capture antibody was added to wells, covered with the lid and incubated overnight at 4°C.

EliSpot assay: The following day, the unbound capture antibody was aspirated off the wells and blotted, and 200 µl of C.T.L media was added and plates were placed at 37ºC, 5% CO_2 in order for wells to reach optimal pH and conditions before addition of cells. Prior to addition of cells wells were aspirated and plates blotted. Both effector and target cells were prepared and resuspended in C.T.L media and seeded into appropriate wells. Plates were incubated at 37ºC, 5% CO_2 for 48 hours after which cells were aspirated off and washed 3 times with 200 µl of wash buffer finally removing any remaining liquid by blotting against tissue paper. Detection antibody was also used at a 1:60 dilution from the stock using reagent diluent allowing for 10 mls per plate. The diluted detection antibody was added at a volume of 100 µl to wells, covered with the lid and incubated overnight at 4°C.

EliSpot detection: The following day, plates were washed as described as above using wash buffer. Strepavidin-AP was used at a 1:60 dilution from the stock using reagent diluent and seeded at 100 µl into each well and incubated for 2 hours at RT. Plates were again washed and 100 µl of BCI/NBT solution was added to each well and the plates wrapped in foil to allow colour development for 20-30 minutes. The colour development was then stopped by rinsing wells using ddH_2O and blotting plate to remove any excess liquid, and the plastic backing of the plate removed. Plates were then left to dry at RT for at least 2 hours before spots could be quantified using an EliSpot reader.

2.2.9.6 ^{3}H thymidine incorporation assay for murine T cells

Plate preparation: Splenocytes from the immunised mice were harvested and centrifuged at 400 g, 4ºC for 3 minutes resuspended in T cell media counted using trypan blue. Cell concentration was adjusted to $5x10^4$ cells per 100 µl per well using T cell media and seeded into appropriate wells of a 96 well round bottom plate. DCs pulsed with relevant peptide(s) were then added at $5x10^3$cells per 50 µl per well alongside plated T cells in quadruplicate sets of wells.

^{3}H thymidine incorporation and detection: Cultures were incubated for approximately 60 hours at 37ºC, 5% CO_2, and [^{3}H]-Thymidine was added at 37 MBq/well in the last 18 hours of incubation. Plates were 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 minute (cpm) and as means of the quadruplicate wells. Data analysed using the unpaired student's *t* test. Significance levels were assigned as $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$.

2.2.10 *In vitro* restimulation of patient PBMCs and human IFNγ EliSpot assay

2.2.10.1 Preparation of lymphocytes from frozen PBMCs

Frozen vials containing patient PBMCs were thawed rapidly using a hairdryer before being transferred into a 25 ml tube containing pre-warmed complete PBMC media and washed twice by centrifugation at 300 g for 5 minutes. Cell pellets were resuspended in 4 ml of media and incubated for 30 minutes with loosened lid at 37ºC, 5% CO_2. Peptides were prepared in complete C.T.L media at a concentration of 1 µg/ml and 10 µg/ml and added to appropriate wells at 100 µl volume. Cells were then centrifuged as before and the pellet resuspended in complete C.T.L media, counted and seeded into a 96 well EliSpot plate at concentrations between $1\text{-}50x10^4$ cells per well. Following 48 hour incubation at 37ºC, 5% CO_2, cell supernatants were carefully collected and stored at $^{-}$20°C for further analysis and wells were subsequently washed with x3 times with wash buffer before addition of detection antibody and following EliSpot performed (refer to in section 2.2.9.5). Following development, plates were air dried, scanned and spots counted using an Immunospot analyzer, EliSpot Reader (Cellular Technology Ltd).

CHAPTER 3

GENE AND TRANSCRIPT ANALYSIS AND PROTEIN VERIFICATION OF T21

3.1 Introduction

Biomarkers have been defined by the National Cancer Institute as "a biological molecule found in blood, other body fluids, or tissue that is a sign of a normal or abnormal process or of a condition or disease." Molecular biomarkers are widely utilised as inexpensive and non-invasive tools to evaluate biological changes indicative of cancer, leading to early and rapid clinical intervention. Biomarkers have been introduced in early prevention population screening and diagnosis in order to reliably detect the presence and classify the type of cancer. Biomarkers can also further determine patient outcome prior to treatment by identifying aggressive and indolent forms of cancer and also in risk stratification to evaluate occurrence or reoccurrence in populations. In addition to their use as prognostic markers, they can be used as predictive markers to anticipate patient responses following specific treatment. This enables a more informed decision to be made on the most effective treatment options available and in post-treatment monitoring as early predictors of relapse.

Molecular biomarkers such as Her-2 in breast cancer (Sjögren *et al.,* 1998) and the Philadelphia chromosome (Ph) in certain leukaemias (Shtivelman *et al.,* 1985) have unquestionably had a major impact on combating cancer. Despite breakthroughs like these, no other non-hereditary cancer biomarker has emerged demonstrating the same degree of success or has been approved for clinical use in the last 25 years. Often, shortcomings in molecular markers have been attributed to their lack of overall sensitivity and/or specificity, the most common example being prostate-specific antigen (PSA) presently used in screening for prostate cancer.

Molecular biomarkers have provided great opportunities as biological targets for cancer therapies and have paved the first steps towards the development of personalised cancer treatments. For instance the use of anti-cancer drugs such as Herceptin in Her2 positive breast cancer and Imatinib in CML patients carrying *BCR/ABL* gene fusion have translated into powerful tools used to take advantage of the molecular traits exhibited by an individual's tumour. Over the last three decades, a better understanding of the immune system's response to cancer has encouraged the emergence of personalised cancer vaccines as a promising treatment strategy for patients with cancer. This approach has been aided significantly by the identification of tumour associated biomarkers and exploiting their apparent dual role as biomarkers of disease and as targetable tumour antigens due to their aberrant expression in cancer. The appeal of this approach is the use of pre-selected biomarkers as tumour-specific targetable antigens by the tumour-bearers own immune system, bypassing various forms of toxicity associated with many current treatment strategies. Approaches for the serological identification of biomarkers using tumour-specific CTL cloning and SEREX has allowed for the discovery of antigens that are both immunogenic tumour proteins and in the case of SEREX, screen for antigens that elicit high-titre IgG TAA-specific antibody responses, which in turn help identify $CD4^+$ T cells reactive to the antigen in question (Jäger *et al.,* 2000). This has provided the platform for assessment of newly identified antigens for their utility to become part of immunotherapeutic intervention strategies and to this end, in the early 2000's, this laboratory sought to identify prostate cancer specific antigens with relevance as immunotherapeutic targets. During this period, T21 (testis clone 21) was identified as a promising candidate tumour associated antigen as a result of modified SEREX screening of a normal testicular cDNA library expressed in *E.coli* and probed with allogeneic prostate cancer patient serum (Miles *et al.,* 2007). Briefly, sera from prostate cancer

patients was used to screen an allogeneic testicular cDNA library (purchased from Clontech, USA) whereby plaque-forming units (PFU) were screened with pooled sera from four patients with prostate cancer. Reactive clones underwent secondary and tertiary screening to reach monoclonality, *in vivo* excised and converted to plasmid forms. The subsequent cDNA were analysed using restriction mapping and sequencing. The identification and initial screening of T21 using the method described warranted further genomic study to determine its function and investigate its immunological potential. Following sequencing of the *T21* clone, rapid amplification of 5' and 3' cDNA ends (RACE) and nested PCR experiments using testicular cDNA were performed to identify the extension of the cDNA sequence in both directions including the un-translated regions. The identified transcript resulted in a 2797bp length nucleotide sequence and subsequent 3 frame translation of this sequence lead to the confirmation of a novel protein consisting of 534 amino acids (Figures 3.2 and 3.3). *In silico* analysis performed in 2001 sited the *T21* sequence to chromosome 12q21.33 and was found to be composed of 19 exons with 12 exons covering the coding region of the gene with numerous transcript similarities.

Following the identification of *T21*, work was undertaken to determine *T21* mRNA and protein expression in a panel of healthy, benign and cancerous tissues as well as in characterised cell lines (Dr Alistair Rogers, PhD Thesis 2009). This work demonstrated mRNA expression present at a low level in essential tissues and strongly localised to malignant glands compared to control and BPH when the prostate cohort was interrogated. 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 in 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). Further *in vitro* gene knockdown experiments in prostate cancer cell lines performed using siRNA transfection with the oligonucleotide sequence CTAGTGCCAGTATCATTAA designed in exon 6 of *T21*, suggested towards the involvement of T21 in cellular proliferation.

Later *in silico* analysis using sequence NCBI-BLAST and alignment tools revealed that *T21* shared significant sequence similarity with a centrosomal protein called CEP290. This gene is also known as nephrocystin-6, 3H11Ag, KIAA0373, tumour antigen Se2-2 and cancer/testis antigen-87 (CT87). The *CEP290* gene encodes for a centrosomal protein (290 kDa) which is present in the cell centrosomes of dividing cells and has a role in tissue specific cillogenesis (Coppieters *et al.,* 2010). *CEP290* was initially identified by Nagase and colleagues in 1997 as a partial clone corresponding to *CEP290* which they called *KIAA0373* derived from sequencing of human brain cDNA libraries. In 2001, Chen and Shou independently identified a partial sequence demonstrating homology to the same gene (which they termed *3H11Ag*) from a gastric cancer cell line expression library. They determined that the sequence generated a transcript approximately 2.3kb in size and encoded for a 70 kDa protein. Two years later, Andersen and colleagues (2003) performed mass spectrometry-based proteomic analysis of centrosomal fractions during interphase (G_1/S phase) (derived from human lymphoblastic KE37 cells) acquired through successive centrifugation. By correlating known centrosomal proteins and fluorescence-tagged localisation in a human osteosarcoma (U2OS cells) they identified a protein with an approximate relative molecular mass of 290 kDa and subsequently named the protein CEP290. Further analysis determined that the *CEP290* gene consisted of 93.2kb, spanning 54 exons and encoded for a putative protein consisting of 2479 amino acid residues from a full-length 7.9kb mRNA transcript sequence. From the deduced CEP290 protein sequence Sayer and colleagues described 13 putative coiled-coil domains, a region with homology to SMC (structural maintenance of chromosomes) chromosome segregation ATPases, a bipartite nuclear localisation signal, 6 RepA/Rep+ protein kinase inducible domain motifs, 3 tropomyosin homology domains, and an ATP/GTP binding site motif A (Sayer *et al.,* 2006). To date, over 100 mutations of *CEP290* have been identified and studied primarily in the context of cilia related disorders, namely Senior-Loken syndrome (Sayer *et al.,* 2006), Meckel syndrome (Baala *et al.,* 2007), Leber congenital amaurosis (den Hollander *et al.,* 2006), Joubert syndrome (Sayer *et al.,* 2006; Valente *et al.,* 2006) and Bardet-Biedl syndrome (Leitch *et al.,* 2008). It is noteworthy that the presence of CEP290 antibodies has been reported in some cancers (Eichmuller *et al.,* 2001; Chen & Shou, 2001).

In earlier studies of T21, primers designed for PCR experiments and the custom anti-T21 antibody used were common to both T21 and CEP290 suggesting that either of the two proteins could be detected in PCR and protein-based studies. As CEP290 has been found to

be involved in centrosomal function, it could naturally be concluded that its presence would be in some way 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* in both a genomic and transcriptional context to determine the best approach to study the role if any, T21 may play in cancer progression. The current study aimed to fully characterise the *T21* mRNA sequence using both *in silico* and experimental investigations and secondly, confirm the currently hypothetical protein sequence in an effort to study T21 independently from CEP290 and its known variants.

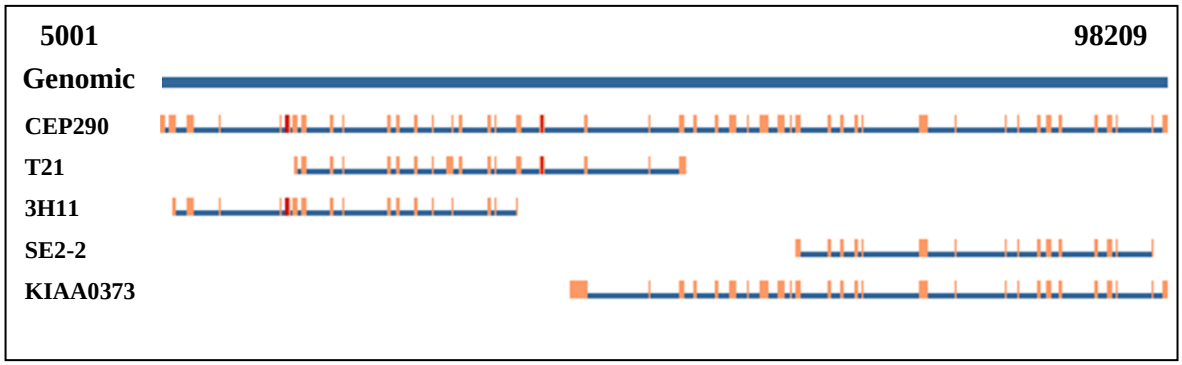
3.2 Results

3.2.1 Genomic investigation into T21

Prior to investigating *T21*, it was important to confirm how the *T21* mRNA sequence aligned to the human genomic sequence from *CEP290* situated on chromosome 12. Study of the extent of mRNA sequence similarity between *T21*, *CEP290* and *CEP290* variants was performed using publically available online curated gene repositories such as NCBI Human Genome Resources (http://www.ncbi.nlm.nih.gov/) and Ensembl Genome browser (http://www.ensembl.org/index.html). Further alignment of exon regions of *CEP290* and its many genomic transcripts that produce putative proteins was also performed to identify any existing sequences that share similarity with *T21*.

To summarise, sequence Basic Local Alignment Search Tool (BLAST) analysis demonstrates a 31% alignment coverage similarity between *CEP290* with *T21* of which there is 96.5% similarity between *T21* and *CEP290* mRNA. Further mRNA to genomic alignment confirmed this similarity (Figure 3.1) and also revealed a potentially unique protein encoding nucleotide sequence consisting of 93bp present in *T21* that was absent in *CEP290* mRNA (Figure 3.2 - highlighted in red). This sequence was subjected to focused searches which revealed 100% sequence homology to genomic *CEP290* demonstrating that the short nucleotide sequence was present in an intronic portion of *CEP290*. After extensive searches of the *T21* mRNA sequence, no further significant similarity to any other DNA or mRNA sequence in the Ensembl and NCBI databases was found, other than that of *CEP290* and its numerous variants, and therefore it is highly likely that *T21* is potentially a novel transcript of the *CEP290* gene found on chromosome location 12q21.32 (ch12:88442,794-88,535,993, complement strand). Moreover, the 93bp sequence was also found to only have homology to that of *CEP290* DNA indicating that its inclusion in the *T21* sequence to be "unique". Further still, this 93bp insertion originating from intron 18 of *CEP290* which, according to previous sequencing investigation, is encoded as part of the T21 protein product. Following nucleotide-to-protein frame translation of T21 and alignment of this protein sequence to CEP290 a 27% alignment coverage similarity between CEP290 with T21 was shown. There is 96.7% similarity between T21 and CEP290 protein but more interestingly there is no significant

similarity of the "uniquely" encoded 31 amino acid T21 sequence with any other organism listed in the Ensembl and NCBI databases (Figure 3.3).



Designation	*Name / Accession No.*	*Function*	*Comments*	*Reference*
CEP290	*Centrosomal Protein 290 kDa (CEP290) / NM_025114.3*	*Present in the cell centrosomes of dividing cells and has a role in tissue specific cillogenesis*	*Well characterised at the genomic level used as a reference standard in the RefSeqGene project*	
T21	*Prostate cancer antigen T21 / AY590151.1*	*Unknown*	*Identified by modified SEREX-defined screening a testis cDNA library using sera from prostate cancer patients*	Miles *et al.*, (2007)
3H11	*Monoclonal antibody 3H11antigen / AF317887.1*	*Localised to the cytoplasm and nucleus*	*MAb 3H11 generated from mice immunised with human gastric cancer cell lines*	Chen and Shou (2001)
SE2-2	*CTCL tumour antigen SE2-2 / AF273044.1*	*Unknown*	*Identified by a SEREX-defined screening a testis cDNA library using sera from CTCL patients*	Eichmuller *et al.*, (2001)
KIAA0373	*KIAA0373 / AB002371.1*	*Role in centrosomes determined by protein correlation profiling using mass spectrometry*	*Identified using cDNA libraries derived from human brain*	Andersen *et al.*, (2003) Nagase *et al.*, (1997)

Figure 3.1 – mRNA to genomic alignment of mRNA transcripts. *Above diagram compiled using NCBI affiliated online alignment tool http://www.ncbi.nlm.nih.gov/spidey with accompanying table detailing putative proteins found to have sequence similarity to CEP290. Blue lines illustrate genomic sequence and orange boxes are representative of exon sequence location and relative sequence sizes.*

```
   1    CCGCGGGGAT CATTATCAAC TTCAAGTGCA GGAGCTTACA GATCTTCTGA AATCAAAAAA
  61    TGAAGAAGAT GATCCAATTA TGGTAGCTGT CAATGCAAAA GTAGAAGAAT GGAAGCTAAT
 121    TTTGTCTTCT AAAGATGATG AAATTATTGA GTATCAGCAA ATGTTACATA ACCTAAGGGA
 181    GAAACTTAAG AATGCTCAGC TTGATGCTGA TAAAAGTAAT GTTATGGCTC TACAGCAGGG
 241    TATACAGGAA CGAGACAGTC AAATTAAGAT GCTCACCGAA CAAGTAGAAC AATATACAAA
 301    AGAAATGGAA AAGAATACTT GTATTATTGA AGATTTGAAA AATGAGCTCC AAAGAAACAA
 361    AGGTGCTTCA GCCCTTTCTC AACAGACTCA TATGAAAATT CAGTCAACGT TAGACATTTT
 421    AAAAGAGAAA ACTAAAGAGG CTGAGAGAAC AGCTGAACTG GCTGAGGCTG ATGCTAGGGA
 481    AAAGGATAAA GAATTAGTTG AGGCTCTGAA GAGGTTAAAA GATTATGAAT CGGGAGTATA
 541    TGGTTTAGAA GATGCTGTCG TTGAAATAAA GAATTGTAAA AACCAAATTA AAATAAGAGA
 601    TCGAGAGATT GAAATATTAA CAAAGGAAAT CAATAAACTT GAATTGAAGA TCAGTGATTT
 661    CCTTGATGAA AATGAGGCAC TTAGAGAGCG TGTGGGCCTT GAACCAAAGA CAATGATTGA
 721    TTTAACTGAA TTTAGAAATA GCAAACACTT AAAACAGCAG CAGTACAGAG CTGAAAACCA
 781    GATTCTTTTG AAAGAGATTG AAAGTCTAGA GGAAGAACGA CTTGATCTGA AAAAAAAAAT
 841    TCGTCAAATG GCTCAAGAAA GAGGAAAAGA AGTGCAACTT CAGGATTAAC CACTGAGGAC
 901    CTGAACCTAA CTGAAAACAT TTCTCAAGGA GATAGAATAA GTGAAAGAAA TTTGGATTTA
 961    TTGAGCCTCA AAAATATGAG TGAAGCACAA TCAAAGAATG AAATCATAGC ACAGGAATTC
1021    TTGATCAAAG AAGCAGAGTG TAGAAATGCA GATATAGAGC TTGAACATCA CAGAAGCCAG
1081    GCAGAACAGA ATGAATTTCT TTCAAGAGAA CTAATTGAAA AAGAAAGAGA TTTAGAAAGG
1141    AGTAGGACAG TGATAGCCAA ATTTCAGAAT AAATTAAAAG AATTAGTTGA AGAAAATAAG
1201    CAACTTGAAG AAGGTATGAA AGAAATATTG CAAGCAATTA AGGAAATGCA GAAAGATCCT
1261    GATGTTAAAG GAGGAGAAAC ATCTCTAATT ATCCCTAGCC TTGAAAGACT AGTTAATGCT
1321    ATAGAATCAA AGAATGCAGA AGGAATCTTT GATGCGAGTC TGCATTTGAA AGCCCAAGTT
1381    GATCAGCTTA CCGGAAGAAA TGAAGAATTA AGACAGGAGC TCAGGGAATC TCGGAAAGAG
1441    GCTATAAATT ATTCACAGCA GTTGGCAAAA GCTAATTTAA AGATAGACCA TCTTGAAAAA
1501    GAAACTAGTC TTTTACGACA ATCAGAAGGA TCGAATGTTG TTTTTAAAGG AATTGACTTA
1561    CCTGATGGGA TAGCACCATC TAGTGCCAGT ATCATTAATT CTCAGAATGA ATATTTAATA
1621    CATTTGTTAC AGGAACTAGA AAATAAAGAA AAAAAGTTAA AGAATTTAGA AGATTCTCTT
1681    GAAGATTACA ACAGAAAATT TGCTGTAATT CGTCATCAAC AAAGTTTGTT GTATAAAGAA
1741    TACCTAAGTG AAAAGGAGAC CTGGAAAACA GAATCTAAAA CAATAAAAGA GGAAAAGAGA
1801    AAACTTGAGG ATCAAGTCCA ACAAGATGCT ATAAAAGTAA AAGAATATAA TAATTTGCTC
1861    AATGCTCTTC AGATGGATTC GGATGAAATG AAAAAAATAC TTGCAGAAAA TAGTAGGAAA
1921    ATTACTGTTT TGCAAGTGAA TGAAAAATCA TTTATAAGGC AATATACAAC CTTAGTAGAA
1981    TTGGAGCGAC AACTTAGAAA AGAAAATGAG AAGCAAAAGA ATGAATTGTT GTCAATGGAG
2041    GCTGAAGTTT GTGAAAAAAT TGGGTGTTTG CAAAGATTTA AGGAAATGGC CATTTTCAAG
2101    ATTGCAGCTC TCCAAAAAGT TGTAGATAAT AGTGTTTCTT TGTCTGAACT AGAACTGGCT
2161    AATAAACAGT ACAATGAACT GACTGCTAAG TACAGGGACA TCTTGCAAAA AGATAATATG
2221    CTTGTTCAAA GAACAAGTAA CTTGGAACAC CTGGAGTGTG AAAACATCTC CTTAAAAGAA
2281    CAAGTGGAGT CTATAAATAA AGAACTGGAG ATTACCAAGG AAAAACTTCA CACTATTGAA
2341    CAAGCCTGGG AACAGGAAAC TAAATTAGGT AATGAATCTA GCATGGATAA GGCAAAGAAA
2401    TCAATAACCA ACAGTGACAT TGTTTCCATT TCAAAAAAAA TAACTATGCT GGAAATGAAG
2461    GAATTAAATG AAAGGCAGCG GGCTGAACAT TGTCAAAAAA TGTATGAACA CTTACGGACT
2521    TCGTTAAAGC AAATGGAGGA ACGTAATTTT GAATTGGAAA CCAAATTTGC TGAGGTTTGA
2581    TATTATAAGT TTTATCATAC AATTATAGAA TAAAGAATTA GTTTTGGTAG ACATTGTATT
2641    ATTGTTAAGT GGTTTGTCTG GATCTCTGAA ATATCTTATT AATATAGTGC CTATGTTTTG
2701    TGTAATAAAT AAATAAAAGA TTTAAATCTG AATTGTTTAA AAGGAAAAAA AAAAAAAAAA
2761    AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAA
```

Figure 3.2 – Published T21 complete mRNA sequence. *Full 2797bp T21 nucleotide sequence (GenBank: AY590151.1) with complete coding sequence highlighted in blue (pos.976-2580), the T21 unique region highlighted in red (pos.997-1089) and underlined the sequence obtained from the cDNA insert (pos.1101 - 1640) (Miles et al., 2007). Also a diagrammatic representative of Figure 3.9A.*

1	**MSEAQSKNEI**	**IAQEFLIKEA**	**ECRNADIELE**	**HHRSQAEQNE**	**FLSRELIEKE**
51	**RDLERSRTVI**	**AKFQNKLKEL**	**VEENKQLEEG**	**MKEILQAIKE**	**MQKDPDVKGG**
101	**ETSLIIPSLE**	**RLVNAIESKN**	**AEGIFDASLH**	**LKAQVDQLTG**	**RNEELRQELR**
151	**ESRKEAINYS**	**QQLAKANLKI**	**DHLEKETSLL**	**RQSEGSNVVF**	**KGIDLPDGIA**
201	**PSSASIINSQ**	**NEYLIHLL**QE	LENKEKKLKN	LEDSLEDYNR	KFAVIRHQQS
251	LLYKEYLSEK	ETWKTESKTI	KEEKRKLEDQ	VQQDAIKVKE	YNNLLNALQM
301	DSDEMKKILA	ENSRKITVLQ	VNEKSFIRQY	TTLVELERQL	RKENEKQKNE
351	LLSMEAEVCE	KIGCLQRFKE	MAIFKIAALQ	KVVDNSVSLS	ELELANKQYN
401	ELTAKYRDIL	QKDNMLVQRT	SNLEHLECEN	ISLKEQVESI	NKELEITKEK
451	LHTIEQAWEQ	ETKLGNESSM	DKAKKSITNS	DIVSISKKIT	MLEMKELNER
501	QRAEHCQKMY	EHLRTSLKQM	EERNFELETK	FAEV	

Figure 3.3 – Published T21 protein sequence. (*GenBank: AAT01278.1) The resulting 3 frame translation of T21 sequence derived from the nucleotide sequencing illustrating a 534 amino acid protein with a theoretical molecular mass of approximately 62 kDa. Anti-T21/CEP290 antibody epitope region is shown underlined (pos.335-351) and the T21 unique region highlighted in red (pos.8-38).Translated cDNA sequence identified by SEREX in bold (pos.1-219)*

The premature termination of the *T21* protein is due to the splicing of intron 29 (173bp) from *CEP20* on to the C-terminal end of *T21*, proceeding exon 12 (Figure 3.4). As a consequence, this splicing inclusion leads to the immediate encoding of a nucleotide triplicate "TGA" serving as a unique stop codon in *T21* and resulting in a truncated protein which remains in-frame with the CEP290 protein sequence.

Further examination of the exon 18 – exon 19 junction of *CEP290* using online alternative splice site prediction algorithms (http://www.wangcomputing.com/assp/index and http://www.cbs.dtu.dk/services/NetGene2/) both predicted an alternative acceptor site (3'Exon-GGCAGAACAG^GTAGTGTAAA-5'Intron) and an alternative donor site (3'Exon-TTTCTTTAG^AATGAAATCA-5'Intron) which flanks the 93bp intronic sequence forming a unique alternative cassette exon (Figure 3.4). Two consensus branch point sequence signals were also identified further upstream of the uniquely encoded exon, required for effective splicing of pre-mRNA to mature RNA.

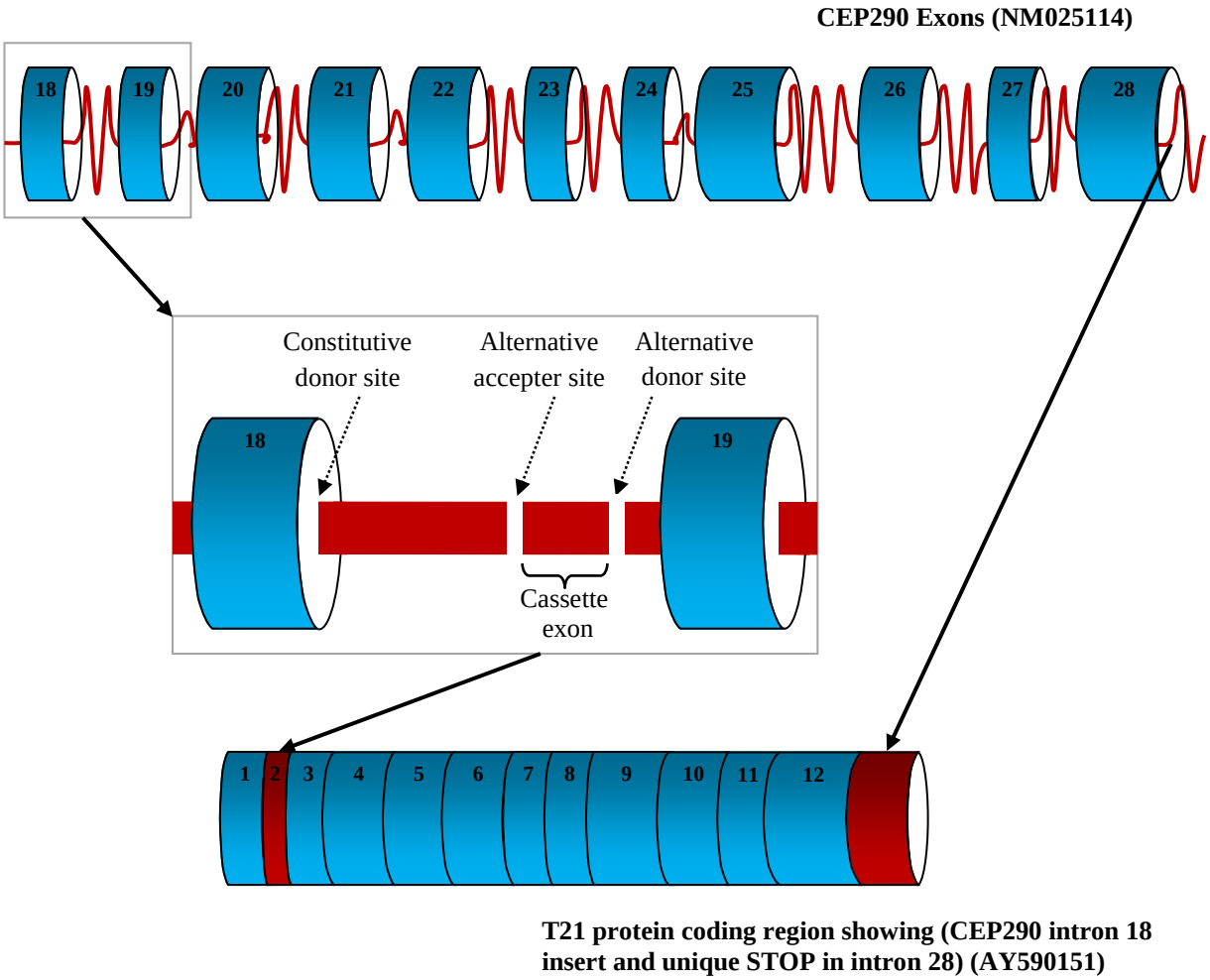


Figure 3.4 – Diagrammatic representation of T21 and CEP290 exon alignment. *The theoretical alternative splicing event leading to the inclusion of the 93bp unique sequence from the CEP290 gene sequence at exon junction 18 (top) to T21mRNA sequence (bottom). Also illustrated is the generation of the unique stop codon in T21 from the splicing of intron 28 of CEP290.*

The CEP290 protein itself is highly conserved across species particularly in primates, who share between 96–99% sequence similarity to human *CEP290*. A lesser degree of sequence similarity is observed in other mammals for example 92% in *Felix catus* and C*anis lupus familiaris*, 91% in *Equus caballus*, 87% in *Rattus norvegicus* and *Mus musculus* and in the Actinopterygii subclass, *Danio rerio* share 58% similarity. It is of note that despite high conservation among species, there is no evidence indicating encoding of the unique sequence. Although primates (such as *Pan troglodytes*) maintain the intronic sequence in the genomic sequence, however transcription to mRNA and translation to protein is not observed.

In order to verify these new findings, primers within the 93bp "unique region" were designed and used in a PCR to confirm the existence of this sequence. Initially, the PCR was performed using cDNA derived from prostate cancer derived cell lines PC3 and DU145. Due to the possibility of genomic DNA contamination (leading to the likely amplifying intronic region of *CEP290*) of the RNA preparations, RNA purification columns were used instead of routine acid guanidinium thiocyanate-phenol-chloroform extraction using STAT-60. The resulting 100bp band observed established the presence of the unique region within *T21* (Figure 3.5A) and the presence of this unique region was later confirmed in normal tissue samples (data not shown). In addition, amplification of the region flanking the *T21* unique sequence using primers designed to amplify the exon 18 – exon 19 junction containing the unique sequence showed two discreet bands corresponding to *T21* (287bp) and *CEP290* (194bp), indicating that both variants are present within a given sample preparation. Interestingly, the band corresponding to the *T21* transcript appeared fainter suggesting a difference in the abundance of mRNA between the *T21* and *CEP290* transcripts (Figure 3.5B).

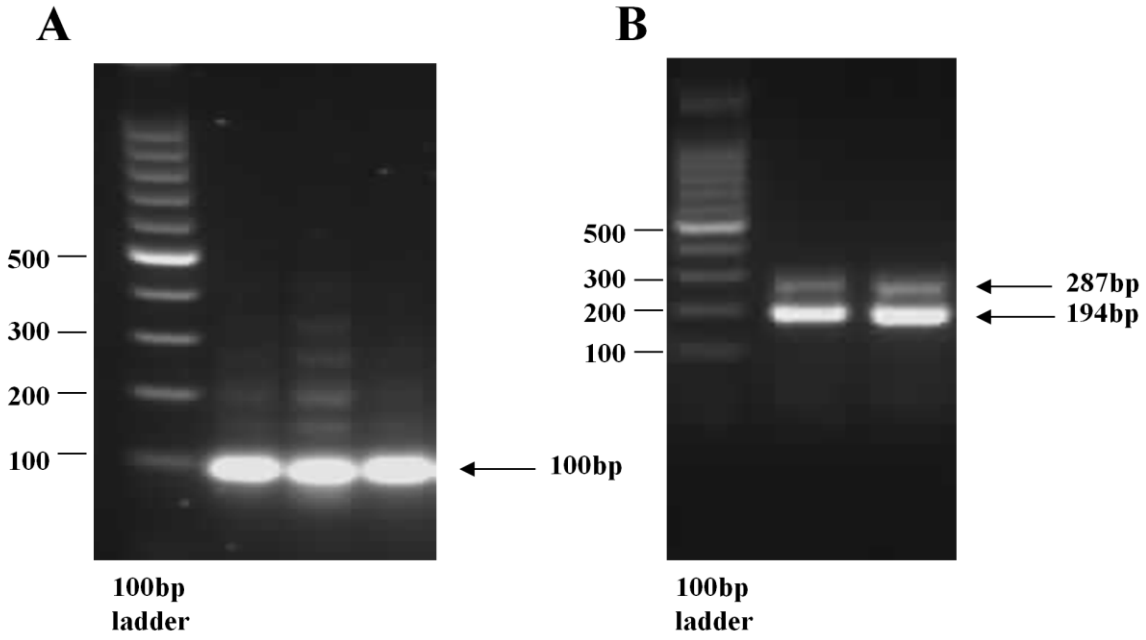


Figure 3.5 – Verification of T21 unique region product amplified using qPCR. *Gel image showing qPCR product amplification (100bp) of T21 unique region from the PC3 cell line derived cDNA in equalling loaded wells (**A**) and similarly two wells illustrating the amplification of both T21 (287bp) and CEP290 (194bp) PCR products using primers designed to flank the unique region (**B**) using PC3 cDNA. Non template control wells were also performed (data not shown).*

3.2.2 Transcriptional evidence to establish T21 protein translation

Based on the findings of *T21* and its similarity to the *CEP290* transcript, a number of questions still remained unresolved. Therefore, a more in-depth comparative study of both the *T21* and *CEP290* transcripts was deemed necessary. The alignment of the nucleotide sequences to the online database tools gave rise to other important differences which could alter the amino acid coding sequence and crucially, the expected theoretical protein length. Sequence alignment of the predicted 5' untranslated region (UTR) of *T21* also highlighted a crucial difference between the two sequences, in which there is an absent adenine residue at position 864 of *T21* when aligned with the matching exon 17 of *CEP290* (highlighted in yellow in Figure 3.6A). It was unclear as to whether this was indeed a real deletion or a sequencing error, therefore further examination using a targeted PCR approach was needed. To this end, primers were designed within the upstream untranslated 5' prime region of *T21* up to and including the unique region (highlighted in green Figure 3.6A). The amplified 639bp product was then excised from the PCR gel and sequenced, verifying the presence of the missing adenine (Figure 3.6C). The addition of this single nucleotide into the existing sequence and the resulting output from 6' frame nucleotide-to-protein translation indicated that the previously translated sequence could be coded for as a protein with a theoretical size of 97 kDa, which added further urgency to investigation of the upstream sequencing as the protein initiation sequence remained unconfirmed.

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. Confirmation of the completed 3' end RACE PCR sequencing was performed when the sequence was first identified (Dr Amanda Miles, PhD Thesis 2004) and again in the current study. The presence of the unique stop codon was confirmed in a PCR experiment with forward primers designed within the unique region and reverse primers designed to include the unique stop sequence codon yielding a band at the expected 1508bp size and confirming the presence of the unique stop codon (Figure 3.7A).

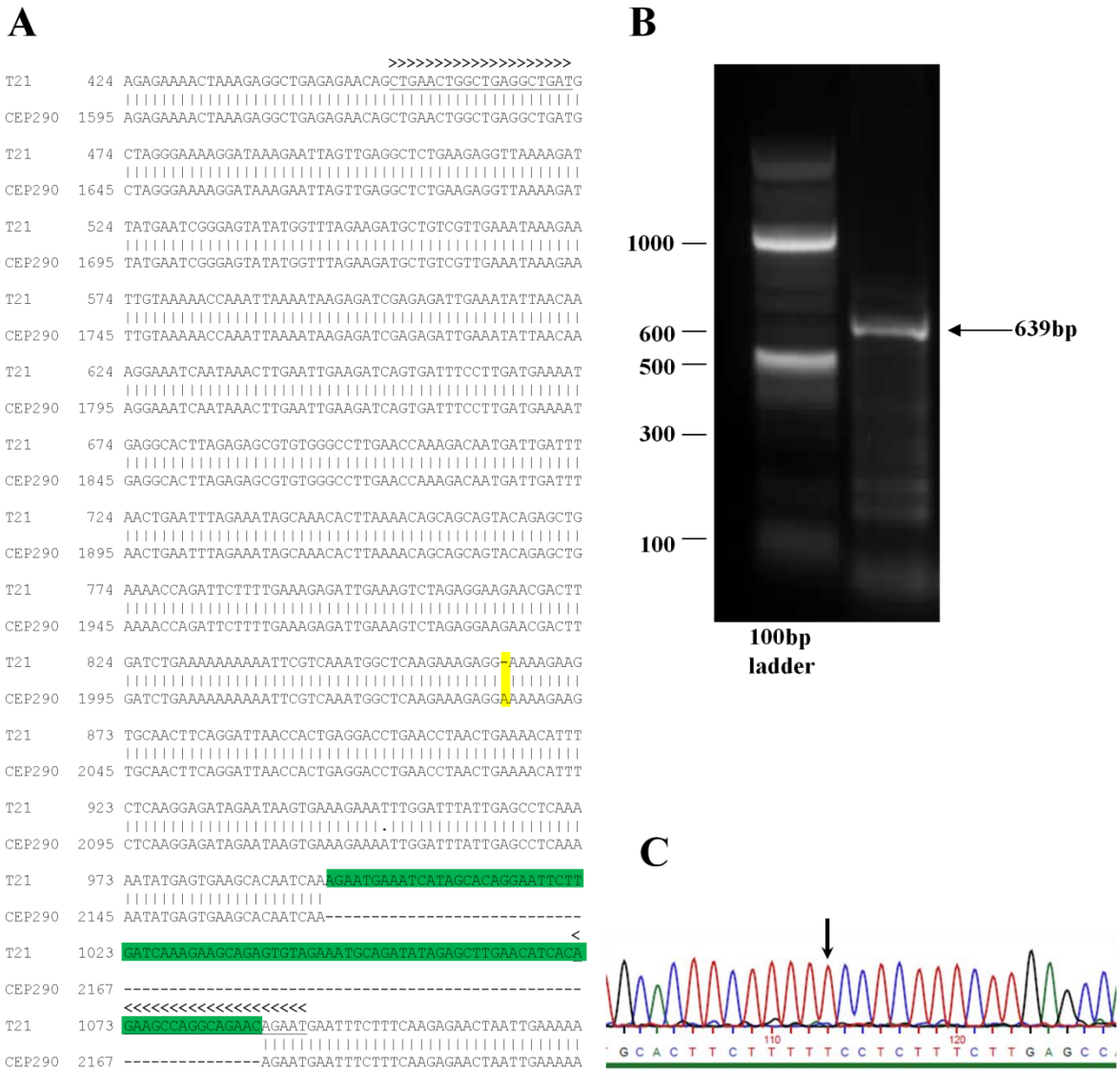


Figure 3.6 – PCR showing previously missing nucleotide present in T21. *Nucleotide sequence alignment of T21 and CEP290 illustrating the absence of a nucleotide in T21 which is present in CEP290 (yellow and the primers designed within the exon 14 of CEP290 to the unique region (green) of T21 (underlined) (**A**). Primers were used to perform qPCR product amplification (639bp) from cDNA derived from normal human prostate mRNA (**B**). Sequencing chromatography confirming the insertion of the T nucleotide not included in the first published sequence (**C**).*

With the sequence obtained from the previous 5' terminal RACE PCR and the knowledge that the sequence may now continue upstream of *T21* uninterrupted, forward primers were designed upstream of the known *T21* sequence located in exon 1 *CEP290* and reverse primers located in the unique region of *T21*. The resulting PCR generated a 1931bp product which indicated that *T21* continues further upstream suggesting that the *T21* transcript may share the same translation AUG codon to that of *CEP290* (Figure 3.7B). Sequencing of the product band also showed the same adenine discrepancy in the sequence data, however despite numerous attempts full length sequencing remained incomplete due to the large product size meaning that further attempts at sequencing would be required to answer this question in its entirety.

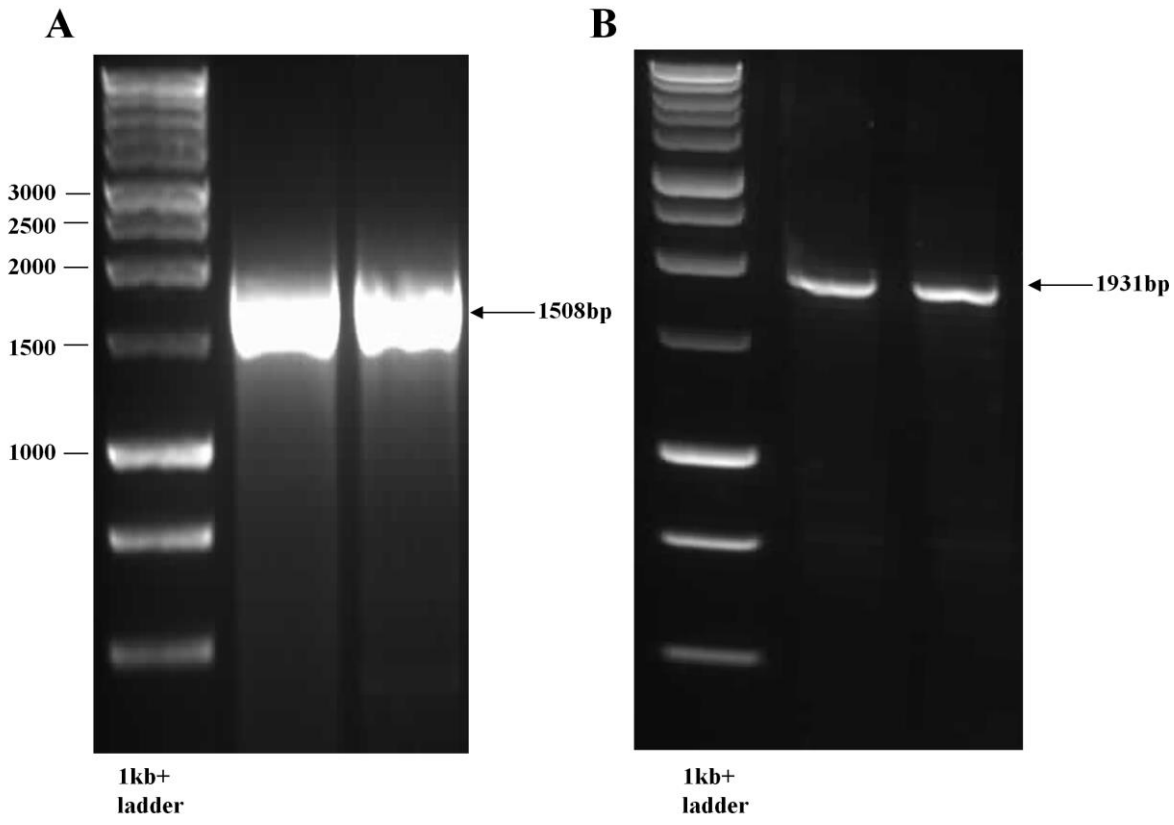


Figure 3.7 – PCR gel images showing amplification of T21 specific products using qPCR. *Gel image showing qPCR product amplification (1508bp) encompassing the T21 unique region and the unique stop codon from cDNA derived from normal human prostate mRNA (**A**). PCR gel image demonstrating amplification of a PCR product (1931bp) using primers designed within the first exon of CEP290 to the unique region of T21 (**B**).*

In order to perform a completed overview of the *T21* mRNA analysis, it was important to perform a PCR that would generate (if present) the predicted full length *T21* transcript extending from the new start region (in exon 1 of *CEP290*) through to the unique *T21* stop codon. By using the appropriate primers situated at the start of *CEP290* and the end of *T21*, a band corrosponding to the transcript would be apparent at 3406bp (Figure 3.8). In addition to the expected band, an additional band was observed at approximatly 1100bp therefore the gel bands were excised and purified and were used in a second round of amplification using primers that flanked the unique region of *T21*. No product bands were observe in the unexpected 1100bp product and was therfore considered to be non-specific amplicon. Interestingly, a band specific for *T21* and a band specific for *CEP290* were observed in the 3406bp sample suggesting there may be multiple amplicons of similar size present within the observed 3406bp band, hence explaining the difficulty in achieving full length sequencing experienced earlier. The opportunity to isolate one *T21* specific product was taken by using the 3406bp band and performing a second round of PCR amplification using primers designed from the start of *CEP290* and one within the *T21* unique sequence resulting in a 1931bp band. Despite multiple attempts and using various sequencing primers, full length sequencing of the band was not achieved.

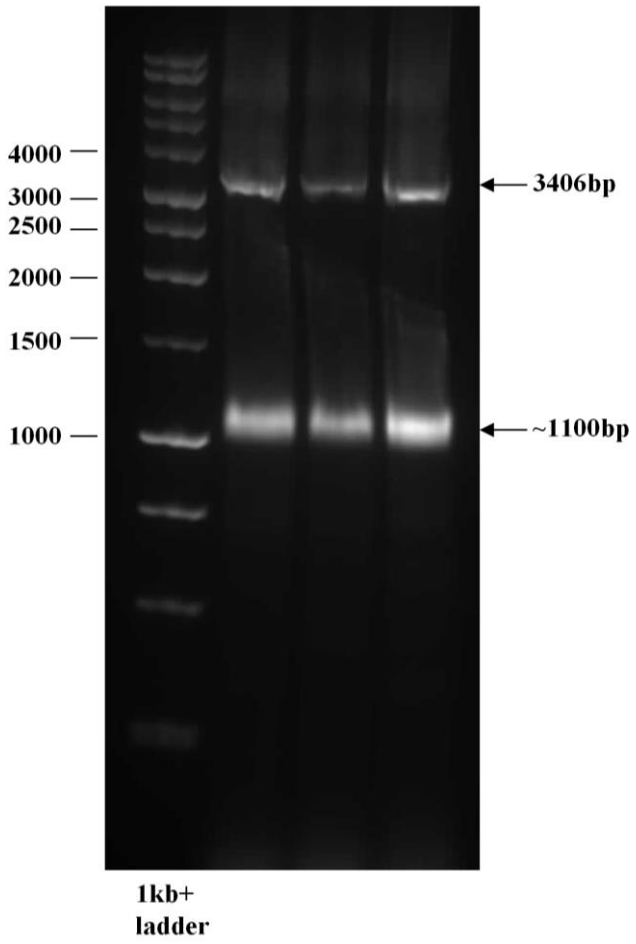


Figure 3.8 – PCR gel image showing amplification of extended T21 specific products. *Gel image showing qPCR product amplification (3406bp and approx. 1100bp) from the beginning of CEP290 to T21 unique stop codon performed using normal human prostate mRNA derived cDNA.*

There is now accumulating evidence at the mRNA level to suggest that the T21 protein sequence extends in the 5' terminus, aligning with the CEP290 start codon. It was therefore important to systematically examine mRNA transcript and protein variants of CEP290 using online databases which allowed for the alignment of known (experimentally verified) and predicted sequences of CEP290. It was then important to verify how the protein sequence of T21 also aligned against these reported variants. This analysis was performed for two reasons, firstly to identify any previously reported instances of the 93bp unique region in T21 and secondly to highlight any existing partial sequences that have homology to the remainder of the T21 sequence and looking into the evidence for them (Figure 3.9).

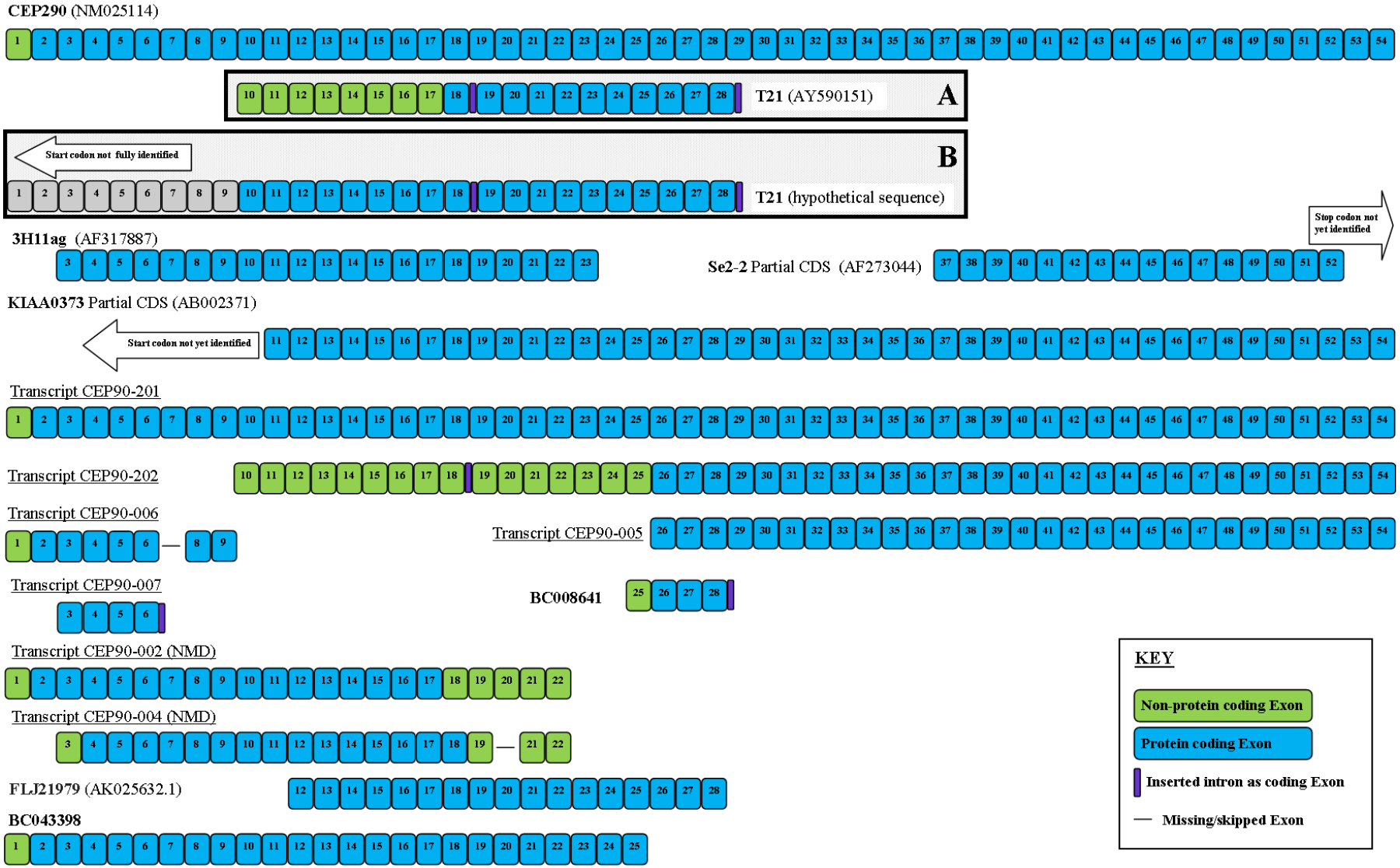


Figure 3.9 – A detailed overview of published CEP290 transcripts. *Online searches performed using NCBI and its affiliated sites, Ensembl and Uniprot were manually collated to illustrate alignment of mRNA transcripts to CEP290 detailing names and NCBI accession numbers. Additional protein encoding exons (blue) and non-coding exons (green) and sequence alignment to show inserts/missing exons are highlighted. In addition, the alignment of CEP290 and the published T21 sequence and the hypothetical alignment of T21 are illustrated in boxes (**A**) and (**B**) respectively.*

The alignments detailed in Figure 3.9 illustrated many alternative splicing events occurring within the *CEP290* gene which in turn give rise to many transcript variants, some of which have been identified to have associations to various cancers. More specifically, the previously mentioned *311HAg* sequence identified in 2001 shows an absence of the 93bp unique region present in *T21* and therefore can be considered as an additional independent transcript. Similarly, *Se2-2* can be considered a separate variant due to it sharing no sequence similarity to T21. These two examples are evidence that *CEP290* itself may indeed be a cancer associated protein leading to anti-CEP290 antibody production, which has so been identified in gastric (Chen & Shou, 2001), CTCL tumours and also leukaemia and melanoma cell lines (Eichmuller *et al.,* 2001).

Intriguingly the discovery of a transcript containing the exact unique region was identified (Transcript *CEP290-202*). This brought to light the possibility of detecting both transcripts during experiments designed to be *T21* mRNA or protein specific. The consequences of this transcript were reassessed when taking into consideration that at this time, transcript *CEP290-202* stands as a prediction only with no experimental evidence to suggest it exists. Secondly, the results observed in Figures 3.7B and 3.8 suggest that *T21* extends further upstream than that of *CEP290-202*. Furthermore, unlike *T21*, *CEP290-202* continues uninterrupted downstream to the final sequence termination signal in exon 54 of *CEP290* suggesting they are separate variants of the same gene. Further analysis suggests that transcripts *CEP290-202* and *-005* correspond to the second reported protein isoform of *CEP290* (UniProt Identifier O15078-2) of which functionally little is known as there is currently no experimental confirmation available. Importantly, the findings suggest that the unique region identified in *T21* exists in a non-protein encoding capacity in *CEP290-202* therefore targeting the polypeptide sequence of the unique region should theoretically be specific to the T21 protein sequence.

Not only was it necessary to consider the implications of these findings for future analysis of *T21* but was also imperative to assess the identification of *T21* retrospectively. A brief summary of the identification process of *T21* initially by RACE PCR performed in 2004 for the purpose of this investigation is stated below interjected with potential implications following the recent sequence elucidation.

First, 5' end RACE PCR was performed using normal testis cDNA using the SMART-RACE kit both purchased from Clontech, four bands were achieved which may have been due to the non-specific amplification of *CEP290* mRNA and possibly transcript *FLJ21979* (Figures 3.9 and 3.10). Nested PCR was performed using the initial RACE PCR product using nested primers from the commercial kit and a gene-specific reverse primer. However this primer was designed well inside the unique region giving rise to two product bands corresponding to two of the bands observed in the initial RACE. The subsequent sequencing of the largest band extended to the top of the known T21 sequence to the unique region and overlapped with the partial sequence obtained from the cDNA insert. Although seemingly complete, the initiation sequence to commence protein translation was not identified and relied on the basis of 3-frame nucleotide-to-protein translation, therefore can be considered as potentially unresolved.

Next, 3' RACE was performed in the same manner in which three bands were observed. This time gene-specific primers were positioned within the unique region and would in theory extend *T21* specifically. Following nested PCR using primers designed outside of the unique region, two bands were observed representing two bands observed on the initial RACE PCR (Figure 3.10). This sequence extended down from the unique region, however this did not include the unique stop codon and therefore translation into protein showed no termination sequence present. Further unsuccessful attempts in 3' RACE prompted an *in silico* investigation using expressed sequence tag (EST) mining which identified a sequence in a GenBank entry (*BE537521*) aligning with the end of 3' RACE sequence. Primers were designed within this GenBank entry sequence and using a 5' primer which overlapped the unique region by 4 base pairs. Forward sequencing data of this product showed the *T21* unique region amplification, however reverse sequencing proceeded from the 3'primer up through the sequence failed to show overlap with the forward sequence. This sequence did overlap with the sequencing data achieved from the initial 3' RACE and the cDNA insert sequence. Sequencing also revealed base discrepancies meaning that the additional bases in *T21* resulted in a non-coding sequence, and was therefore considered to be an incomplete sequence. Re-mining EST databases with the now extended sequence resulted in the identification of GenBank entry *BC008641* (a clone of *CEP290* which contains a unique stop). PCR using the *BE537521* specific forward and a *BC008641* specific reverse primer which included 12 bases specific for the unique stop codon was performed and produced a weak band. On this occasion, sequencing demonstrated forward

and reverse sequencing overlap to include the unique region and confirming the stop sequence achieving full length protein coding of *T21* (Figure 3.9). Incidentally, *BC008641* derived from human placental choriocarcinoma tissue mRNA forms a truncated portion of *CEP290* and was noted as having a bona fide poly A tail.

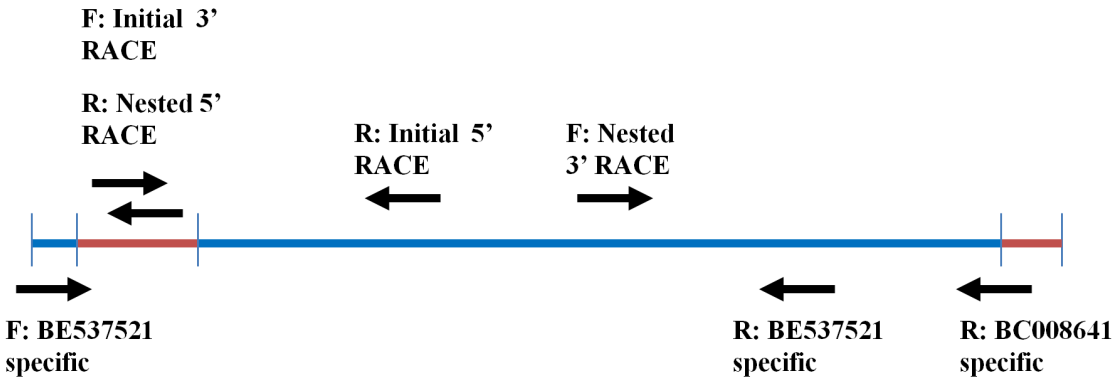


Figure 3.10 - Schematic showing previous RACE primer design. *T21 coding region (blue) containing within, T21 93bp unique region (red, left) and the alternatively spliced exon containing the unique TGA codon (red, right). Arrows representative of primer locations for initial and nested RACE PCR's performed and primers to verify sequence similarity to identified EST's.*

Retrospectively, a number of conclusions could now be made from the previous study. Firstly, in both 5' and 3' end experiments, primers designed, were (by chance) within the unique region in either initial RACE or in nested PCR's, establishing that the sequence amplified was *T21* (or possibly *CEP290-202*) specific and not *CEP290*; moreover, the product sequencing also consistently showed the inclusion of the unique region. However, 5' terminal extension can be considered as incomplete due to the new knowledge of the sequencing discrepancy and therefore possible incorrect assignment of the initial AUG codon in the *T21* mRNA. Finally, the *T21* 3' end sequencing could be considered complete due to EST data mining and sequence alignment verified with experimental sequencing evidence and thus excluding *CEP290-202* as a candidate. However, a question remains as to whether there are multiple gene transcripts within the *T21* unique region which could terminate prior to the identified unique stop codon. An amplification of both 5' and 3' ends could *i)* reveal the genuine AUG codon upstream of the current transcript, *ii)* verify the known stop codon and *iii)* uncover multiple mRNA sequences that contain the unique region.

Preparation of RNA was performed using GeneRacer kit (Invitrogen) which is based on RNA ligase-mediated and oligo-capping RACE methods resulting in selective ligation of *T21* unique region specific primers to the 5' ends of decapped mRNA using T4 RNA ligase. The kit specifically amplifies mRNA that has a poly A tail and is reverse transcribed, therefore by eliminating truncated messages and ensuring that only full-length transcripts are amplified as detailed in section 2.2.3. The RACE PCR experiments were performed using normal prostate cDNA purchased from Clontech. Following many attempts, the resulting PCRs consistently produced similar sized bands to that of the RACE experiments performed in 2004 (Amanda Miles, PhD thesis, 2004), with both 3' and 5' end terminals failing to extend any further than the previously acquired sequences (Figure 3.11).

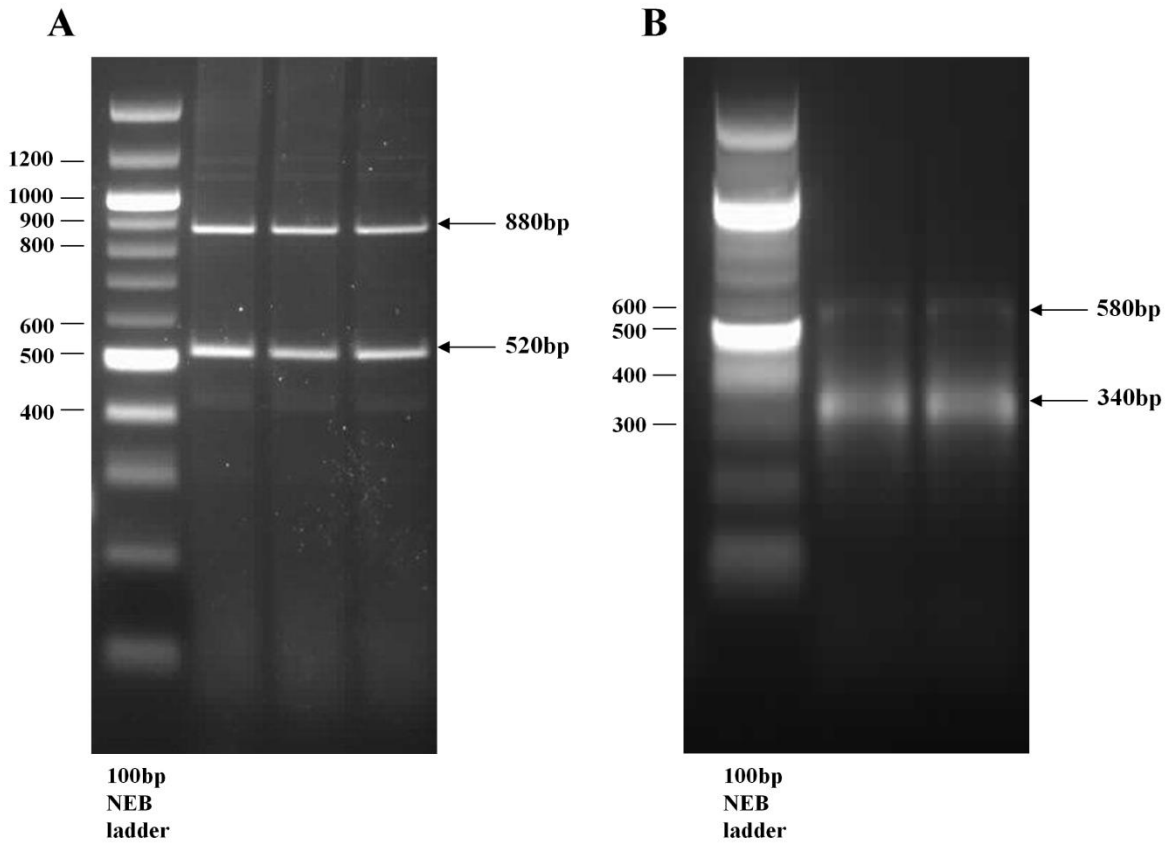


Figure 3.11 – 5' and 3' RACE PCR of T21. *Figure 3.10(**A**) illustrates the products achieved following 5'RACE PCR using T21 unique region specific reverse primer. Lanes 1-3 represent equal loadings of the 5'RACE PCR product onto a 1% agarose gel. Two intense bands were obtained at approximately 880bp and 520bp (representative of three independent experiments). Figure 3.10(**B**) illustrates the products achieved following 3'RACE PCR using T21 unique region specific forward primer. Lanes 1 and 2 represent equal loadings of 3'RACE PCR product onto a 1% agarose gel. Two bands were obtained at approximately 580bp and 340bp.*

Following inconclusive RACE PCR experiments, northern blotting was considered to be an appropriate approach to verify the existence of a *T21* mRNA transcript. In addition, with the possibility of an additional transcript containing the unique region it was important to confirm exactly how many potential variants containing the unique sequence were present. Northern blotting was performed using short custom made anti-sense oligonucleotide RNA probes (Sigma, UK) labelled using single biotinylated cytidine (bis)phosphate nucleotide to the 3' terminus using T4 RNA ligase (Thermo Scientific, UK). Next, labelled RNA probes were hybridised to the target total mRNA and loaded onto polyacrylamide gels for electrophoresis and the separated RNA blotted using semi-dry transfer at 200 mA for 30 minutes onto positively charged nylon membranes. Next fixation of RNA to the membrane was performed by baking at 80°C for 15 minutes followed by detection using the Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific, UK).

Before commencing, optimisation experiments were performed to confirm ideal component concentrations, running conditions and to refine technical procedure. Following running of mRNA, gels were incubated with GelRed and UV light to visualise the quality and quantity mRNA determined by separated ribosomal RNA subunits observed as prominent bands representing ribosomal subunits 28S (5kb) and 18S (2kb).

The experimental blots were performed using 2 µg of total normal prostate mRNA (Figure 3.12). A single band specific to *T21* was detected using a probe designed to the 93bp unique region (probe 1). Importantly probe 4, designed at the 3' end of the *T21* mRNA transcript to include the unique stop sequence also detected a band of the same size as observed in probe 1 indicating the existence of *T21* as an actual transcript. Interesting, another band detected by probe 4 indicated a smaller transcript that may form part of another truncated *CEP290* product such as *BC008641*. A probe designed at the 5' end of the known *T21* transcript was also designed with the intention of observing multiple separating bands allowing size confirmation of *T21* and estimation of the number of variants *CEP290* may have. However, adequate band resolution was not achieved. Additionally, a *CEP290* specific probe designed downstream of *T21* and away from many other mRNA variants of *CEP290* (probe 2) demonstrated a single band, unexpectedly only slightly larger in size that that of *T21* perhaps due to the variants present encoding for the truncated isoform of *CEP290* (Figure 3.12).

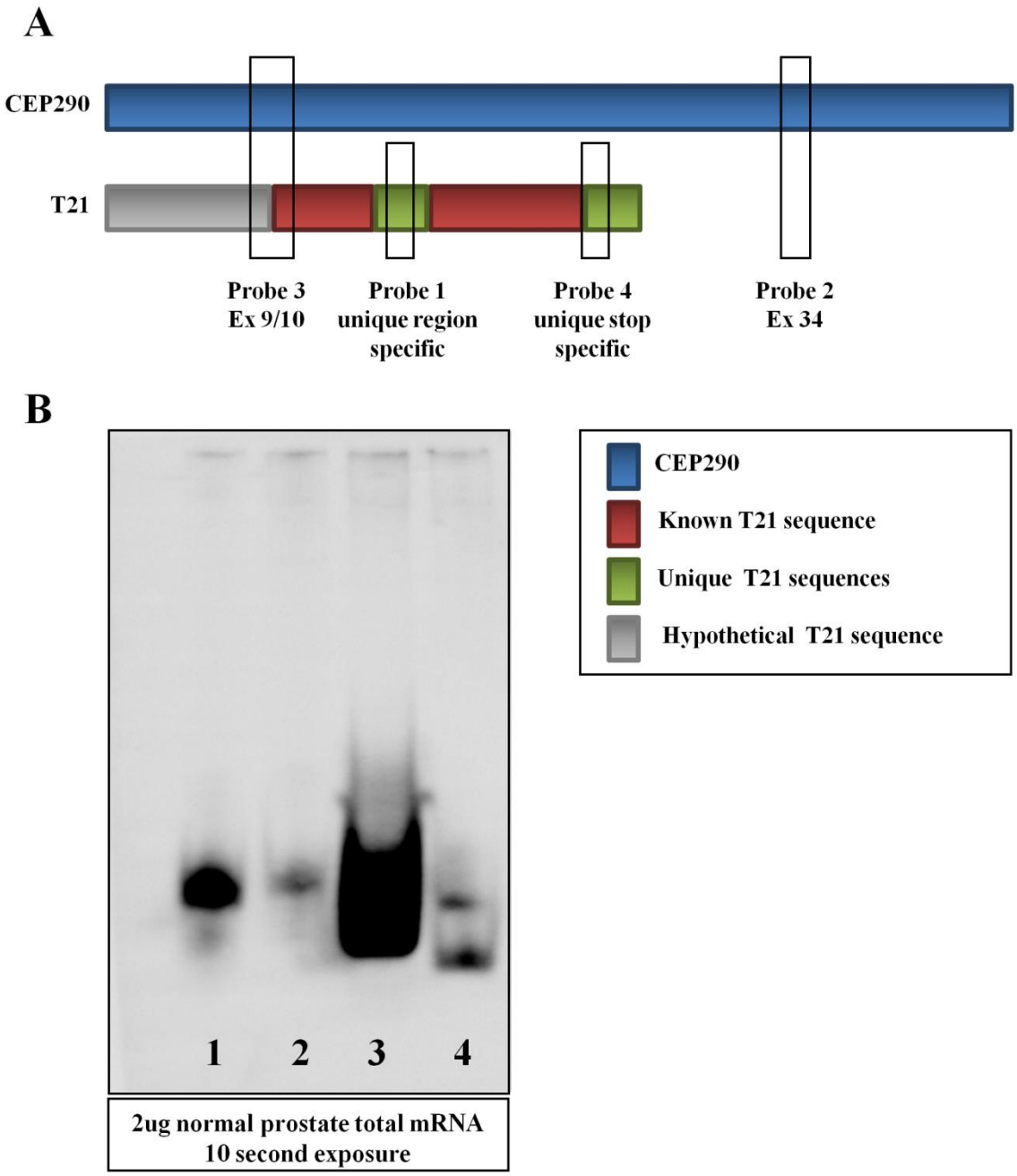


Figure 3.12 – Detection of CEP290 and T21mRNA by northern blot analysis. *Four RNA molecular probes designed along CEP290 and T21mRNA sequences (exon annotation referring to CEP290) (**A**). Northern blot performed using 2 µg of prostate total mRNA pre-hybridised with probes on 12% PAG (**B**), lanes 1 - 4 represent probes 1, 2, 3, and 4. (n=3).*

3.2.3 Protein investigation to establish T21 translation

Previous protein based investigations on T21 had been performed using a custom-made polyclonal mono-specific rabbit antibody (Pacific Immunology, USA). The epitope selection was based on several predicted characteristics of T21 including immunogenicity, hydrophobicity/hydrophilicity and protein folding. It is now known that this antibody was raised against a 18mer peptide sequence sharing 100% homology to CEP290 (underlined in Figure 3.3) and other CEP290 protein variants, therefore the antibody binding would not be unique to T21 (termed throughout this study as anti-T21/CEP290 antibody). In order to further investigate T21 at the protein level, independently from CEP290, a new polyclonal anti-T21 antibody (anti-T21u antibody) was produced (Pacific Immunology, USA). On this occasion, the mono-specific anti-T21u antibody was raised against a pre-selected 14mer peptide sequence within the newly identified unique region of T21 (ADIELEHHRSQAEQ), which unavoidably meant bypassing antigen prediction algorithms in favour of sequence specificity to T21. The procedure detailed by the company entailed the following; rabbits were immunised with a synthetic peptide sequence with 3 further boosts, and subsequently bled and the sera recovered from which antibodies were isolated using the same immunisation peptide sequence covalently coupled to affinity columns allowing for purification of mono-specific anti-T21 antibodies. Final elutions were assessed using peptide ELISA assays ensuring antigen binding specificity and quantify purified antibody. Prior to antibody production, protein BLAST analysis revealed, as expected, 100% sequence homology to T21 only, however other proteins matching between 5-8 consecutive amino acid residues could unavoidably form potential antibody targets. With this consideration, the generation of a mono-specific polyclonal antibody in this way increases the probability of T21 specific binding occurring due to the presence of multiple T21-specific peptide binding regions.

Preliminary validation assessment of the anti-T21u antibody to determine the specificity and reproducibility of the antibody was performed systematically using multiple applications. These included blocking experiments using the antibody specific peptide to confirm specificity of the antibody (ELISA, WB and IF), comparison of anti-T21u antibody to isotype control to determine levels of background IgG antibody binding (WB and IF) assessment of a large panel of cancer cell lines and tissues (ELISA, WB, IF and IHC) to optimise antibody conditions specifically for each application (detailed in section

2.1.5). In addition, as a positive confirmation, immunoprobing was performed using a previously purchased custom recombinant T21 protein (GTP Technology) purified from *E.coli* with an N-terminal His tag *(*sequence shown in Figure 3.3). Band separation and probing of the recombinant protein with the anti-T21u antibody detected a band at 68.58 kDa (Figure 3.13A), this finding concurred with the protein band detected by the company following anti-His western blot analysis (see appendix Figure II).

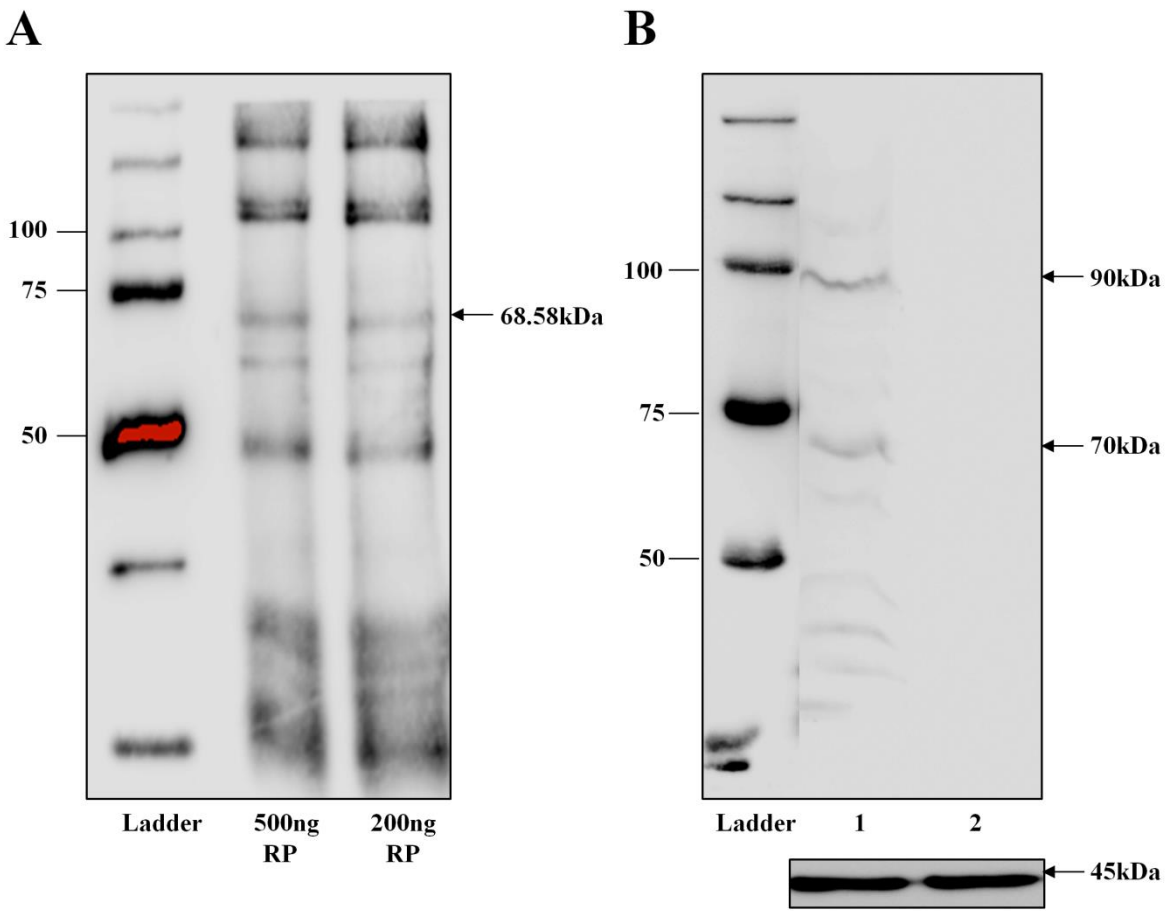


Figure 3.13 –T21 protein detection using T21u antibody. *Western blot analysis performed using recombinant T21 protein (RP) with anti-T21u antibody detected a discreet band at 68.58 kDa concurring with anti-His western blot analysis supplied with the RP from GTP technology (**A**).*
*Western blotting performed using 30 µg of total protein lystate derived from PC3 cells and probed using the anti-T21u antibody detected two prominent bands at 90 kDa and 70 kDa as shown in lane 1 (**B**). Lane 2 represents peptide blocking of antibody prior to probing PC3 cell lystate. An anti-β-actin antibody was used as a protein loading control observed at 45 kDa.*

The probing of a recombinant protein confirmed that the new T21 specific anti-T21u antibody was able to identify the T21 protein sequence, subsequently evidence that T21 was indeed endogenously expressed in cells was sought. Western blot analysis was performed using the prostate cancer cell PC3, lead to the observation of two discreet bands appearing at approximately 70 kDa and more distinctly at 90 kDa (Figure 3.13B). The molecular weight observed did not correspond to the predicted sequence size (+100 kDa) and reasons for the observation of two bands instead of the anticipated one specific band must be considered. For example, many proteins undergo extensive modifications during protein synthesis resulting in mass changes in proteins when compared to their actual sizes. The migration of proteins may be affected by post-translational modification, post-translational cleavage, splice variants and isoforms and the composition of amino acids altering the relative charge. As mentioned earlier antibodies may also bind to other linear epitopes present in the denatured protein lysate.

These findings led to the opportunity to further investigate T21 protein in cell lines to both address the currently hypothetical status of this protein and thereafter investigate its expression in tissues and elucidate its potential function. With this said, cell lysates obtained from cell lines identified by immunoblotting and ELISA to express T21 protein using T21u antibody were chosen in order to isolate the protein by native immunoprecipitation (IP). Here, a Dynabead magnetic separation method was selected over agarose/sepharose slurry due to higher quality and specificity of recovery when performing small scale isolation. Additionally sepharose beads are considered unsuitable for downstream mass-spectrometry applications (personal communication Dr David Boocock). The resulting lysate elutates were subjected to western blot analysis which demonstrated the presence of one discreet band at approximately 90 kDa. The T21 specificity of this band was confirmed by its absence in an IgG control probed with anti-T21u antibody (Figure 3.14). Interestingly the previously identified band (at approximately 70 kDa) was not observed in the immunoprecipitated eluate suggesting that the presence of this band maybe due to an exposed epitope(s) occurring during protein denaturation which results in the appearance of a band.

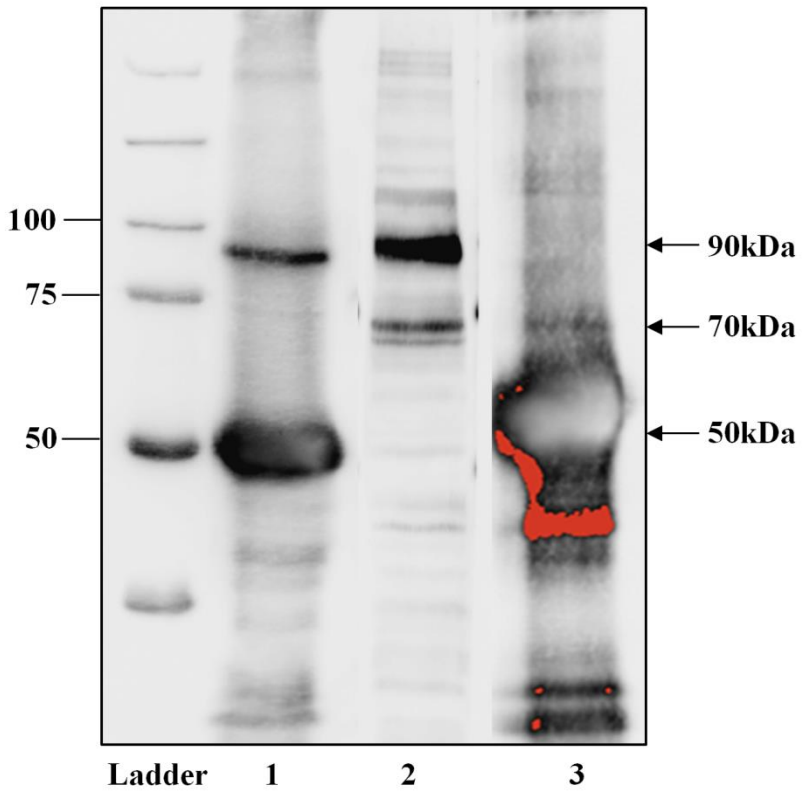


Figure 3.14 – Western blot of T21 protein expression following immunoprecipitation. *Western blot analysis performed using breast cancer derived cell line lysate (T47D) following immunoprecipitation (IP) using anti-T21u antibody. IP shows a specific band observed at 90 kDa which was not observed using an anti-rabbit IgG antibody IP which was probed with anti-T21 antibody during immunoblotting. Bands observed at 50 kDa represent heavy chain of antibody. Lane 1 = T21-IP, lane 2 = input cell lystate and lane 3 = IgG control-IP (n=3).*

In an attempt to confirm the presence of T21, proteins present in the IP elution underwent a ZipTip clean up procedure before being tryptically digested and subject to MS/MS analysis using an Ultraflextreme MALDI-TOFTOF (Bruker Daltonics). This procedure was performed using samples derived by direct elution and also by excision of the protein band from polyacrylamide gel following electrophoresis and coomassie brilliant blue staining. To determine the feasibility of this approach in respect to identifying T21, MS/MS analysis using mascot software 2.2 search engine (http://www.matrixscience.com/) using the previously mentioned recombinant T21 protein (customised by GTP Technology) lead to the identification of CEP290 sequences. A specifically created "in-house database" consisting of only the hypothetical T21 sequence identified the T21 unique region sequence (performed by Miss A. Yvonne Dede). This second "in-house" approach was used due the difficulty in identifying such a short 31 amino acid residue sequence unique

to T21 limiting tryptic digestion sites causing limited specific peptide sequence identification and in turn, reducing the overall significance of these hits. However, despite using the same MS/MS approach as used to identify tryptic peaks specific to the T21 recombinant protein, T21 sequence identification using immunoprecipitated elution's remained elusive with no significant identities against T21 or the homologous CEP290 sequence being observed in the excised gel samples. Furthermore there were no identifiable ion peaks in the direct elution sample (data not shown).

In an alternative approach also using IP, western blot analysis were performed on anti-T21u antibody directed elutions which were then probed using either anti-CEP290 or anti-CEP290/T21 antibodies as both of these antibodies would theoretically be directed in-frame upstream (CEP290 antibody) or downstream (T21/CEP290 antibody) of the unique sequence in the T21 protein sequence (Figure 3.15). On this occasion, cross-probing in this manner failed to produce a T21 specific band (using anti-CEP290 antibody) and what remains inconclusive evidence of single 90 kDa band in the case of anti-T21/CEP290 antibody. In addition, IP's performed using anti-CEP290 and anti-T21/CEP290 antibodies which were subsequently probed using anti-T21u antibody proved equally unsuccessful (data not shown).

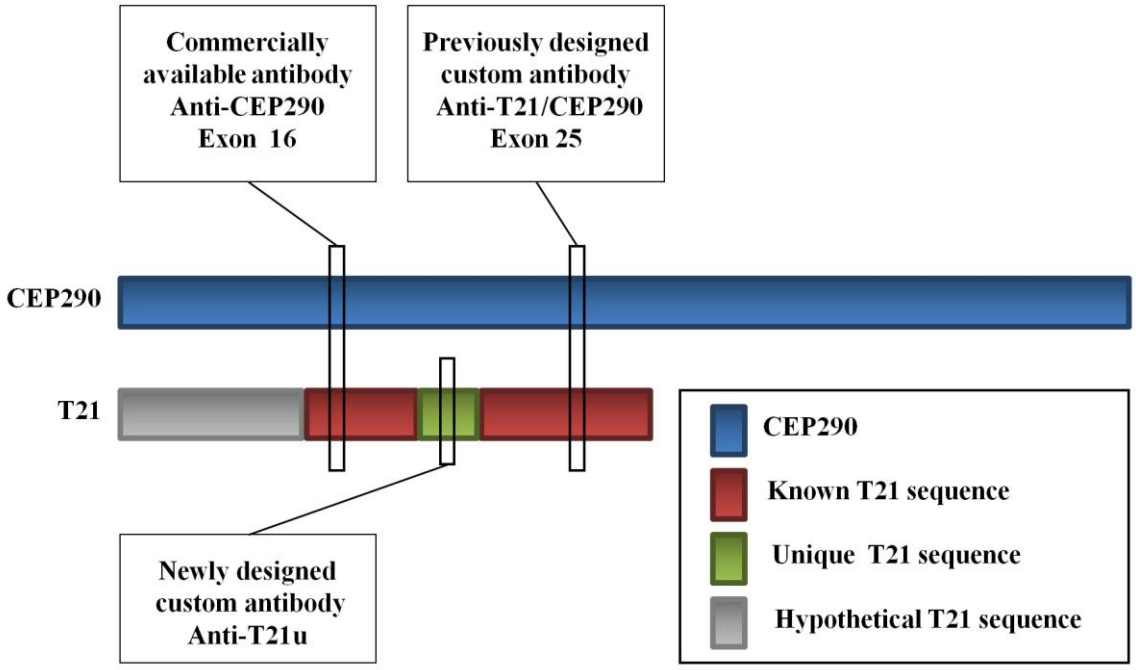


Figure 3.15 – Schematic of T21 and CEP290 antibodies used for this study. *Three antibodies specific to CEP290 and T21 proteins were used during this study (exon annotation referring to CEP290).*

3.3 Discussion

The identification of novel TAAs present opportunities to specifically trigger tumour-killing mediated by TAA primed T cells, which can be incorporated into a whole host of immunotherapeutic treatment approaches. Many researchers have adopted the SEREX approach in order to identify cancer restricted, immunorelevant tumour antigens, with one promising sequence identified by this laboratory being T21 (Miles *et al.*, 2007). Upon the discovery that this sequence may indeed have an association by virtue of significant sequence homology to another protein named CEP290, genomic, transcriptional and protein studies were undertaken in order to *i)* determine the genomic and transcriptional relationship between *CEP290* and *T21*, *ii)* perform retrospective analysis to the identification of the *T21* sequence and subsequently determine the full length transcriptional sequence of *T21* and finally *iii)* to address the currently hypothetical status of the T21 protein sequence and establish whether it is an independent transcript to CEP290 and its known variants.

Gene alignment to the published *T21* transcript confirmed a 31% alignment coverage similarity between *T21*with *CEP290* of which 96.5% similarity between *T21* and *CEP290* mRNA was observed. Furthermore, no significant homology of the *T21* unique region to any other organism in the Ensembl and NCBI databases was observed. It can therefore be considered that *T21* is a transcript of the *CEP290* gene found on chromosome 12q21.32. Moreover, it was concluded that a 27% alignment coverage similarity is shared between T21 with CEP290 of which there is 96.7% similarity between T21 and CEP290 protein sequences. This demonstrates that *T21* shares in-frame nucleotide-to-protein translation with *CEP290* suggesting that the antibody used in previous investigations of T21 would recognise peptide binding sites of both CEP290 and T21 (referred to throughout this investigation as CEP290/T21 antibody).

In addition to *in silico* gene analysis, approaches were taken to investigate the transcriptional output of *T21* centred on the primary identification, results and the sequence submitted to GenBank in May 2004. As a result of this study, the *T21* sequence was found to contain a 93bp insertion proceeding exon 18 of *CEP290* from *CEP290* intron 18/19. This subsequently encodes for the incorporation of a 31 amino acid sequence which is

specific to T21 and not to CEP290. *In silico* alignment of the known transcripts of *CEP290* indicated that a transcript called *CEP290-202* may indeed encode for this region, however it is located upstream of the protein encoding sequence. This 93bp insertion was thereafter referred to as "the unique region" due to its exclusive protein encoding in T21.

Importantly, this was not the only difference of note, as the T21 protein prematurely terminates after exon 28 of *CEP290*. Further alignments confirmed that skipping of a splicing event occurs after exon 28 of *CEP290* resulting in the continued transcription into the intronic sequence and as a consequence an immediate sequence termination signal is encoded for, giving rise to a truncated T21 protein sequence. These findings were also confirmed experimentally, giving evidence of a novel, previously unreported, CEP290 protein variant containing an alternatively spliced exon. Interestingly, the *T21* and *CEP290* mRNA were shown to be co-expressed in tissue, suggesting that T21 does not have a substitution affect on CEP290. Like proteins that encode point mutations, differentially expressed proteins and fusion proteins; alternative splicing events and intron retentions are a unique source of potential antigens due to their association to tumours and the sometimes contributing affect in malignant transformation. These factors make these proteins a potentially important, but often overlooked, source for immunotherapeutic targets. Early examples include a study which identified an intronic sequence which forms part of an antigenic peptide sequence for melanoma ubiquitous mutated-1 (Coulie *et al.,* 1995); others that have shown intron encoded peptides include GnT-V (Guilloux *et al.,* 1996), TRP-2-INT2 (Lupetti *et al.,* 1998) and gp100-in4 (Robbins *et al.,* 1997) capable of HLA-restricted recognition by $CD8^+$ T cells resulting in killing of melanoma tumour cells.

Previously reported investigations of disease-related *CEP290* mutations deal primarily with cilliopathies with little understanding as to how *CEP290* mutations generate multiple and varying disorders and phenotypes commonly affecting brain and retina development, spinal cord (CNS) and kidney function (Coppieters *et al.,* 2010). *CEP290* mutations are frequent causes of Leber congenital amaurosis (LCA) as den Hollander and colleagues identified key mutations by sequencing all 53 coding exons from LCA-affected patients by way of RT-PCR using overlapping primer sets (den Hollander *et al.,* 2006). One particular genetic defect is responsible for approximately 20% of all cases of the disease, the defect is caused by an intronic mutation (*c.2991+1655A>G*) which is responsible for creating a strong splice donor site and the insertion of a 128bp cryptic exon between exons 26 and 27,

leading to a premature stop downstream of exon 26 (p.Cys5998X). This results in the production of an abnormal truncated protein and consequently a complete or partial loss of function. Interestingly the *T21* unique splicing event itself has not been identified as a transcriptional variant in any published allelic variants of *CEP290* which have been associated with disease states. Other investigations demonstrated that CEP290 has been previously been identified as a SEREX-defined TAA (Eichmuller *et al.,* 2001) and showed increased expression in several cancers (Chen & Shou, 2001), however both instances were shown to be independent of T21, either due to the location of the identified antigen (Se2-2) or the exclusion of the unique 93bp sequence indicating an alternative variant being identified and assessed (3H11Ag).

The unexpected discovery of the unique region and a novel stop signal in T21 have proven invaluable in comparing *T21* and *CEP290* transcript expression as these differences were exploited to extend the 5' end of the mRNA using RACE PCR and also used for northern blot analysis, both useful tools often applied to determine the presence of transcriptional variants and particularly in determining size and differential expression of several unknown or novel SEREX identified antigens (Jäger *et al.,* 2002; Rogers *et al.,* 2002; Krackhardt *et al.,* 2002). In the case of the latter, RNA probes designed specifically at the unique region and at the unique stop meant *T21* could be verified as an independent transcript confirming the presence of an alternatively encoded exon and unique stop sequence confirmed using both normal tissue and cancer cell line mRNA. Initially, northern blotting was performed using DNA templates generated using *T21* or *CEP290* specific primers designed to amplify between products (sized between 200 – 800bp) from normal prostate total mRNA. Next, the DIG Northern Starter Kit (Roche Applied Science, UK) was used for *in vitro* transcription of the template DNA in the presence of digoxigenin-11-UTP (DIG), a non-radioactive label which incorporates uridine nucleotides, using T7 RNA polymerase to generate DIG labelled single stranded RNA probes. Following this, a formaldehyde agarose gel containing GelRed solution loaded with 2 µg of RNA was run at 3 V/cm for 2 hours and the gel briefly visualised for the two ribosomal bands. The separated RNA was blotted onto a positively charged nylon membrane by capillary transfer overnight and the RNA fixed to the membrane by UV-crosslinking method. Membranes were then subject to overnight hybridisation of the labelled probe followed by a series of stringency washes followed by development using anti-DIG-AP antibody and CDP-star solution for signal detection using

chemiluminescence. This method was chosen due to safety issues, costing and superior sensitivity over radioactivity; however despite many attempts and altering various parameters suggested by the kit manufacturer, probing failed due mainly to procedural difficulties in either the probe construction or the detection stages (data not shown). A major disadvantage of separating mRNA using polyacrylamide is the limited band resolution achieved compared to using formaldehyde/agarose gels, which is why the approach described above was attempted in the first instance. The secondary objective of determining the transcript size of *T21* remained unanswered, however on the findings presented here, there is evidence that the *T21* transcript exists.

Further study to conclude the regulatory mechanisms for this splicing event occurring could be of interest and allow for a more complete molecular characterisation of *T21*. Previous experiments revealed incomplete 5' terminal end extension of the mRNA; further to this, PCR amplification of *T21* from the unique region upstream to exon 1 of *CEP290* demonstrated that *T21* may extend to the start of *CEP290* and additional sequencing analysis of this product revealed an additional adenine residue present in *CEP290* which was previously omitted from the published *T21* sequence. The consequence of this nucleotide addition draws questions as to the initiation signal of T21 protein; however this study failed to fully confirm the extended upstream sequence from the *CEP290* ATG to the unique region and therefore unable to determine protein start ATG in order to identify the transcriptional start.

Cancer progression has been long associated with genetic alterations which consequently result in aberrantly expressed proteins (detailed in chapter 1). These cancer-associated alterations can occur at various stages of transcription as a consequence of altered pre-mRNAs and the use of alternative promoters and splicing events which alter coding regions ultimately impacting on protein functionality. For this reason, further insight into the regulatory mechanisms of *T21* mRNA production is essential to understanding its biological significance in cells. Despite *CEP290* being a well characterised gene, the promotor region remains unknown. Further experimental investigation involving specific promotor silencing could determine whether *T21* and *CEP290* share the same promotor or are indeed independently regulated. With this considered, RACE PCR using *T21*-specific forward and reverse primers were employed in an attempt to translate upstream of *T21* to obtain full length cDNA but despite several attempts, 5' terminal translation could not be

achieved. This is not surprising as RACE PCR of the 5' end can be technically problematic due to the presence of fragment RNA, premature termination of cDNA due to formation of secondary structures and amplification bias favouring both highly abundant and short amplicons during PCR.

As previously mentioned CEP290 is so named because of its role in centrosome and cilia development. The centrosome is the primary microtubule-organising centre (MTOC) found in cells, consisting of a pair of centrioles surrounded by an amorphous protein lattice collectively termed the pericentriolar material (PCM) component, which is responsible for nucleation and anchoring microtubules forming mitotic spindles during cell division (Bettencourt-Dias & Glover, 2007). Ontological evaluation of the *CEP290* gene reveals protein and microtubule binding properties that not only localise to the centrosomes but also extend to the formation of microtubule basal bodies and connecting cilia of photoreceptor, hence the association of *CEP290* deregulation seen in many cilia-related diseases. Moreover, CEP290 was found to be associated with G_2/M transition of the mitotic cycle through co-operation with ciliary GTPase Rab8A, a member of the *RAS* oncogene family (Tsang *et al.,* 2008). The protein alignment from the cDNA sequence suggests that *T21* would theoretically retain conserved regions of activity from CEP290 protein, but contextually, the whole of *T21* transcriptional sequence remains unknown therefore it would be counter-intuitive to begin bioinformatics-based prediction analysis of its potential function. However, focus on *CEP290* and the unique region revealed sustained evolutionary conservation of the *CEP290* gene, as demonstrated in higher mammals and (relevant for the latter phase of this study) revealed 87% similarity between murine CEP290 and human CEP290 proteins with no sequence similarity to the T21 unique region. Conservation of the *T21* unique region intron sequence was evident in primate species such as chimpanzee, gorilla and monkey, however no inclusion into putative proteins were observed. Due to there being no significant protein encoding sequence similarity of the T21 unique region to any other organism in the Ensembl and NCBI databases, from this it could be concluded that this variation is unique to humans.

Previous protein investigations were performed using a polyclonal anti-CEP290/T21 antibody. Once again the unique region offered the perfect opportunity to target T21 specifically for transcript-to-protein verification. Following the production of a mono-specific polyclonal anti-T21 antibody recognising epitopes within the unique region (anti-

T21u antibody), western blot analysis performed on various cancer and normal cell line lysates identified two distinct bands at approximately 90 kDa and 70 kDa, both of which were specific to the peptide used to raise the antibody. Critically, immunoprecipitation followed by western blotting using anti-T21 antibody revealed a single 90 kDa band from which it can be inferred that only this protein holds the correct conformational structure to bind the antibody of interest. Future usage of this antibody demonstrated its suitability for both linear and native protein analysis applications. However, this study fell short of positively identifying T21 using 1D SDS PAGE peptide profiling coupled with mass spectrometry and by cross immunoprecipitation with anti-T21 antibody.

As a consequence of these findings, previous investigation into the validity of T21 as a potential prostate cancer biomarker and/or an immunotherapeutic target now remain unresolved. A new study to investigate the expression of T21 in normal and cancer tissue would therefore demonstrate whether T21 holds potential for clinical applications. Additionally, investigations into T21 as a biomarker of disease 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.

CHAPTER 4

EXPRESSION OF T21 IN TISSUES AND ITS ROLE IN TUMOUR PROGRESSION

4.1 Introduction

The molecular composition of *T21* and its origins as a variant of *CEP290* have been discussed in the previous chapter. To date little is known in regards to the function of T21 although previous investigations have hypothesised that due to the shared protein similarity to CEP290, T21 may play a role in centrosomal function and cilia development. The deregulation of the cells centrosomes has long been proposed as a contributing factor towards genomic instability and tumourogenesis (Boveri, 1914 (translated by Harris, 2008)) and so the function and interactions of CEP290 should be further examined.

The centrosomal localisation of CEP290 has been found to be dynamically expressed throughout the cell cycle (Sayer *et al.,* 2006) with redistribution to the cytoplasm prior to prometaphase. During the interphase, CEP290 localises in two or four centrosomal bodies observed in the G_1 and G_2 phases of the cell cycle. In the G_0 phase, CEP290 is present at the mother and daughter centrioles sites, the former acting as the basal body from which primary cilia is assembled (Tsang *et al.,* 2008). Using various co-immunoprecipitation techniques, a number of groups have identified several centrosomal and ciliary-related

proteins which interact with CEP290. In 2006, Sayer and colleagues identified an interaction between CEP290 (exons 2-21) and activation transcription factor 4 protein (ATF4), which regulates the expression of genes involved in oxidative stress, cell differentiation, metastasis angiogenesis and drug resistance. It is induced by tumour microenvironmental factors in responses to cell stress signals in processes related to tumourogenesis (Ameri & Harris, 2008; Horiguchi *et al.,* 2012). Other co-immunoprecipitation studies revealed associations with several microtubule-based transport proteins including centrin, dynactin subunits p150, γ-tubulin, kinase family member 3A (KIF3A), kinesin-associated protein 3 (KAP3), ninenin, pericentriolar material 1 (PCM1), centrosomal protein 110 (CP110), pericentrin, RPGR and RPGR-interacting protein 1 (RPGRIP1) and Rab8 (Chang *et al.,* 2006; McEwen *et al.,* 2007; Kim *et al.,* 2008). Furthermore, *CEP290* gene silencing studies in human retinal pigment epithelial cells resulted in the disorganisation of the cytoplasmic microtubule network and alterations in the formation of primary cilia through Rab8a, a small GTPase required for ciliary membrane elongation at the centrosomes. Knockdown of *CEP290* also resulted in the disruption in the migration of mother centrioles to the cell cortex. However both studies noted that ablation of *CEP290* did not impede centrosomal formation or cell cycle progression (Kim *et al.,* 2008; Tsang *et al.,* 2008).

Tsang and colleagues also identified regions of CEP290 that are necessary for sufficient binding to its interacting proteins; for CP110: amino acids 1-336, 221-366 and 362-822. For Rab8a: amino acids 1208-1695, (Tsang *et al.,* 2008) which are beyond the C terminal of T21 (at amino acid position 602-1103 of CEP290), however the sequence could hypothetically extend further upstream. *In silico* searches using the NCBI conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) shows that CEP290 consists of two SMC (structural maintenance of chromosome) regions one upstream of the known T21 sequence, and one called SMC_prok_B within T21 which is structurally associated with the organisation and segregation of partitioning chromosomes during cell division (Marchler-Bauer *et al.,* 2011).

To date, investigation of T21 expression in normal and tumour tissue and studies into the possible role in cancer progression was carried out without prior knowledge of the unique region and the similarity shared between T21 and CEP290. As a consequence, experiments designed using the anti-T21/CEP290 antibody, PCR primers and interfering

RNA oligonucleotides which have now been confirmed to be common to both T21 and CEP290, do not reveal whether T21 is indeed a potential biomarker for cancer or a target for immunotherapy. Therefore the primary objective of this study was to assess the expression profile of T21 using healthy normal tissues following which cancer samples derived from tumour tissues and cancer cell lines were analysed to determine whether T21 may have potential as a tumour restricted immunotherapeutic target. Expression at the mRNA level was determined using both qPCR and RT-Q-PCR and protein expression and localisation were determined using immunoblotting and immunostaining techniques respectively. The second objective involved conducting a series of experiments using immunofluorescence and gene silencing techniques designed to determine whether T21 and CEP290 share functional characteristics and provide an insight into their relationship and T21 involvement in cancer progression.

4.2 Results

4.2.1 Expression analysis of T21 mRNA in normal and cancer tissues

Earlier studies of mRNA expression of *T21* reported its expression to be restricted to testis and to various tumour types confirming its application as a potential biomarker of disease and as a possible target of immunotherapy. More recently, sequence similarity to *CEP290* and the newly identified unique region have drawn these findings into question. To address this, *T21* and *CEP290* mRNA expression was assessed using RNA from a panel of pooled normal human tissues (purchased from Clontech, USA). Briefly, 2 µg of total RNA was reverse transcribed to generate cDNA after which newly designed primers flanking the *T21* unique region (Figure 3.5B) were used to perform semi-quantitative RT-PCR using the conditions described in chapter 2 in comparison to the expression of *β-actin* (house keeping control). In contrast to previous findings, T21 expression was observed in brain, placenta, prostate and testis tissue (Figure 4.1a). In addition, the ubiquitous expression of *CEP290* throughout all normal tissues indicated that there is no shared expression profile or association in relation with *T21* expression.

In order to assess mRNA *T21* expression alone, a more sensitive approach was adopted to quantify mRNA using real time quantitative RT-Q-PCR. Primers designed within the unique region were used to specifically amplify *T21* (Figure 3.5A). In accordance with standardised guidelines in the literature (Bustin *et al.,* 2009), primers were optimised to achieve maximal efficiency and the relative abundance value of specific gene expression was determined by dividing with the value derived by that of averaged housekeeping genes (Pfaffl, 2001). Here, two different housekeeping genes were used, 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. In this experiment the relative expression of *T21* was detectable at considerably higher levels in normal prostate, stomach and spinal cord when compared to testis, which was set to an arbitrary value of one (Figure 4.1b). Similar experiments were performed following extraction of mRNA from a panel of cell lines derived from prostate, breast, melanoma and head and neck cancers. Care was taken to avoid DNA contamination (and possible amplification of intronic

CEP290) of the RNA extracted from cell lines, therefore as a precaution, RNA spin columns were used instead of routine acid guanidinium thiocyanate-phenol-chloroform extraction using STAT-60. *T21* mRNA expression was detected in prostate cancer cell lines LNCaP, DU145 and PC3 (all derived from disseminated tumour cells at metastatic secondary sites). In comparison, a lower level of expression was observed in cell lines from normal immortalised prostate cells (PNT1α and PNT2), and also in OPCT-1, a cell line derived from primary prostate tumour (Figure 4.2a). Of the other cancer cell lines assessed, *T21* was highly expressed in breast (T47D and MCF-7), metastatic melanoma (MM27) and head and neck (A253) (Figure 4.2b). Further experiments were attempted using samples from prostate cancer patients used in previous studies, however the poor quality of the mRNA meant they were unsuitable for any further analysis (results not shown).

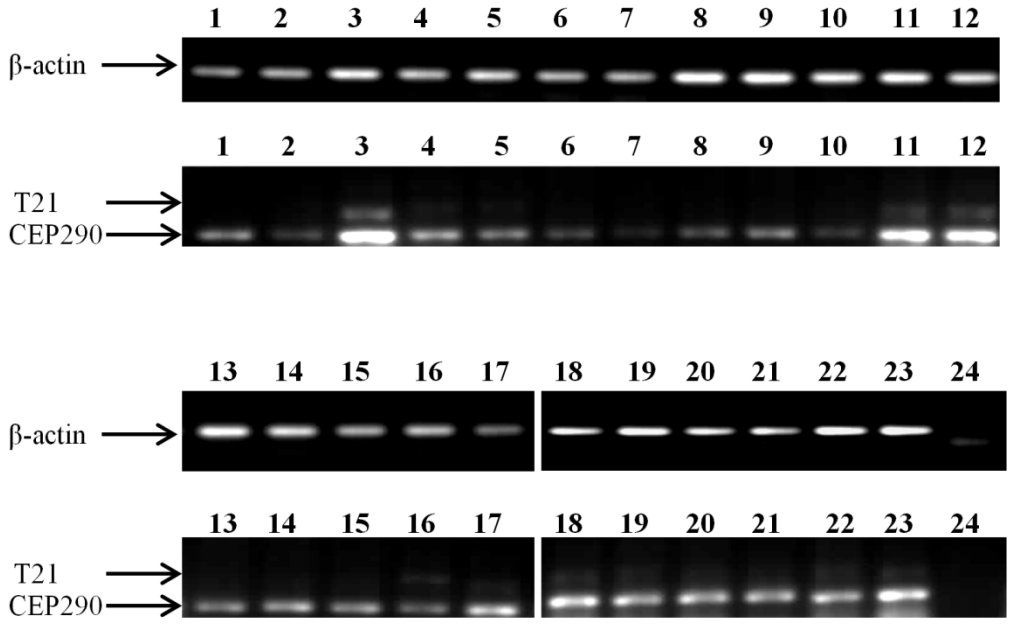


Figure 4.1a – PCR gel images for T21 mRNA expression in normal human tissue. *Semi-quantitative PCR of β-actin loading control (116bp) and T21(287bp)/CEP290(194bp) mRNA expression in commercially acquired normal human tissue total RNA panel. Lanes represent - Adrenal gland (1), Colon (2), Brain, cerebellum (3), Brain (4), Foetal brain (5), Foetal liver (6), Heart (7), Kidney (8), Liver (9), Lung (10), Placenta (11), Prostate (12), Salivary gland (13), Skeletal muscle (14), Spleen (15), Testis (16), Thymus (17), Uterus (18), Ovary (19), Mammary gland (20), Retina (21), Spinal cord (22), Stomach (23) Negative control (24) (representative of two experiments).*

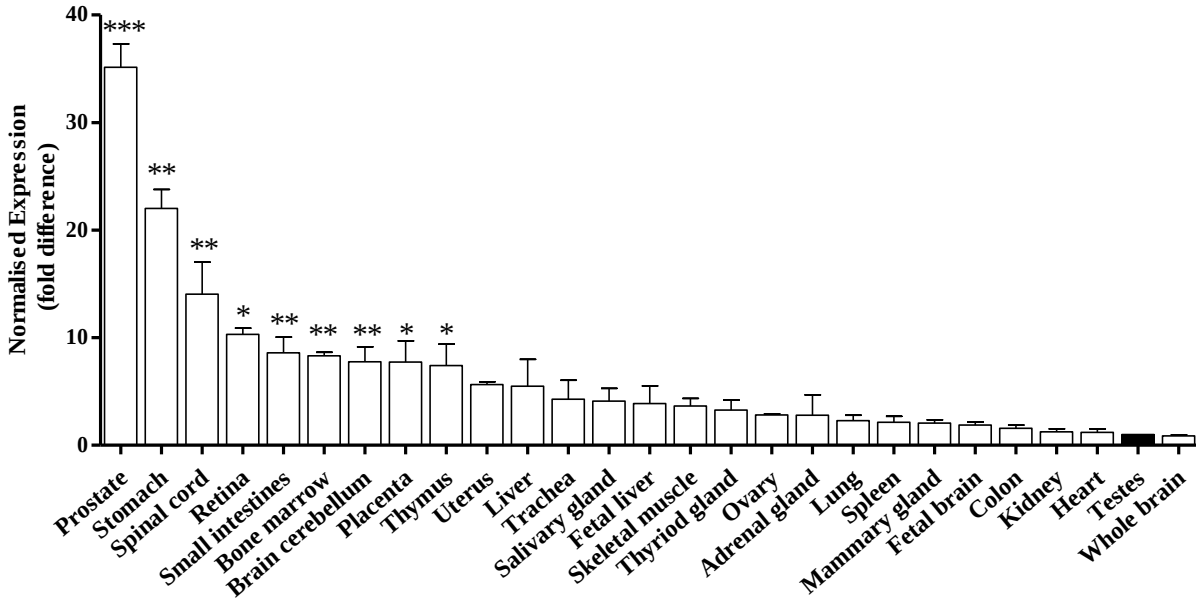


Figure 4.1b – Quantitative RT-PCR for T21 mRNA expression in normal human tissue. *Real time PCR expression of T21 mRNA in commercially acquired normal human tissue panel. Experiment was carried out three times in duplicate (n=3 technical repeat experiments) represented with standard error of mean with data expressed as relative fold change to the normalised expression of testis mRNA expression given as an arbitrarily value of 1. Statistical significance according to expression in testis indicated (*p<0.05) (**p<0.01) (***p<0.001) as determined by student's t test. Samples derived from different lot panel to those analysed and shown in Figure 4.1a.*

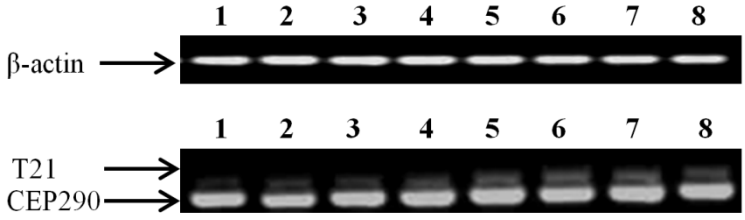


Figure 4.2a – PCR gel images showing T21 mRNA expression in normal prostate and prostate and breast cancer cell lines using qPCR. *Semi-quantitative PCR of β-actin loading control (116bp) and T21(287bp)/CEP290(194bp) mRNA expression using prostate and prostate cancer derived cell lines. Lanes represent – Commercially available normal prostate (1), Normal prostate cell lines PNT1α (2) and PNT2 (3), Prostate cancer origin cell line OPCT-1(4), Prostate cancer cell lines derived from secondary metastasis DU145 (5), LNCaP (6), PC3 (7), Breast cancer origin T47D (8) (n=2).*

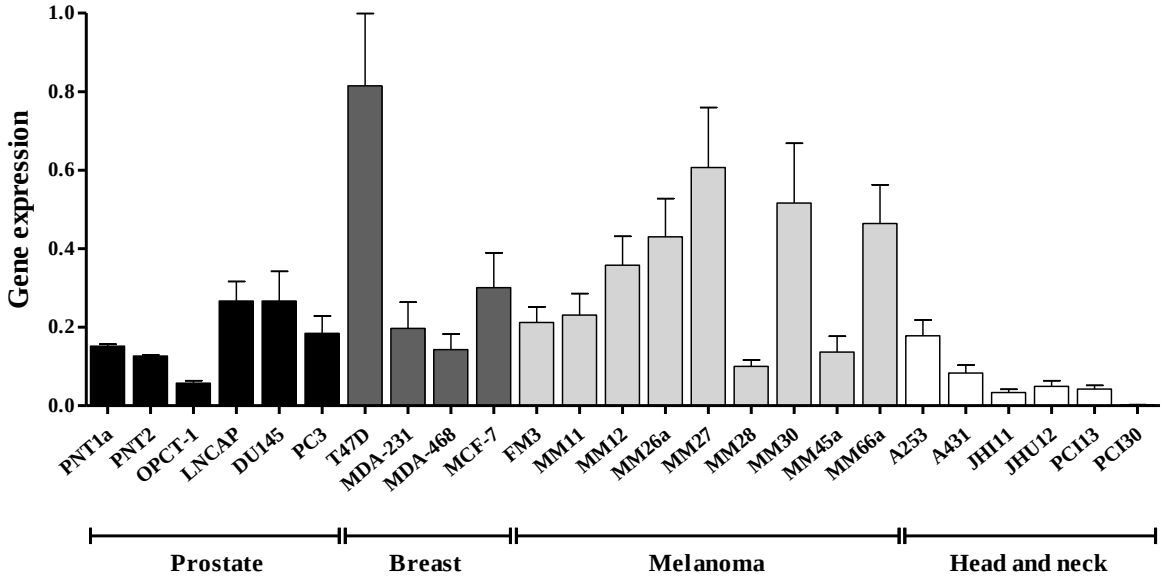


Figure 4.2b – Quantitative RT-PCR for T21 mRNA expression in human cancer cell lines. *Real time PCR expression of T21 mRNA in various cancer cell lines (PNT1α and PNT2 derived from normal prostate) derived from prostate, breast, melanoma and head and neck origin. Experiment was carried out three times in duplicate (n=3) represented with standard error of mean with data calculated by d^ct and represented as relative gene expression.*

4.2.2 Expression analysis of T21 protein in normal and cancer tissues

Despite being a useful tool, mRNA expression analysis is not always predictive of protein abundance (Guo *et al.,* 2008) nor does it give insight into cellular localisation of proteins. Commercially available tissue microarrays were immunohistochemically stained for T21 protein expression using the anti-T21u antibody. Protein expression was determined by staining intensity designated using a scoring ranging from 0 = no staining, 1 = mild staining, 2 = moderate staining to 3 = strong staining. In normal tissues cores (purchased from US Biomax), staining intensities were limited to no or mild expression with kidney, prostate and testis showing moderate expression in some cases. Representative images for each normal tissue type are illustrated in Figures 4.3a and b and are fully detailed in Table 4.1. It is of particular note that T21 protein could not be clearly detected in the thymus suggesting the T21-specific T cells may not be subjected to central tolerance mechanisms and will be discussed further in the next chapter.

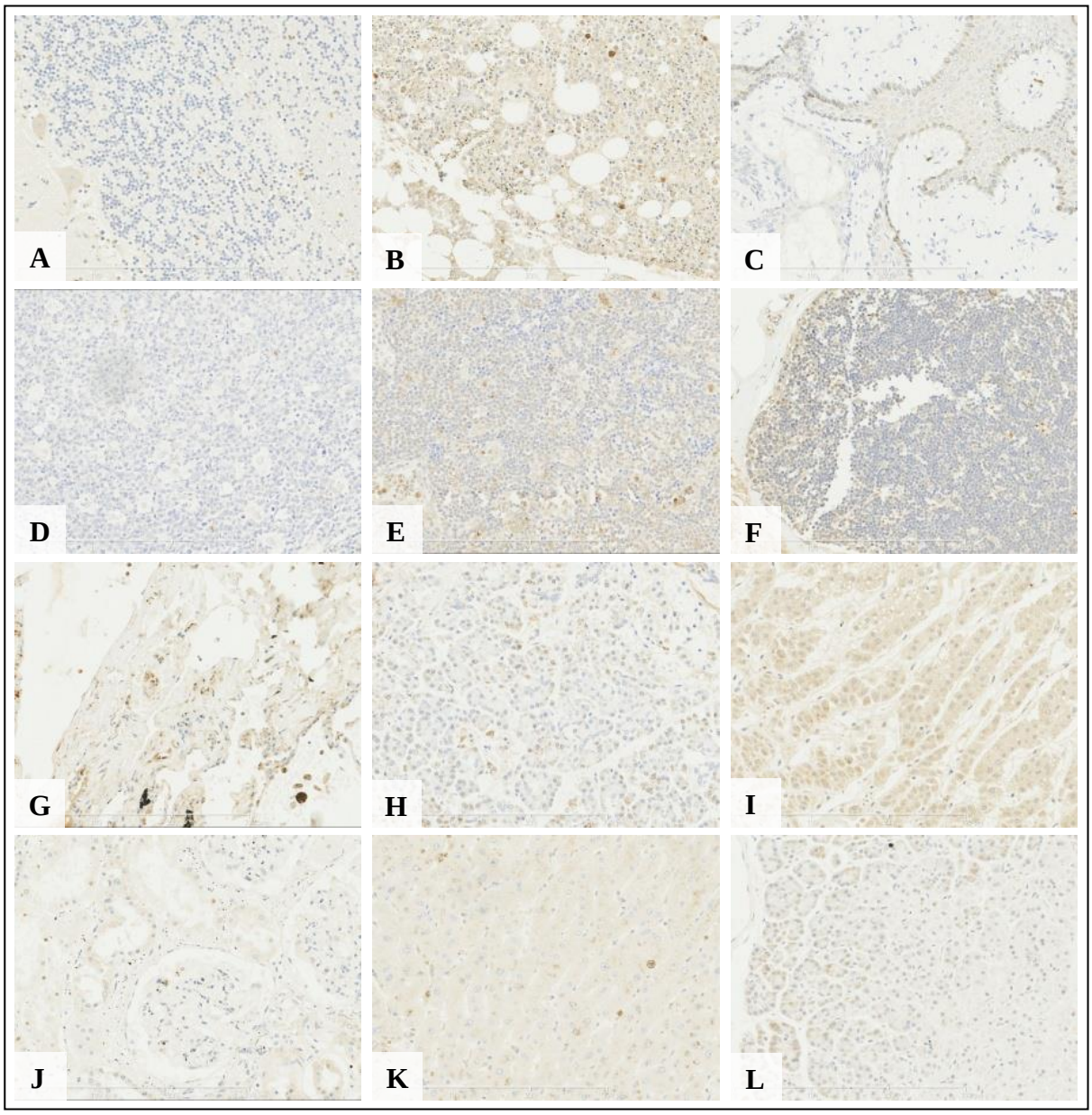


Figure 4.3a – Images immunohistochemical staining for T21 protein expression in normal tissue microarrays. *Cerebral cortex (A), Bone (B), Skin (C), Tonsil (D), Lymph node (E), Thymus (F), Lung (G), Thyroid (H), Adrenal gland (I), Kidney (J), Liver (K) and Pancreas (L). Representative images at objective magnification x20.*

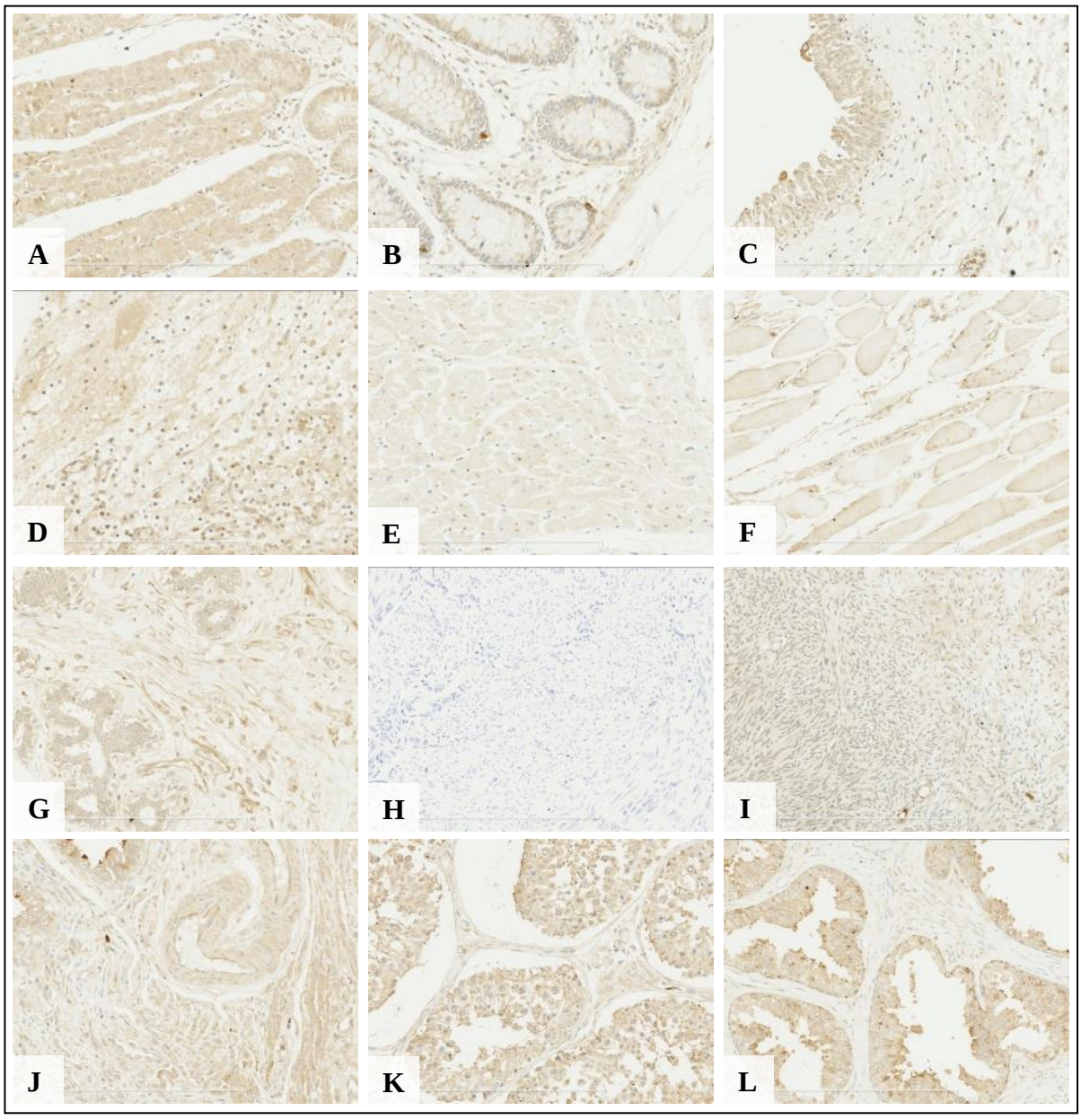


Figure 4.3b – Images immunohistochemical staining for T21 protein expression in normal tissue microarrays. *Stomach (A), Colon (B), Bladder (C), Urethra (D), Heart (E), Stratified muscle (F), Breast (G), Uterus (H), Ovary (I), Fallopian tube (J), Testis (K) and Prostate (L). Representative images at objective magnification x20.*

In addition to examining the expression of T21 protein in normal tissues, further assessment was performed using tissues derived from various malignancies (purchased from US Biomax) summarised in Table 4.1A alongside normal tissues. An increase of expression was observed in malignant tissues derived from colon, lung, bone, prostate and uterus compared to their corresponding normal tissues. The overall staining distribution indicated that of the normal tissues scored, 94% had no staining/mild staining with the remaining 6% having moderate staining. In comparison, collective malignant tissues

scored 78% no staining/mild and 23% moderate to strong staining. Taken together, the data suggests that T21 may be present at increased levels in some tumours namely in colon, uterus, lung and prostate, indicating that further analysis into these tumour types is necessary to determine whether T21 expression is indeed associated with disease progression.

A

	Anti-T21u Ab									
	Normal tissue					Malignant tissue				
	staining intensity					staining intensity				
Tissue type	**#**	**0**	**1**	**2**	**3**	**#**	**0**	**1**	**2**	**3**
Adrenal	1	1				-				
Bladder	1		1			3	3			
Bone	1		1			6	2	3	1	
Breast	4	4				2	2			
Cerebral	7	7				7	7			
Colon	9	7	2			3	1		2	
Fallopian	1		1			-				
Heart	3	3				-				
Kidney	3	1	1	1		3	3			
Liver	3		3			3	2	1		
Lung	3	1	2			6		3	2	1
Lymph node	1	1				4	3	1		
Muscle	1	1				-				
Ovary	3	3				3	1	2		
Pancreas	3	1	2			3	3			
Parathyroid	1	1				-				
Pituitary	1		1			-				
Placenta	1	1				-				
Prostate	3	2		1		3			3	
Skin	3	1	2			3	2		1	
Small Int.	1	1				3	2	1		
Spinal	1	1				-				
Spleen	1	1				3	2		1	
Stomach	1		1			3	3			
Testis	3		1	2		3		2	1	
Thymus	1	1				-				
Thyroid	3	3				2	1		1	
Tonsil	3	2	1			-				
Umbilical	1	1				-				
Urethra	1		1			-				
Uterus	4	2	2			3	1		2	

B

	CEP290 (The Human Protein Atlas)									
	Normal tissue					Malignant tissue				
	staining intensity					staining intensity				
Tissue type	**#**	**0**	**1**	**2**	**3**	**#**	**0**	**1**	**2**	**3**
Adrenal	1				1	-				
Bladder	1			1		-				
Bone	-					-				
Breast	4	1		2	1	12		2	8	2
Cerebral	3	2	1			-				
Colon	2			2		12		1	8	3
Fallopian	2			2		-				
Heart	1	1				-				
Kidney	2			1	1	11	10	1		
Liver	2	2				12	6	3	3	
Lung	2	1	1			11	1	8	2	
Lymph node	1	1				12	10	2		
Muscle	1	1				-				
Ovary	2	1	1			11		1	8	2
Pancreas	2	1		1		11		3	8	
Parathyroid	1		1			-				
Pituitary	-					-				
Placenta	2		1		1	-				
Prostate	1				1	12		1	10	1
Skin	3	1	1	1		12	4	2	6	
Small Int.	1			1		-				
Spinal	-					-				
Spleen	2	1	1			-				
Stomach	2				2	9	5	1	3	
Testis	2			1	1	11	5	3	3	
Thymus	-					-				
Thyroid	1			1		4		2	1	1
Tonsil	1			1		-				
Umbilical	-					-				
Urethra	-					11	4	4	3	
Uterus	4		2	2		-				

Table 4.1 – Score summary of immunohistochemistry tissue sections for T21 protein expression. *Table representing staining intensities observed in normal and cancer tissues using purchased TMAs from US Biomax (Cat. MNO341, MNO381 and ME241) (**A**). As a comparison, tissue staining data published by The Human Protein Atlas project (http://www.proteinatlas.org) using an anti-CEP290 antibody (purchased from Abbott laboratories)(**B**). A numerical scoring method assigning 0=no staining, 1=mild staining, 2=moderate staining and 3=strong staining used. Numbers in cells represent number of tissues scored and shaded areas represent no matching cancer tissue available.*

Previous staining of normal tissues using custom anti-T21/CEP290 antibody (purchased from Pacific Immunology) demonstrated that protein expression was restricted to stomach, ovary, breast and prostate tissues (Miles *et al.,* 2012). Moreover, data published online by The Human Protein Atlas project (http://www.proteinatlas.org) showing protein expression profiles for CEP290 in normal human tissues and various cancer types demonstrated important staining differences between T21 (using anti-T21u antibody) and CEP290. The staining distribution from The Human Protein Atlas project indicated that of the normal tissues scored, (22/46) 48% no staining/mild, 35% (16/46) moderate and the remaining (8/46) 17% having strong staining. Of these normal tissues, adrenal, breast, kidney, placenta, prostate, stomach and testis demonstrated strong staining. In comparison, cancer tissues scored (79/151) 52% no staining/mild, (63/151) 42% moderate and (9/151) 6% strong staining. Within cancer tissues, breast, colon, ovary, prostate and thyroid tissues demonstrated strong staining, equally moderate to strong staining was demonstrated in almost all cancer derived cell lines (data available at http://www.proteinatlas.org). Despite high levels of CEP290 distributed throughout normal tissues (Figure 4.1B), anti-CEP290 auto-antibodies have been reported in patients with cancer (Eichmuller *et al.,* 2001; Chen & Shou, 2001), which may indicate that CEP290 related immune tolerance mechanisms have been overcome in order to generate an immune response. These expression profiles patterns vary between the two proteins; however both are upregulated in some cancers.

Following these observations, further investigation of the expression of T21 in prostate cancer patients was undertaken in collaboration with Dr Desmond Powe (Department of Histopathology, Queens Medical Centre, Nottingham University Hospitals NHS Trust, Nottingham, UK). Before commencing the study, the anti-T21u antibody was optimised together with assay conditions (buffers and incubation times) on tissue core off-cuts taken from the same patient cohort in formalin fixed paraffin embedded (FFPE) tissue microarray (TMA) blocks. Optimal concentration conditions were determined to be 1:50 dilution of a 0.4 mg/ml stock solution left overnight at 4°C. The patient cohort comprised of ten TMA FFPE blocks constructed of benign prostatic hyperplasia (BPH) and clinically confirmed cancer patients tissue cores collected between 1998 and 2000 as a wider study by the Nottingham Prostate Research Group. Ethical approval for generic consent for prospective collections and for the use of pathology archival material has been granted by the Greater Manchester National Research Ethics Service, 2010. For this study, patient cohorts consisted of 79 histopathologically confirmed prostate cancer and 57 benign cases

(mean age = 70.8, median age = 71). Semi-quantitative scoring intensity scale ranging from 0 for no staining, 1 for weak, 2 for moderate and 3 for strong staining observed was applied to determine the degree of IHC staining (Figure 4.4). The association between T21 and clinical variables were statistically modelled using Kaplan-Meier plots and Cox regression hazards risk model (SPSS software version 20.0 from SPSS Inc. USA) calculated using univariate chi-square analysis, a statistical significance considered as $p \leq 0.05$ was applied to the data.

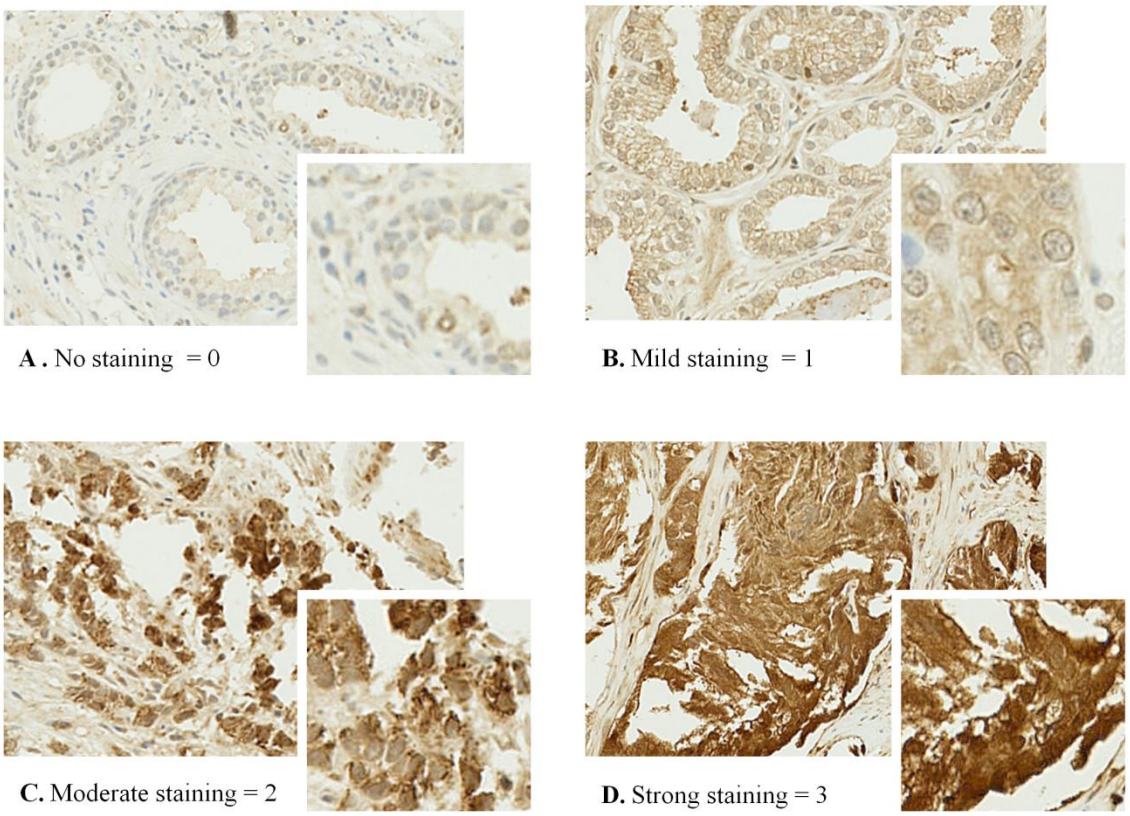


Figure 4.4 – Immunohistochemical staining for T21 protein expression in prostate tissue. *Images illustrating scoring method used for prostate tissue. Representative images at objective magnification x20 and x40 inset.*

In line with previous observations, T21 staining was detected mostly in the cytoplasm and for some samples in the nuclei and membrane of prostate glands. No clear centrosomal localisation was observed. Within is cohort of TMA samples, 88.9% of patients showed positive staining for T21 of which 58.7% were prostate cancer patients (Table 4.2A). However, no clear distinction could be determined between benign and prostate cancer

patients when assessing the presence or absence of T21. Further examination of disease comparative positive staining distribution revealed that the majority of benign patients had mild staining, whereas prostate cancer patients had mild/moderate staining intensity. In fact, of all of the moderately stained tissues, 71% were of prostate cancer origin (Table 4.2B). Next, statistical analysis was performed using only the cancer cohort. To summarise, no statistical associations were observed between intensity of T21 protein expression and age and survival from diagnosis (Table 4.3 and Figure 4.5). Further examination of high and low expression of T21 between variables was performed by grouping (0+1 intensities = low expression) and (2+3 intensities = high expression). Once again, no significant association was seen between T21 expression and survival from initial diagnosis (Figure 4.6), the time taken to develop of distant metastasis from initial diagnosis (Figure 4.7), between low and high Gleason grade, PSA, the presence or absence of vascular invasion, tumour percentage, metastasis, androgen deprivation therapy (ADT), or prostate cancer related death (Table 4.3).

A – Presence and absence of staining		**Negative**	**Positive**	**Total**
		0	**All intensities**	
Benign	**Freq.**	**7**	**50**	**57**
	Percent	**46.6**	**41.3**	**41.9**
Malignant	**Freq.**	**8**	**71**	**79**
	Percent	**67.4**	**58.7**	**58.1**
Total	**Freq.**	**15**	**121**	**136**

B – Positive staining intensity distribution		**Positive intensities**			**Total**
		1	**2**	**3**	
Benign	**Freq.**	**33**	**13**	**4**	**50**
	Percent	**48.5**	**28.3**	**57.1**	**41.3**
Malignant	**Freq.**	**35**	**33**	**3**	**71**
	Percent	**51.5**	**71.7**	**42.9**	**58.7**
Total	**Freq.**	**68**	**46**	**7**	**121**

Table 4.2 – Scoring of immunohistochemistry prostate sections for T21 protein. *Table outlining benign and malignant disease cases stained and scored for T21 expression showing absence of expression (negative) and expression observed at any score intensity (positive) (**A**). A numerical scoring method assigning 0=no staining, 1=mild staining, 2=moderate staining and 3=strong staining used (illustrated in Figure 4.4) showing the distribution of staining among positive intensities (**B**).*

Variables		Freq.	Negative	Positive intensities			p-value
			0	1	2	3	
Age	<49	79	0	1	0	0	0.763
	50-64		1	6	6	0	
	65-74		3	12	18	2	
	>75		4	16	9	1	
Survival	Months	53	7	22	22	2	0.280
Survival	Months	53	29		24		0.436
Distant Mets	Months	27	12		15		0.444
Gleason grade	1 – 6	96	20		21		0.240
	7 – 10		32		23		
PSA	< 5ng	69	16		14		0.530
	> 5ng		20		19		
Vascular invasion	Neg.	68	30		26		0.535
	Pos.		6		6		
Cancer	Percent	67	36		31		0.231
Metastasis	Months	96	52		44		0.358
ADT	w/o	65	5		4		0.639
	With		31		25		
Death	CaP related	57	13		9		0.469
	Unrelated		19		16		

Table 4.3 – Association between T21 expression and other clinicopathological variables.

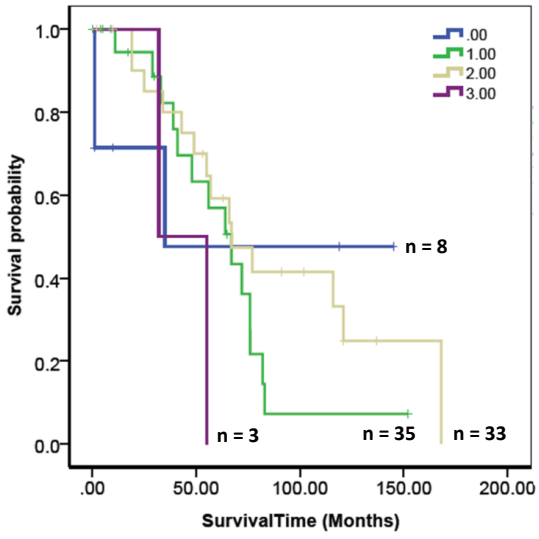


Figure 4.5 – Kaplan-Meier survival time analysis using all intensities. *Survival analysis representing clinically confirmed prostate cancer cohort. A numerical scoring method assigning 0.00=no staining, 1.00=mild staining, 2.00=moderate staining and 3.00=strong staining is given. Results indicate no statistical significance.*

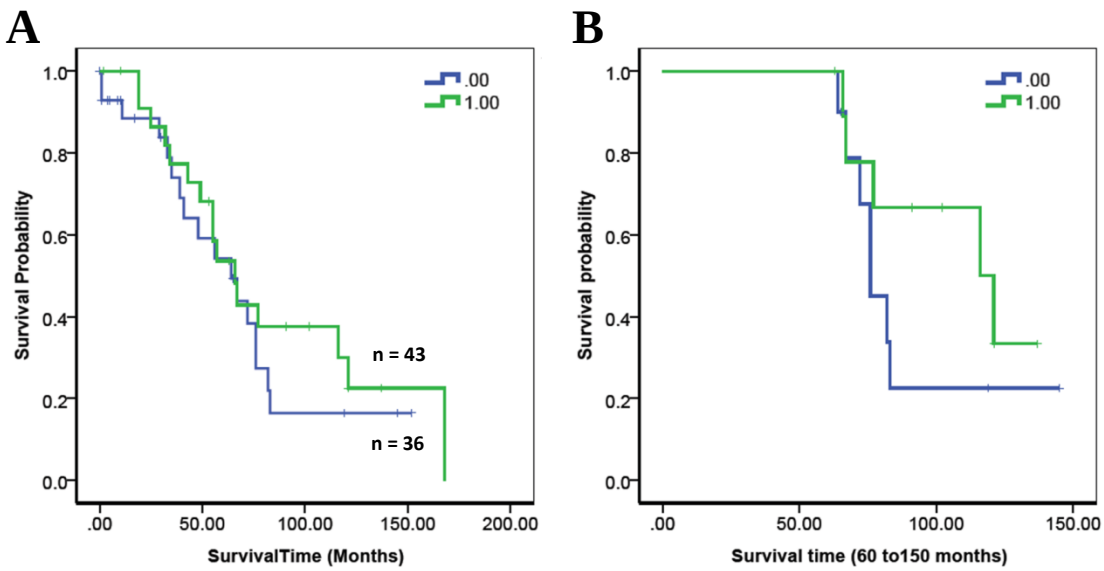


Figure 4.6 – Kaplan-Meier survival time analysis using high and low intensities. *Survival analysis representing clinically confirmed prostate cancer cohort between 0 to 170 months (A) and between 60 to 150 months. A numerical scoring method assigning 0.00=no/mild staining and 1.00=moderate/strong staining is given. Results indicate no statistical significance.*

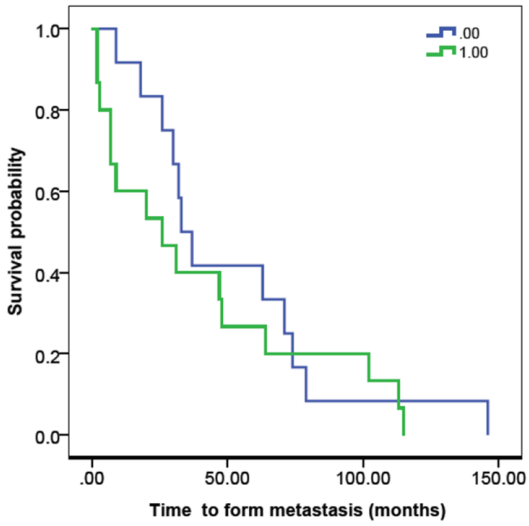


Figure 4.7 – Kaplan-Meier survival analysis from diagnosis to time to form metastasis using high and low intensities. *Survival analysis representing clinically confirmed prostate cancer cohort between 0 to 140 months from initial diagnosis to forming distant metastasis. A numerical scoring method assigning 0.00=no/mild staining and 1.00=moderate/strong staining is given. Results indicate no statistical significance.*

4.2.3 Expression analysis of T21 protein in cancer cell lines

As mentioned previously, *T21* had been identified from immunogenic screening using sera taken from patients with advanced prostate cancer therefore further investigation of this antigen was performed using cell lines derived from prostate cancer. The initial expression profile of the cancer cell line panel (Figure 4.2b) demonstrated that two breast cancer cell lines (T47D and MCF-7) in particular demonstrated extremely high levels of *T21* compared to prostate cell lines therefore it was decided to screen all available prostate and breast cancer cell lines further for T21 and CEP290 protein expression. Following optimisation of immunoblotting procedures (detailed in chapter 2) cell line derived lysates were subjected to western blot analysis using the anti-T21u antibody. Previous investigation revealed a T21 specific band at approximately 90 kDa (refer to section 3.2.3). The results demonstrated T21 to be detectable when loading 50 µg of total protein lysates in prostate cancer cell lines PC3, LNCaP and faintly observed in DU145 (Figure 4.8A). The T21 specific band was not detected in PNT1α, PNT2 (normal immortalised prostate cell lines) and OPCT-1 (primary prostate cancer). Examination of the breast cancer cell lines showed little expression in SkBr3 and MDA468 and -231, however MCF-7 and T47D did demonstrate protein expression of T21 (Figure 4.8B). These findings were also confirmed when performing ELISA (data not shown).

The expression of CEP290 was also assessed in both prostate and breast cancer cell lines using an anti-CEP290 antibody (Everest Biotech) (Figure 3.15). As with other commercially available anti-CEP290 antibodies assessed during this investigation (data not shown) multiple bands were detected when performing western blotting using this antibody. Bands were observed at 250, 200, 150 and 75 kDa making it difficult to determine CEP290 expression. The results suggest that CEP290 is expressed in all cell lines at varying levels (Figure 4.9), which supports the observations of the mRNA expression profiles described earlier. Significantly, no band was detected at 90 kDa which would be suggestive of T21 expression, since the anti-CEP290 antibody (Everest Biotech) used may "theoretically" detect T21 (Figure 3.15). Attempts were made to isolate a CEP290 specific band using immunoprecipitation and magnetic bead separation followed by detection of peptide fragments using mass spectrometry; however these experiments proved unsuccessful (data not shown).

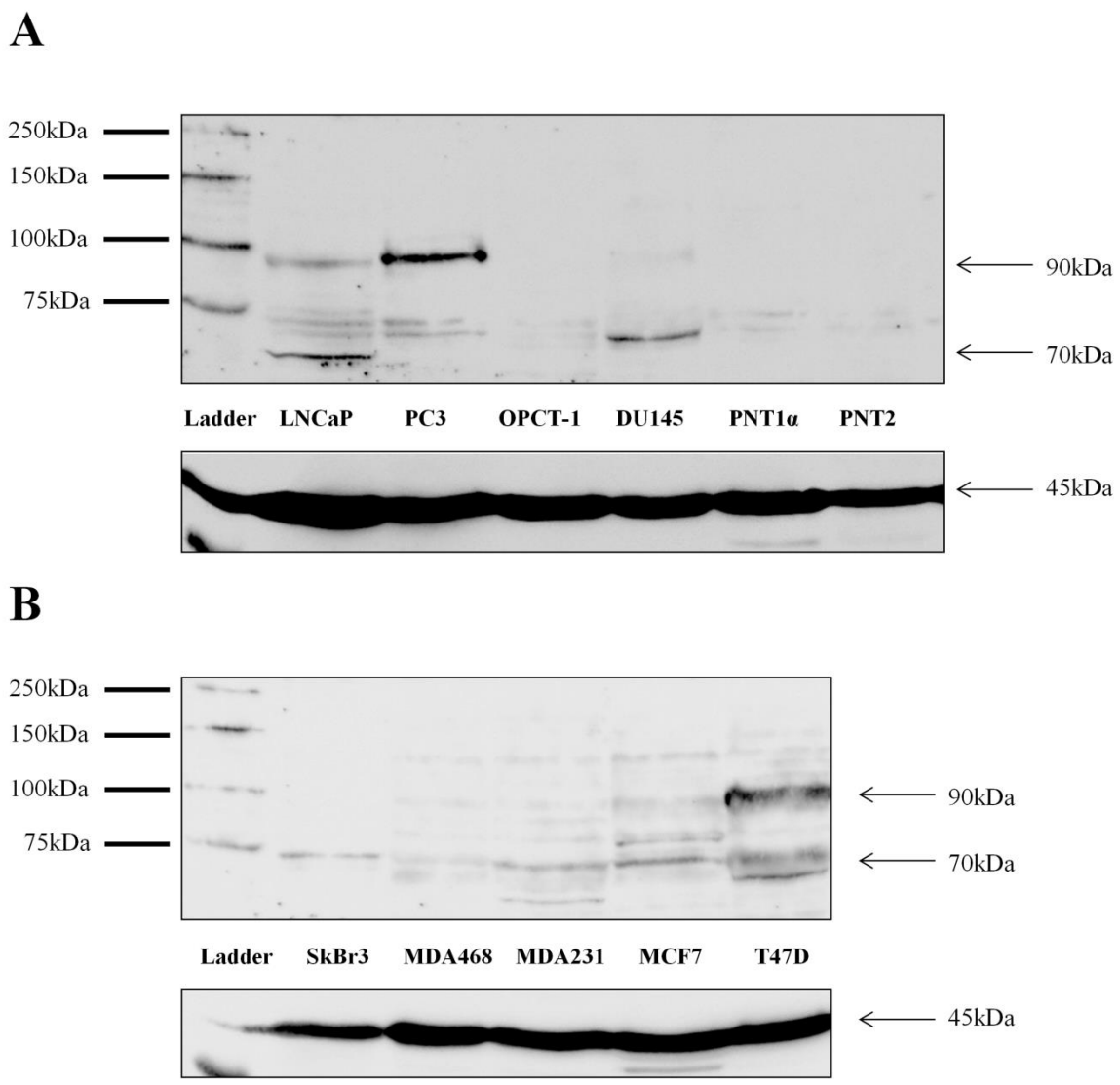


Figure 4.8 – Western blot analysis of T21 protein expression in cancer cell lines. *Protein expression of various prostate derived cell lines (**A**) and breast cancer derived cell lines (**B**) showing 2 bands (90 kDa and 70 kDa) performed using western blotting and using β-actin (45 kDa) as representative protein loading control (n=2).*

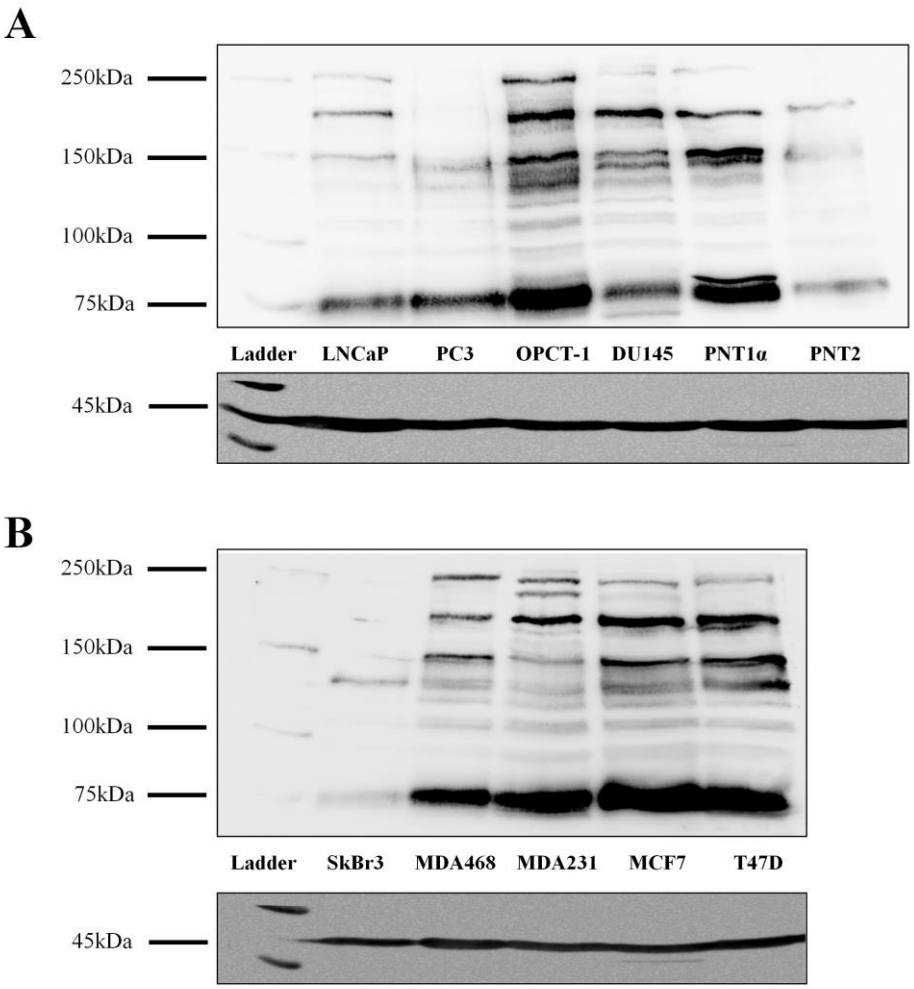


Figure 4.9 – Western blot analysis of CEP290 protein expression in cancer cell lines. *Protein expression of various prostate derived cell lines (**A**) and breast cancer derived cell lines (**B**) showing several discreet bands (90 kDa and 70 kDa) performed using western blotting and using β-actin (45 kDa) as representative protein loading control (n=2).*

In addition to western blotting, T21 cellular localisation was investigated using immunofluorescence detection. Antibody staining was optimised, cells were fixed and incubated with anti-T21u antibody and subsequently with fluorescently labelled secondary Alexafluor antibodies and assessed by fluorescence microscope (Olympus BX51) (detailed in section 2.2.5.1). Each assay was performed using the appropriate controls (see appendix Figure IIIa). The results demonstrated overall low levels of cytoplasmic expression in

PNT1α, PNT2, DU145 and OPCT with higher expression observed in LNCaP, PC3 and T47D cells corroborating the expression patterns observed in mRNA and in western blotting (Figures 4.10 and 11). However, it was observed that in some seemingly low expressing cell lines such as PNT1α, PNT2 and MDA468 cells undergoing division demonstrated more intense staining suggesting that T21 may be expressed during the cell cycle (Figures 4.10 and 11). During the course of this investigation, prostate cell lines were assessed using the previously designed custom anti-T21/CEP290 antibody (Figure 3.15). It was observed that the expression was present throughout the cytoplasm and also strongly localised to the centrosomal bodies during each active stage of the cell cycle (see appendix Figure IIIb). As dual-staining of both of these antibodies was not possible (both raised using the same species), parallel co-expression experiments using either anti-T21u or anti-T21/CEP290 antibodies together with antibodies known to associate with cell proliferation, CEP290 and the cell centrosomes was undertaken. Based on the collective findings of the cell line mRNA and protein expression profiles, it was decided that progress with *in vitro* investigation of T21 using the prostate cancer cell line PC3.

Thus, T21 expression was observed alongside the cell cycle marker Ki67, which is present during all active phases of the cell cycle (Figure 4.12). Immunofluorescence analysis demonstrated that the expression of T21/CEP290 (anti-T21/CEP290 antibody) was strongly associated with cells undergoing cell proliferation (anti-Ki67 antibody) whereas intense staining of T21 using anti-T21u appeared not to co-localise to proliferating cells in the same manner (Figure 4.12B). In order to compare T21 and CEP290 expression, cells were dual stained with the previously described anti-CEP290 antibody in parallel with anti-T21/CEP290 antibody (Figure 4.13A) or anti-T21u antibody (Figure 4.13B). As expected the anti-T21/CEP290 antibody strongly co-localised to the CEP290 protein (anti-CEP290 antibody) whereas no strong localisation was detected using T21 (anti-T21u antibody). Centrosomal localisation was validated by staining for the centrosome-specific protein pericentrin, a component of the pericentriolar material expressed in the centrosomes (Figure 4.14A). Dual staining with anti-pericentrin antibody also confirmed that T21 (anti-T21u antibody) did not localise to the centrosomes (Figure 4.14B). From these results it can be inferred that T21 does not share a functional role similar to that of CEP290 at the centrosomal sites during cell proliferation.

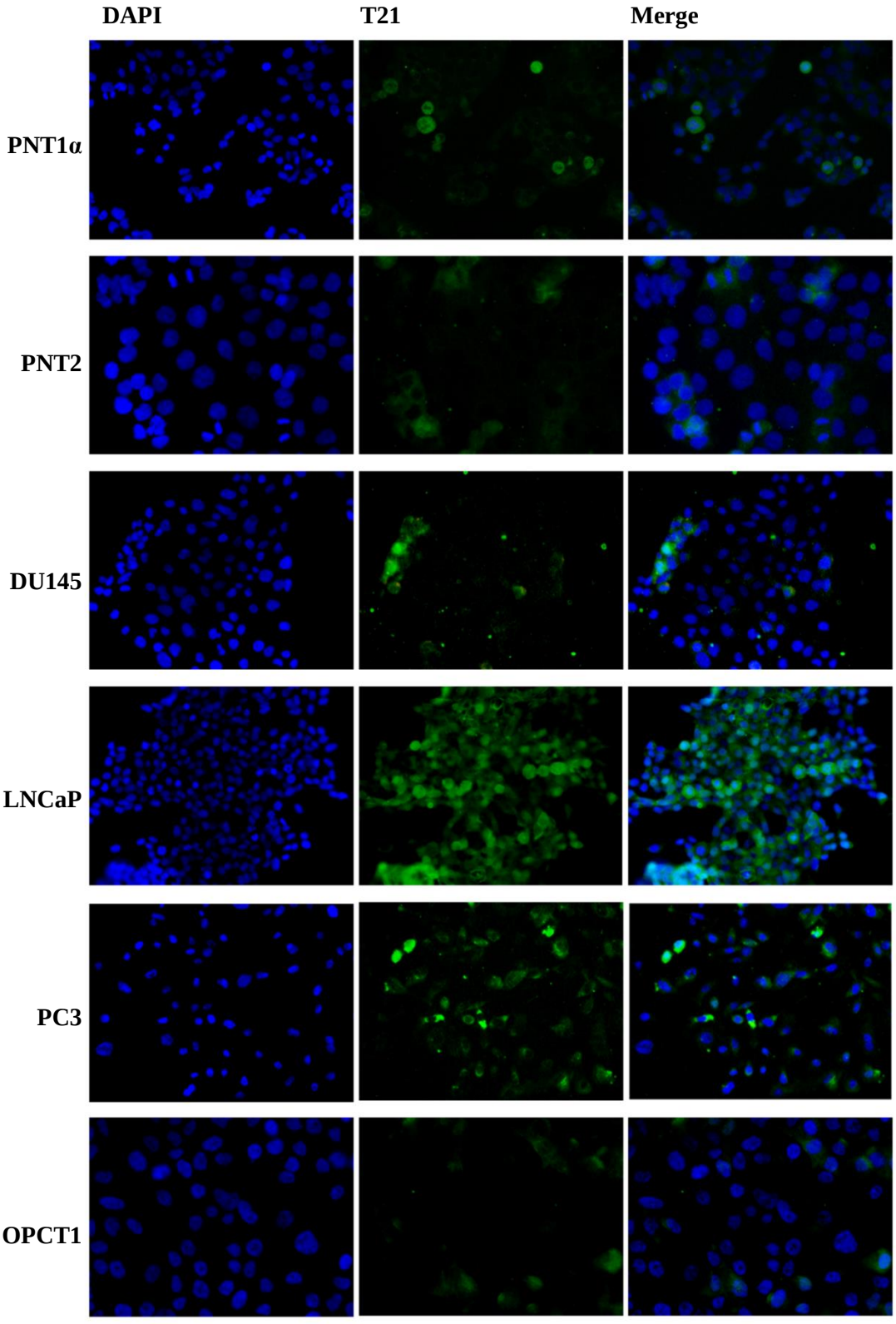


Figure 4.10 – Immunofluorescence analysis showing protein expression of T21 in prostate derived cell lines. *Cells derived from prostate cell lines labelled using custom anti-T21u antibody (green) and secondary stained using anti-rabbit Alexafluor 488 antibody and with nuclear staining using DAPI (blue). Representative images using magnification x20 (n=2).*

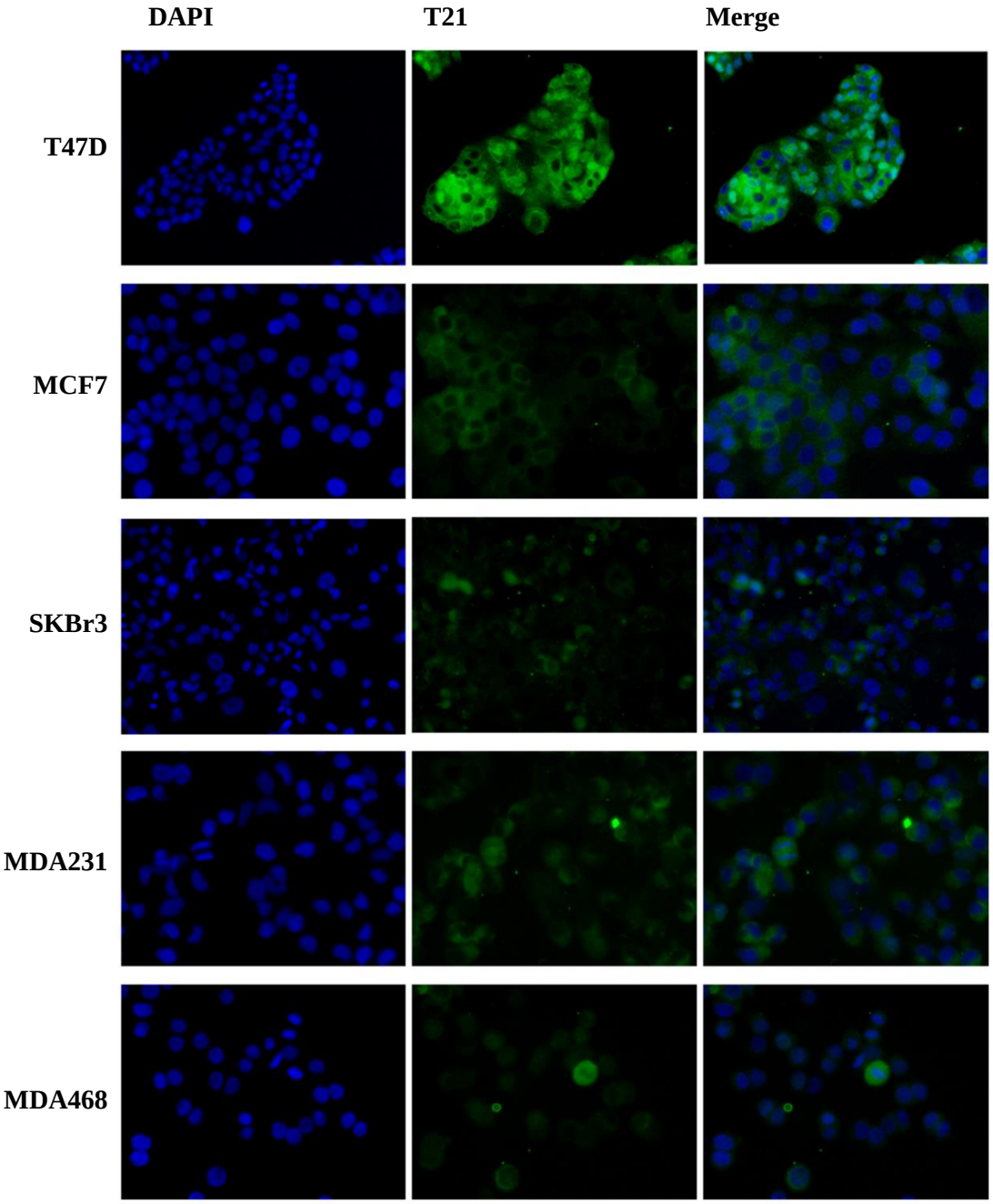


Figure 4.11 – Immunofluorescence analysis showing protein expression of T21 in breast cancer cell lines. *Cells derived from breast cancer lines labelled using anti-T21u antibody (green) and secondary staining as described previously and with nuclear staining using DAPI (blue). Representative images using objective magnification x20 (n=2).*

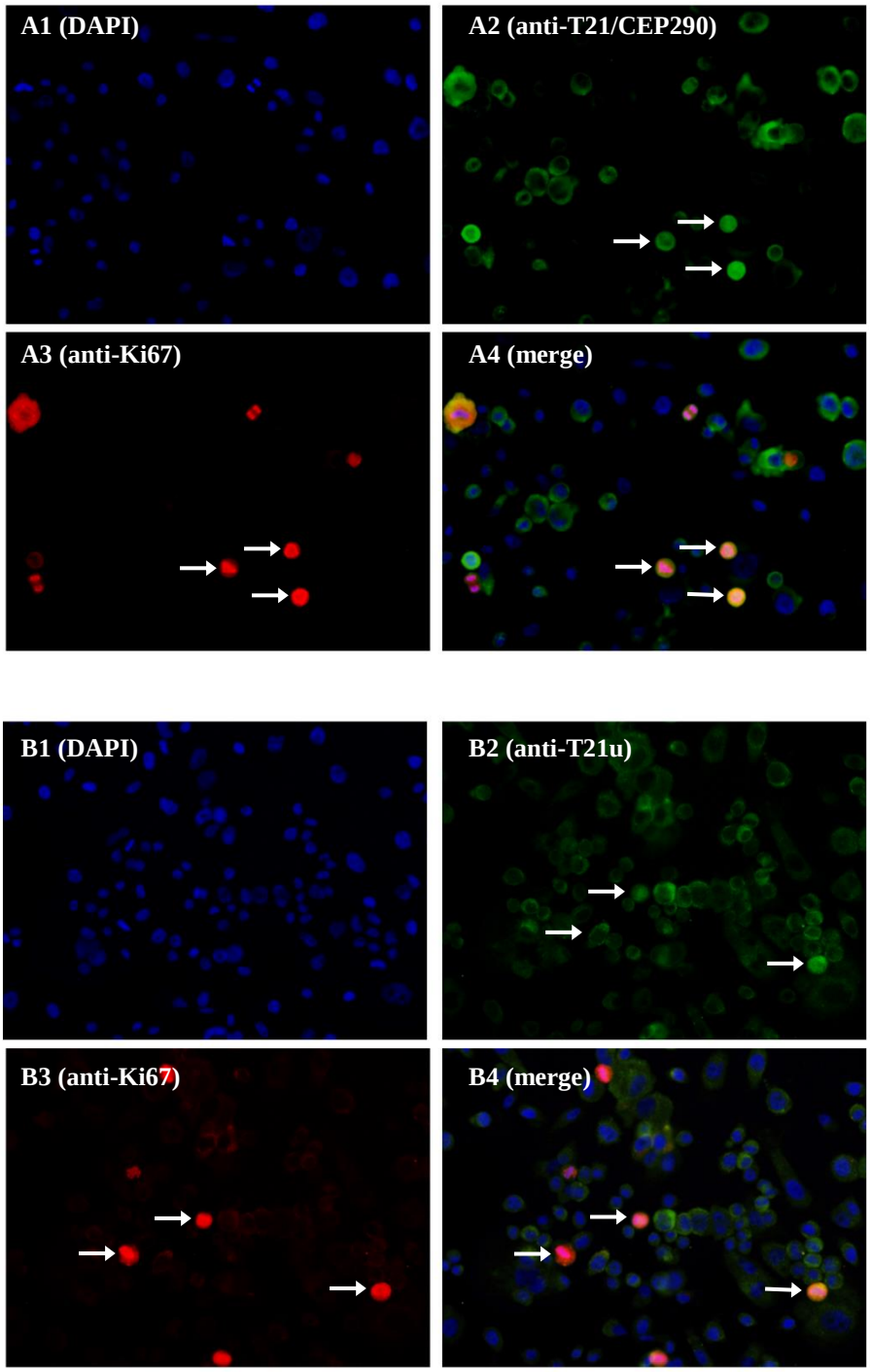


Figure 4.12 – Immunofluorescence composites comparing T21u and T21/CEP290 antibody localised with Ki67 in PC3 cell line. *Cells derived from PC3 cells were dual stained with anti-Ki67 antibody (red) and using either anti-T21/CEP290 antibody (**A**) or anti-T21u antibody (**B**) shown in green and nuclear staining using achieved using DAPI (blue). Cell localisation was observed in using anti-CEP290/T21 antibody and to a lesser degree using T21u antibody (white arrows). Representative images using objective magnification x40 (n=3).*

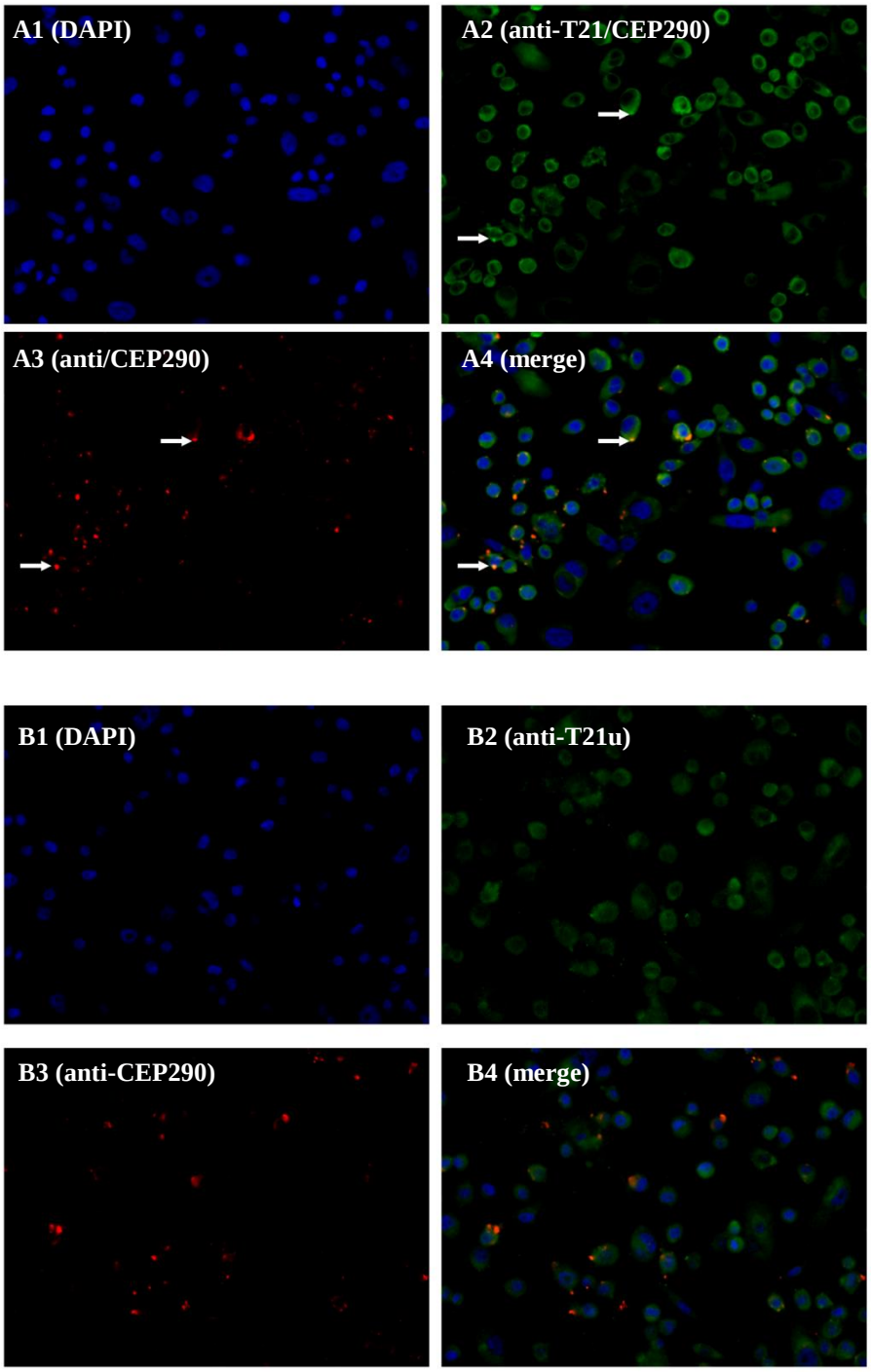


Figure 4.13 – Immunofluorescence composites comparing T21u and T21/CEP290 antibody localised with CEP290 in PC3 cell line. *Cells derived from PC3 cells were dual stained with anti-CEP290 antibody (red) and using either anti-T21/CEP290 antibody (**A**) or anti-T21u antibody (**B**) shown in green and nuclear staining using achieved using DAPI (blue). Centrosomal localisation was observed in using anti-CEP290/T21 antibody (white arrows) but was absent when using T21u antibody. Representative images using objective magnification x20 (n=3).*

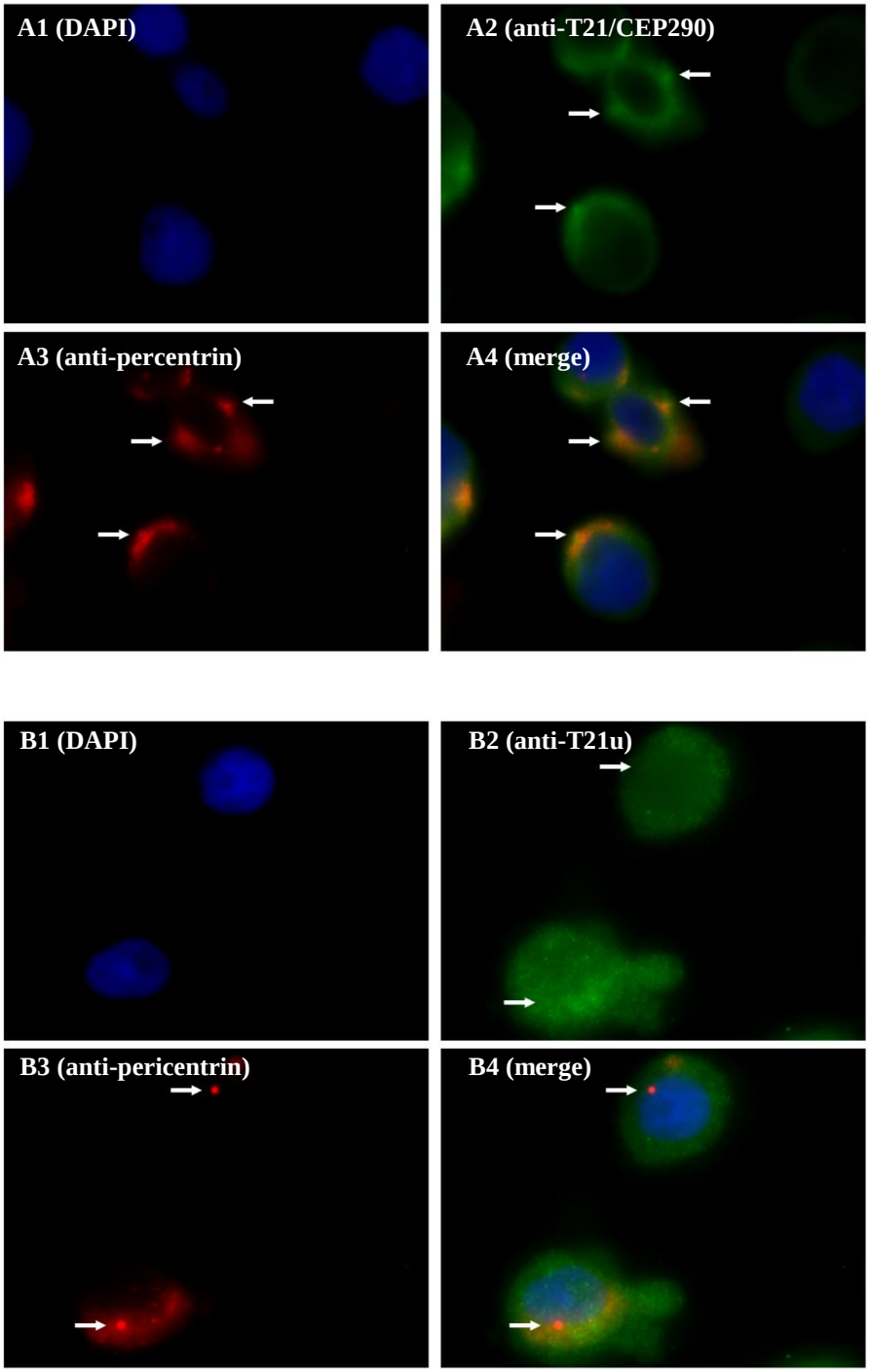


Figure 4.14 – Immunofluorescence composites comparing T21u and T21/CEP290 antibody localised with pericentrin in PC3 cell line. *Cells derived from PC3 cells were dual stained with anti-Pericentrin antibody (red) and using either anti-T21/CEP290 antibody (**A**) or anti-T21u antibody (**B**) shown in green and nuclear staining using achieved using DAPI (blue). No centrosomal co-localisation was detected when using anti-T21u antibody (white arrows). Centrosomal co-localisation was observed using CEP290/T21 antibody (white arrows). Representative images using objective magnification x100 oil emulsion (n=3).*

4.2.4 The role of T21 in tumourogenesis

4.2.4.1 Transient gene silencing of T21 using small interfering RNA

In order to understand further the role of T21 has in cells, experiments were designed to silence gene expression using RNA interference in PC3 cells. Briefly, three gene specific siRNA molecules were designed within the unique region of *T21*. PC3 cells were then cultured in the presence of either control siRNA (ineffective scrambled cassette) or *T21*-specific siRNA along with Interferin, a non-liposomal amphiphile transfection reagent. Total RNA was extracted from cells using RNA spin columns after 24, 48 and 72 hours post transfection to assess *T21* silencing at the mRNA level by RT-Q-PCR using *T21* specific primers. The data showed siRNA *T21-3* to be the most effective in silencing *T21* (81% inhibition) after 24 hours with the remaining two siRNA molecules achieving 58% and 56% knockdown. Moreover, *CEP290* expression assessed using gene specific primers designed within exon 34 of *CEP290* revealed no significant knockdown occurring in any of the *T21*-siRNA treated cells (Figure 4.15A). Maximal knockdown was achieved using *T21-3* siRNA after 24 hours followed by a gradual return of *T21* expression (Figure 4.15B). Interestingly, knockdown was also observed using primers specific to *CEP290* within exon 2 (upstream of the known *T21* start sequence) although failing to reach statistical significant (data not shown). This further supports the premise that the *T21* mRNA sequence may indeed extend further upstream then previously demonstrated. Furthermore, transfection of PC3 cells with siRNA molecules designed within exon 2 of *CEP290* has also independently demonstrated *T21* specific knockdown (performed by Mr B. Alshehri: personal communication).

The transfected cells were observed by microscopy and for changes in cell behaviour and morphology during *in vitro* culture documented. In summary, cells treated with *T21* siRNA did not exhibited any noticeable changes when compared with control siRNA or Interferin treatment other than a seemingly reduced capability to proliferate. Following this observation, experiments assessing cell proliferation were performed using ^{3}Thy incorporation parallel to assessing mRNA *T21* knockdown using siRNA *T21-3*. The data demonstrates a significant reduction of the proliferation observed in cells transfected with *T21* siRNA compared to control siRNA over a period of seven days (Figure 4.16).

To confirm that a reduction in *T21* mRNA leads to a decrease in T21 protein production, *T21* knockdown was performed on a larger scale in order to yield sufficient protein to perform western blotting using the anti-T21u antibody. Although a comparable decrease in T21 protein expression between control siRNA and *T21* siRNA was demonstrated after 72 hours, earlier differences remained inconclusive due to the poor yield of T21 protein (Figure 4.17). The large quantities of siRNA required for this approached proved to be a limiting factor for repeat experiments. Therefore immunofluorescence was used to assess the level of knockdown which revealed a decrease in T21 protein production following cell transfection with *T21* siRNA (Figure 4.18). Further experiments are required beyond the 72 hour timeframe in order to observe if/when T21 protein production is restored.

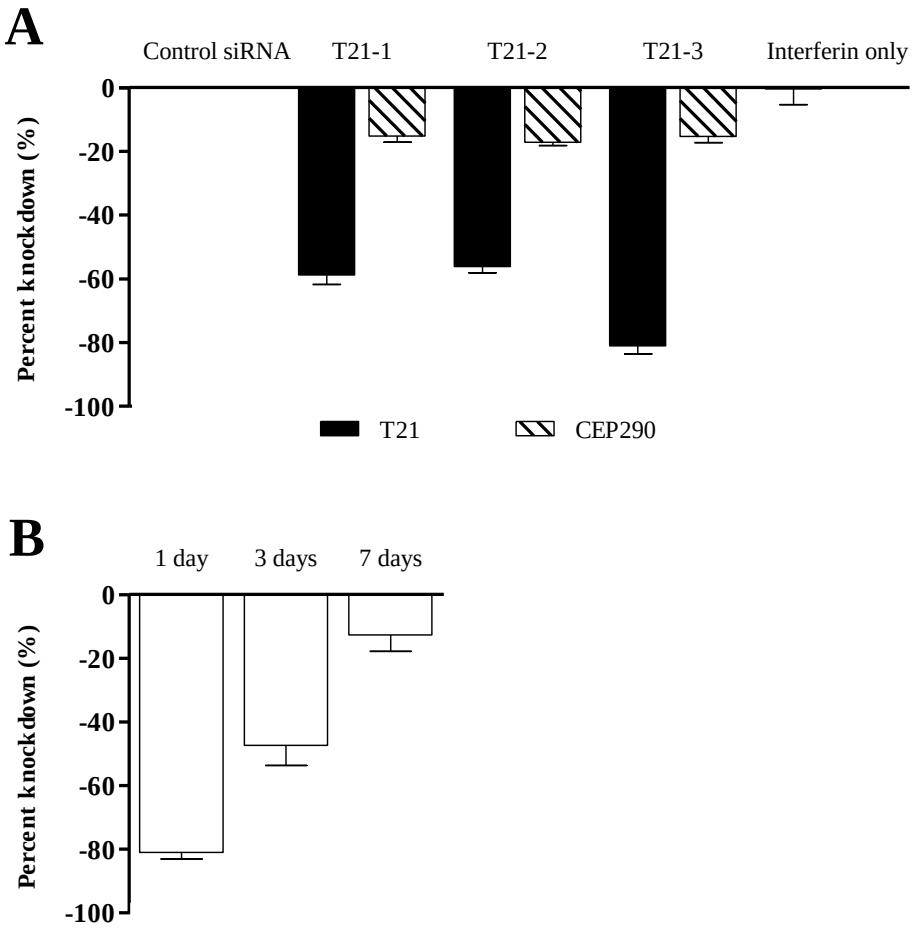


Figure 4.15 – T21 silencing of a T21 positive prostate cancer cell line following T21 specific siRNA transfection. *(**A**) Real time PCR analysis showing percentage of T21 and CEP290 mRNA knockdown following transfection of PC3 cell line with 3 T21 specific siRNA. (**B**) Time course performed by real time PCR showing T21-3 siRNA 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 control siRNA.*

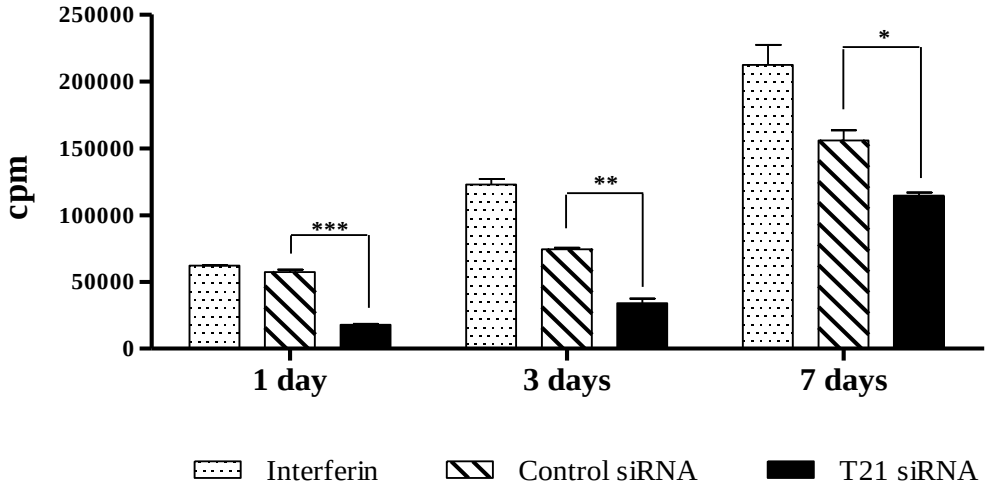


Figure 4.16 – Cell proliferation assay following T21 specific siRNA transfection. *Time course carried out using ^{3}H-Thy incorporation assay demonstrating reduced PC3 cell proliferation observed following T21-3 siRNA silencing over time. Experiments were carried out three times on triplicate wells (n=3) represented with standard error of mean. Statistical significance indicated (*p<0.05) (**p<0.01) (***p<0.001) as determined by Student's t test.*

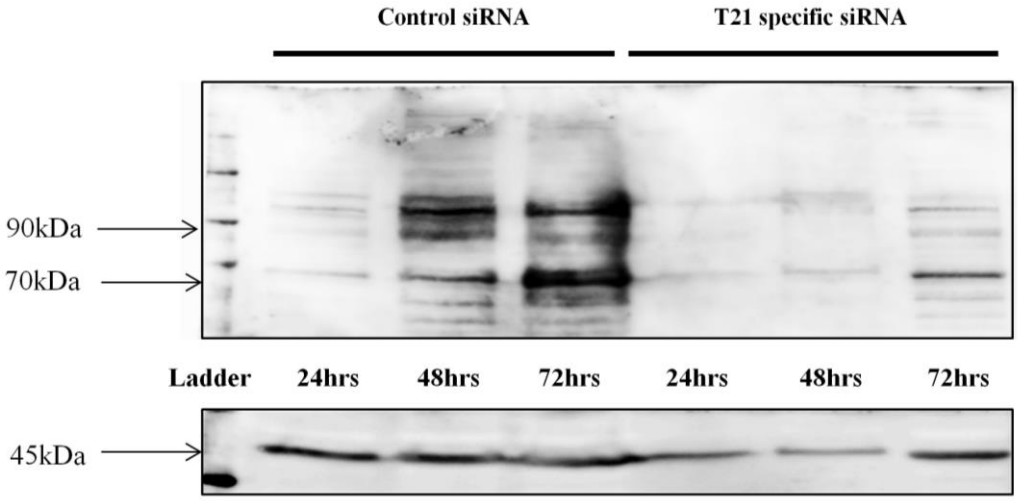


Figure 4.17 – Western blot analysis of T21 protein expression following T21 specific siRNA transfection. *Above, protein expression showing 2 bands (90 kDa and 70 kDa) taken during a time course of 3 days following siRNA transfection using PC3 cell line. Below, β-actin (45 kDa) as representative protein loading control (n=1).*

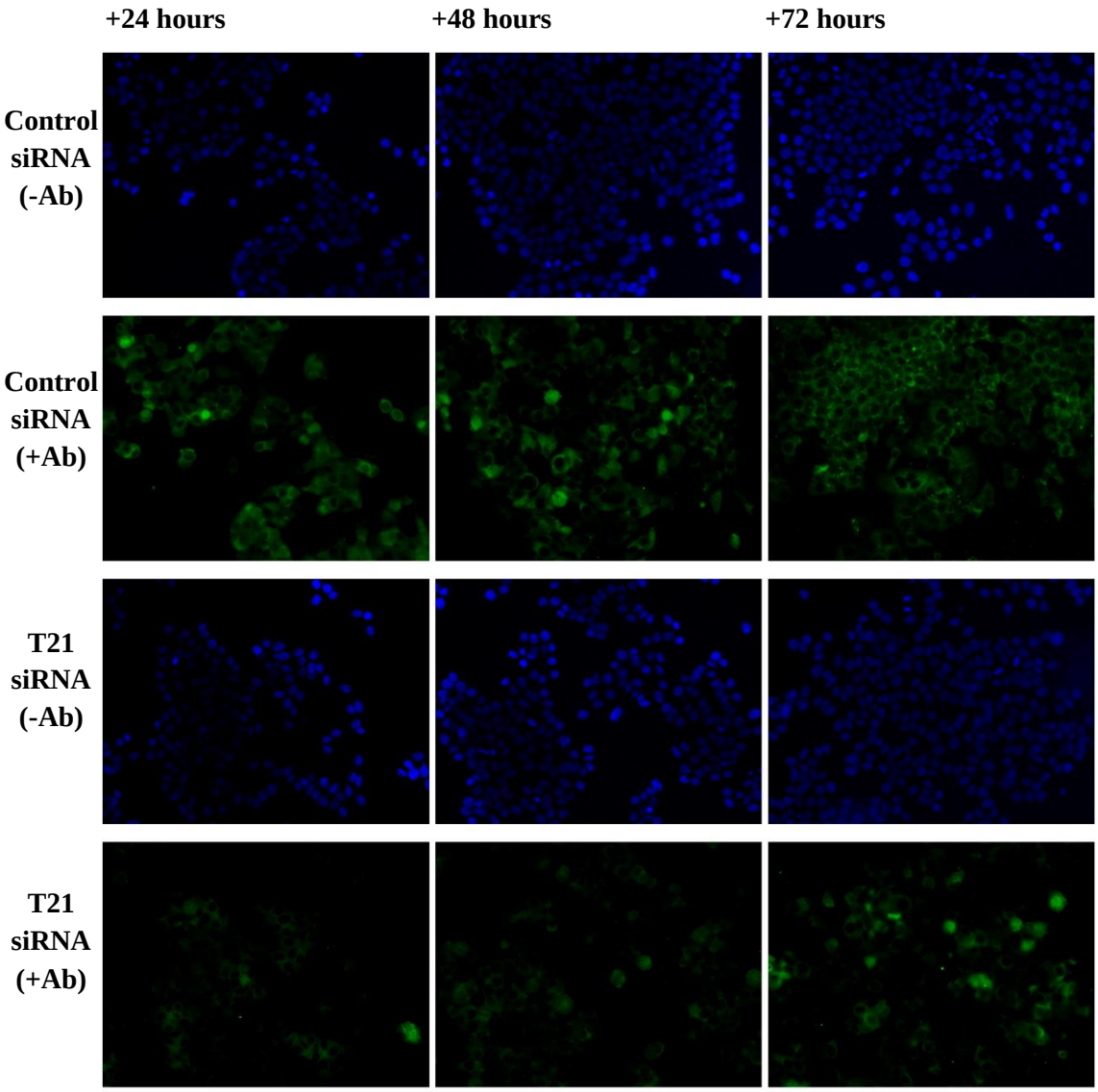


Figure 4.18 – Immunofluorescence analysis showing protein expression of T21 protein expression following T21 specific siRNA transfection. *PC3 cells taken during a time course of 3 days following siRNA transfection were labelled using anti-T21u antibody (green), secondary stained as described previously and with nuclear staining using DAPI (blue). Representative images (n=2) using magnification x20.*

Gene silencing through transfection of interfering RNA by siRNA duplexes or through transcriptionally expressed shRNA can lead to the inadvertent stimulation of interferon response-pathways (Bridge *et al.*, 2003). The level of mRNA expression of interferon-response genes, signal transduced and activator of transcription 1 (*STAT1*) and oligoadenylate synthase 1 (*OAS1*) were assessed and demonstrated no statistical changes between control and siRNA knockdown. This confirmed that the effects observed were *T21*-specific (data not shown).

Collectively, these findings indicated that T21 may not function in the same context as CEP290 within the centrosomes, however the associated reduced proliferative capacity of cells following transient silencing of *T21* indicate a role in driving cell growth. Therefore it was hypothesised that T21 activity may be important during the cell cycle. To assess this further, PC3 cells were once again subjected to siRNA treatment for 24, 48 and 72 hours. During the final two hours of incubation, cells were washed and incubated with 5-ethynyl-2´-deoxyuridine (EdU) and carefully harvested using trypsin/versene and fixed before proceeding to fluorescent staining for EdU (Click-iT EdU cell proliferation assay) and live cells (LIVE/DEAD cell stain) (both purchased from Invitrogen, UK). Cell fluorescence was determined using a Gallios flow cytometer and data obtained analysed using the Kaluza Flow Analysis Software (Beckman Coulter). The results summarised in Table 4.4 showed that in cells treated with *T21* siRNA there was a consistent reduction in cells undergoing G phase of the cell cycle and an increase in G_2/M phase transition compared to the equivalent control siRNA with little percentage variation observed during S phase between the *T21* siRNA and control siRNA treated cells. Due to time and reagent constraints this experiment was performed once. Therefore, the results presented here are preliminary and would require further repetition to gain more conclusive insights.

		Cell cycle phases (% cells)		
		G phase	**S phase**	**G_2/M phase**
+24 hours	**siRNA control**	**63**	**11**	**26**
	siRNA T21	**56**	**14**	**30**
+48 hours	**siRNA control**	**63**	**8**	**29**
	siRNA T21	**60**	**9**	**31**
+72 hours	**siRNA control**	**68**	**6**	**26**
	siRNA T21	**60**	**7**	**33**

Table 4.4 – Cell cycle analysis following T21 specific siRNA transfection. *Time course experiment carried out over 72 hours following siRNA transfection of PC3 cells using T21 specific siRNA and control siRNA (n=1). Cell cycle phases of live transfected cells detected using LIVE/DEAD Cell Stain (Invitrogen) coupled with Click-iT EdU cell proliferation assay (Invitrogen).*

The opportunity arose to study whole genome expression in response to knockdown of *T21* using Next Generation Sequencing (NGS). Briefly, cells treated with either control or *T21* siRNA were lysed directly using RNA STAT-60 and total RNA was extracted using isopropanol precipitation (described in section 2.2.2.1). The RNA quality was assessed using a RNA 6000 pico chip kit (Agilent) and an Agilent 2100 Bioanalyzer (Agilent)

following the manufacturer's instructions. RNA samples with RNA integrity number (RIN) values between 8 and 10 were stored until analysis by NGS. RNA samples with RIN values below 8 were discarded. Next generation sequencing of total RNA samples were outsourced to Source Bioscience, UK. Briefly, 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 clusters 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 UK. Technical repeats were merged and logarithmic (fold-change) values and p values were calculated to determine significant differences. Threshold cut off of values above (up regulated expression) and below (down regulated expression) 1.5 fold-change were applied to determine significant gene regulation changes. Functions of genes undergoing significant changes of expression were then obtained using online databases such as OMIM (http://www.ncbi.nlm.nih.gov/omim/), Uniprot (http://www.uniprot.org/), and GeneCards (http://www.genecards.org/). Although a complete bioinformatics analysis and experimental validation of gene expression is currently still ongoing, genes that were either significantly up regulated (448 genes) or down regulated (434 genes) following *T21* siRNA knockdown was complied. Following a more focused examination of gene functions and literature searches, a more refined shortlist of 20 genes that had roles in the processes involved cancer were chosen to most suitability illustrate the general changes in mRNA expression (Table 4.5).

The absence of genes that are known to interact with CEP290 from the data provides further evidence that T21 may not share a common centrosomal function. The data indicated the up regulation of genes that are involved in the promotion of tumourogenesis through cell proliferation and cell cycle control (*MAPK6*, *PKIA*, *UHMK1*, *CDKN2B*), overriding DNA damage pathways and promoting cell survival (*HIPK2*, *MDM2*, *RBBP8*), and genes that modulate immune responses (*CISH*, *PLAU*, *IL6R*). The up regulation of genes such as *SYCP3*, *CALM3*, *MFAP2* and *RAB3IP* may be indicative of a more specific role T21 plays in centrosomal and structural rearrangement during mitosis. Conversely, genes that were suppressed played regulatory roles involved in tumour progression through regulation of Ras family (*DOK1* and 3 *RAB6B*, *FRS3*), cell stress/DNA repair pathways (*PPM1E*, *CHD8*, *XRCC3*) or the PI3K-Akt pathway (*PI3KIP1*).

Genes up regulated when T21 is expressed:

Gene	Pathway / function		FC
MFAP2	Structural	Interaction with notch-1 inducing signalling	50
CYP2D6	Metabolism	Drug metabolism, alterations/polymorphisms associated with cancer	30
IDI2		Interaction with BCL 6	15.9
HYAL1		Concentration dependent inhibitor or promoter of tumour growth	5.1
CISH	JAK/STAT pathway	Negative regulator of cytokine signalling through STAT5 and implicated in immune regulation of T and NK cells	3.4
SPAG17	Unknown	Sperm-associated antigen 17 –CT antigen and potential immunotheraputic target	2.9
PLAU	Pro-tumour activity	Involved in degradation of the ECM and possibly involved in tumour cell migration and proliferation. Inferred association with Treg suppression	2.2
MAPK6	Promotes cell cycle	Ser/Thr protein kinase linked to promotion of entry in the cell cycle	2.1
CALM3		Regulates the centrosome cycle and progression through cytokinesis	2.1
PKIA		Inhibitor of cAMP-dependent protein kinase activity	2.1
HIPK2	DNA damage	Inhibits cell growth and promotes apoptosis through p53 target genes	2
IL6R	Cell growth	Regulates cell growth and differentiation with a role in the immune response	2
RAP2A	Ras pathway	RAP2, member of RAS oncogene family	1.9
SYCP3	Promotes cell cycle	Synapsis, recombination and segregation of meiotic chromosomes	1.9
UHMK1		Promotes cell cycle progression in G1 phase	1.9
CEP290		Activates ATF4-mediated transcription	1.9
CDKN2B		Cell growth regulator involved in controlling cell cycle G1 progression	1.8
MDM2	p53 pathway	Leads to inactivation of p53, diminishing its tumour suppressor function	1.8
RBBP8	DNA damage	May itself be a tumour suppressor acting in the same pathway as BRCA1	1.8
RAB3IP	Structural	Modulates actin organisation	1.8

Genes down regulated when T21 is expressed:

Gene	Pathway / function		FC
SPATA21	Unknown	Involved in the differentiation of haploid spermatids	-19
CRB2		May play a role in polarized cells morphogenesis associated with cancer	-5.2
BST1	Immuno-modulatory	Involved in pre-B-cell growth	-3.4
DOK3	Ras pathway	Docking protein 3 - negative regulator of Ras	-3.4
BCL2L12	Apoptosis	BCL family act as anti- or pro-apoptotic regulators	-2.7
DOK1	Ras pathway	Docking protein 1 - negative regulator of Ras and TLR4	-2.6
PDZD2	Immune activation / pro–tumour activity	Upregulated in primary Pr tumours, possible role in the early stages of Pr tumourogenesis	-2.3
FGF1		Involved in the regulation of cell survival, division, angiogenesis, differentiation and migration	-2.1
RAB6B	Ras pathway	RAB6B, member RAS oncogene family	-2
FRS3		Function in linking FGF receptor stimulation to activators of Ras	-1.9
PIK3IP1	PI3K-Akt pathway	A negative regulator of PI3K which promotes cell proliferation and survival	-1.9
OAS2	Apoptosis	Roles in mediating resistance to virus infection, control of cell growth, differentiation, and apoptosis	-1.9
PPM1E	Cell proliferation	Negative regulators of cell stress response pathways	-1.9
CHD8		Suppresses p53 - mediated apoptosis, inhibitor of Wnt signalling pathway	-1.8
XRCC3	DNA repair	Involved in the homologous recombination repair (HRR), thought to repair chromosomal fragmentation, translocations and deletions	-1.8
GGT7	Metabolic	Possibly involved in lung carcinogenesis	-1.8
ZBTB7A	Cell cycle	Specifically represses the transcription of the CDKN2A gene	-1.8
TRIM2	Cell survival	Blocks apoptosis response to ischemia by degrading BIM	-1.8
TUBA3D	Structural	Involved in motility and spindle formation	-1.8
SH3GL1		Overexpression may play a role in leukemogenesis	-1.8

Table 4.5 – Select list of genes following siRNA transfection. *A representative list of 20 up- and 20 down-regulated genes following Next Generation Sequencing analysis following 24 hour transient siRNA transfection of PC3 cells. Gene selection was based on a minimal 1.8 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.*

4.2.4.2 Stable gene silencing of T21 using short hairpin RNA

While it is useful to examine the effects of transient suppression of *T21* using siRNA interference, it is necessary to study what effects long term silencing could have. One approach is to use short hairpin RNA interference to stably transfect cells to endogenously suppress gene expression. Hence, the siRNA sequence that induced the most significant knockdown (*T21-3*) was chosen to form a custom pGFP-B-RS mammalian expression vector containing the *T21*-specific sequence (purchased from Origene Technologies) along with a scramble cassette control vector. Next, PC3 cells were transfected with either *T21*-specific shRNA or control vector as detailed in section 2.2.6.2. Transfected cells were grown in media supplemented with Blastcidin (selective antibiotic) over one passage before single colonies were achieved through limiting dilution assays. Once cells were sufficient in number, mRNA was extracted and reverse transcribed to generate cDNA in order to assess for the expression of *T21* using RT-Q-PCR (Figure 4.19). Next, expanded colonies that demonstrated the most stable significant knockdown compared to control cells were sorted for GFP^+ expressing cells using a MoFlo XDP High-Speed Cell Sorter (Beckman Coulter) and returned to culture prior to confirming *T21* mRNA expression (Figure 4.19). As found in earlier studies using transient transfection, stably transfected cells exhibited a reduced proliferative capacity following knockdown of *T21* in comparison to cells transfected with control vector (Figure 4.20). Finally, the expression of T21 protein in transfected cells was assessed using anti-T21u antibody by both western blotting (Figure 4.21) and using immunofluorescence (Figure 4.23). Together these experiments concluded that T21 protein expression was indeed decreased after shRNA transfection, however the effects of the silencing where short-lived returning T21 expression to that of the control expression after a number of passages. This may require further refinement of the antibiotic concentration, timing of fresh antibiotic supplements to the media and further cell sorting to establish a more stably transfected cell line (ongoing work).

As an interesting side note, retrospective western blot analysis performed using the anti-T21/CEP290 antibody on PC3 cells transfected with *T21* shRNA demonstrated the reduction of protein detected in a band at approximately 90 kDa when compared to control. This further would appear to confirm reduced T21 protein expression as a result of T21 specific knockdown. Therefore, this antibody could also be used to detect T21 protein

expression using western blotting technique in addition to the anti-T21u antibody (Figure 4.22).

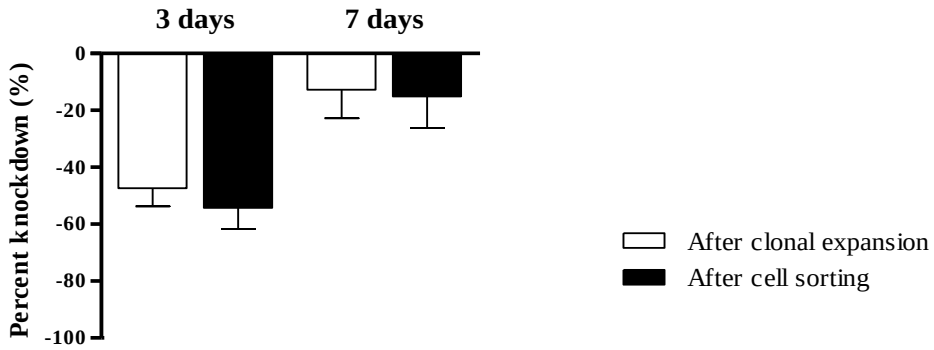


Figure 4.19 –T21 stable knockdown of PC3 prostate cancer cell line following T21 specific shRNA transfection. *Following shRNA transfection, cells underwent clonal expansion via limiting dilution. One clone was selected for further sorting of GFP-positive cells. Real time PCR analysis showing percentage of T21 knockdown following transfection of PC3 cell line with pGFP-B-RS vector containing T21 shRNA showing T21 silencing over time. Experiments were carried out twice in duplicate (n=2) represented with standard error of mean with data expressed as percentage change to the control shRNA.*

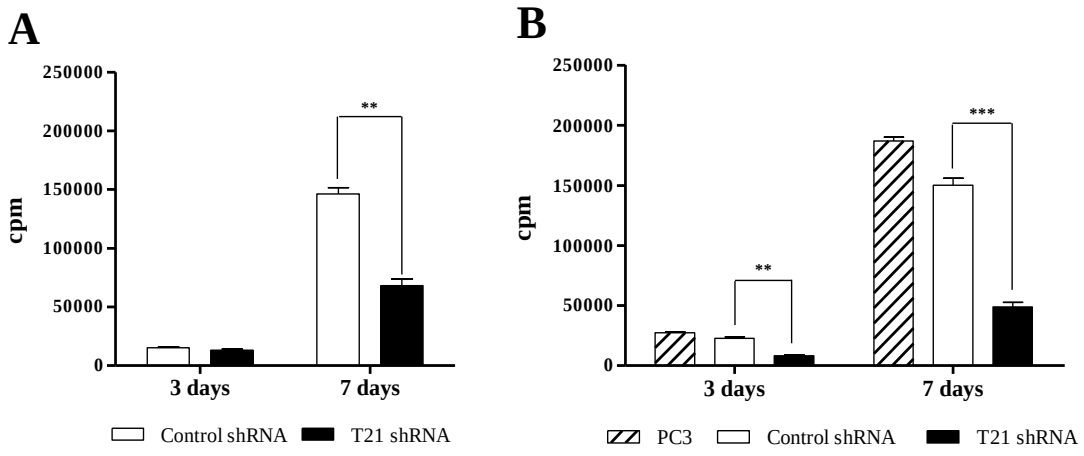


Figure 4.20 – Cell proliferation assay following T21 specific shRNA transfection. *Time course carried out using a ^{3}H incorporation cell proliferation assay demonstrating reduced PC3 cell proliferation observed following T21 shRNA silencing (**A**) and after cell sorting (**B**). Experiments were carried out three times on triplicate wells (n=3) represented with standard error of mean. Statistical significance indicated (**p<0.01) and (***p<0.001) as determined by Student's t test.*

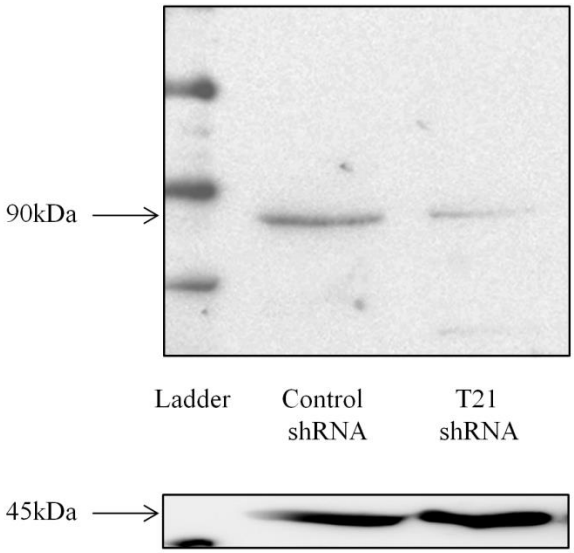


Figure 4.21 – Western blot analysis of T21 protein expression following T21 specific shRNA transfection. *Protein expression following 72 hours post-T21 shRNA transfection of PC3 cell line compared to control cells. Below, β-actin (45 kDa) as representative protein loading control (n=2).*

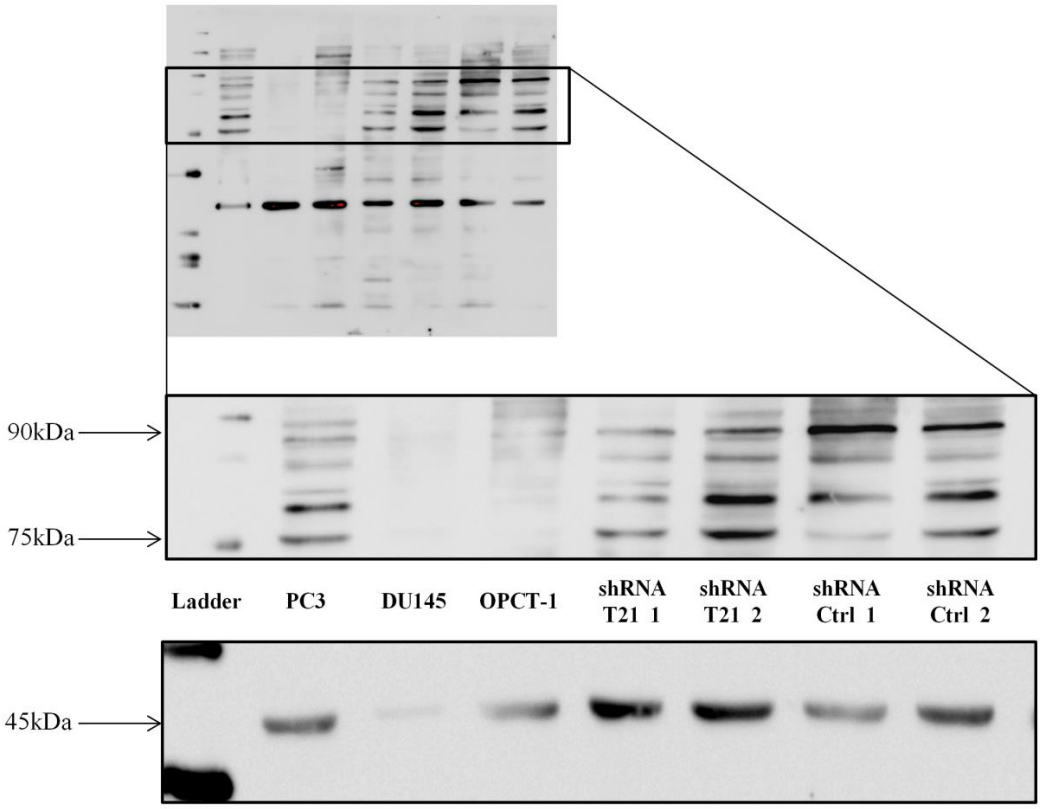


Figure 4.22 – Western blot analysis of T21/CEP290 protein expression following T21 specific shRNA transfection. *Protein expression observed at 90 kDa following 48 hours post T21 shRNA transfection of PC3 cell line compared to control cells. Below, β-actin (45 kDa) as representative protein loading control (n=2).*

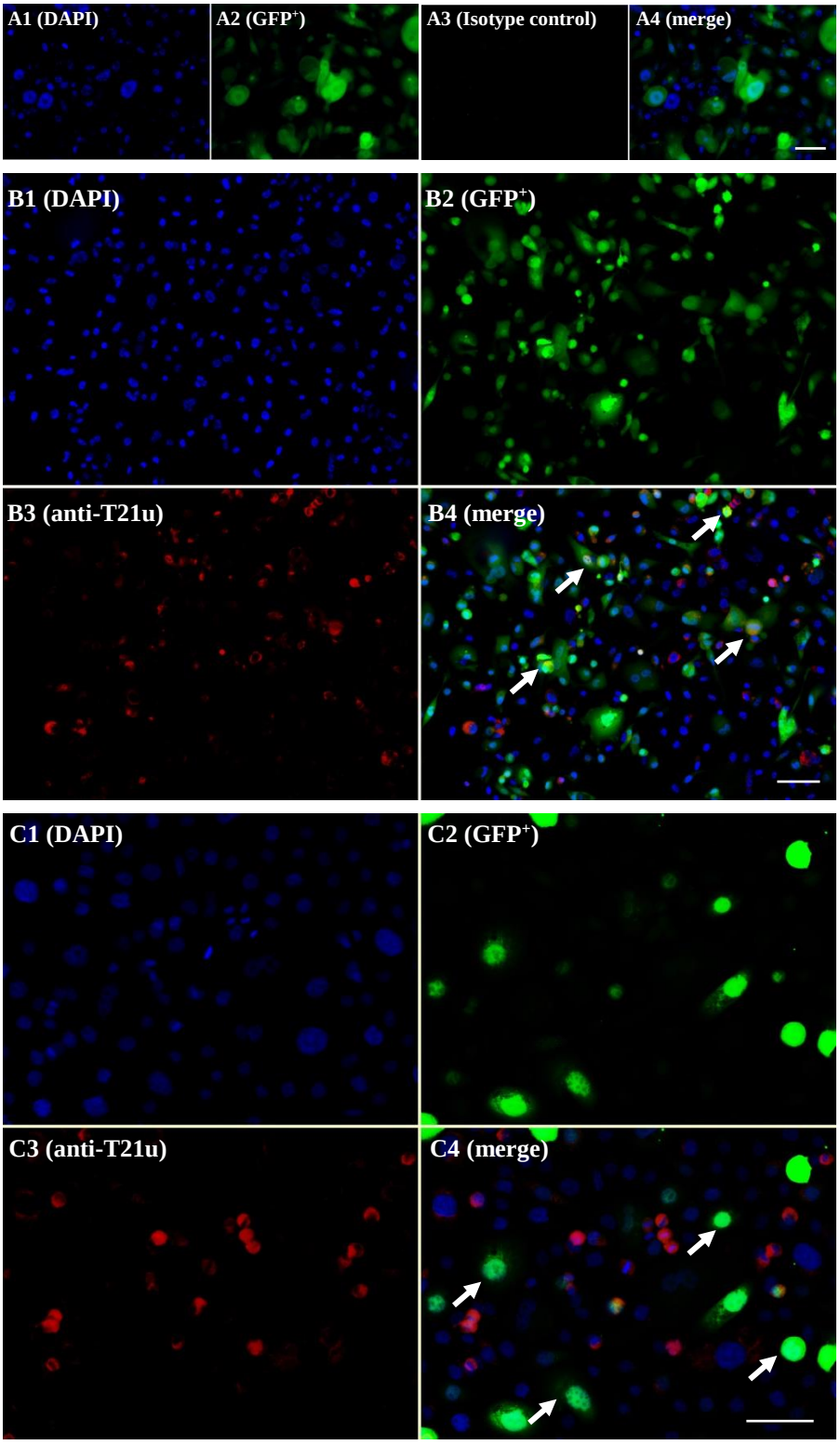


Figure 4.23 – Immunofluorescence images illustrating T21 protein expression after shRNA silencing of PC3 cells. *Immunofluorescence image composites of PC3 cells showing absence of co-expression of T21 protein and GFP following 5 days after T21 specific shRNA transfection (**C**) when compared to control shRNA transfection (**B**) performed using anti-T21 antibody (red). Transfection of constructs observed via expression of GFP (green). The absence of T21 staining was observed using isotype control (**A3**).*

4.3 Discussion

4.3.1 Molecular expression profiles of T21 using human tissues

After previous investigations into T21 protein failed to reveal the true extent of expression in normal and cancer tissue, it has until now remained unclear whether T21 holds potential as a biomarker of disease or has any viability as an immunotherapeutic target. As a consequence, little is known as to what function, if any, T21 may have that could contribute to tumourogenesis. The identification of the unique region within T21 presents the opportunity to study T21 expression without detecting CEP290. Carefully designed *T21* specific oligonucleotides in addition to the anti-T21u antibody within the unique region allowed re-examination of T21 in human cancer tissues and cell lines to be performed. Initially, *T21* mRNA expression was assessed in normal healthy tissues using both semi-quantitative and quantitative PCR. Experiments demonstrated that *T21* mRNA expression was not limited to testis tissue; in fact many tissues demonstrated comparatively high levels of mRNA expression compared to testis, for example normal prostate, stomach and spinal cord tissue. Moreover, protein expression measured in normal healthy individual tissue TMA demonstrated an overall absence or mild expression with except to some tissues (kidney, prostate and testis) showing moderate levels of expression. None of the normal tissues stained for anti-T21u antibody demonstrated strong expression of T21. These results demonstrated that the expression of *T21* at the RNA level was not always consistent to the protein expression observed and that RNA and protein from the same patient tissue would be necessary to confirm these findings. What was apparent from these results was that although T21 was seemingly distributed throughout many normal tissues, the level of expression remained low with exception of normal prostate. This suggests that T21 could be tentatively categorised as a tissue-specific unique TAAs.

As T21 was first identified and verified as a novel TAA by immunological probing of patient sera taken from advanced prostate cancer, the opportunity to study its expression pattern in prostate cancer progression represented an obvious study. Tissue sections taken from benign prostatic hyperplasia (BPH) and clinically confirmed prostate cancer patients were stained using the anti-T21u antibody. As observed with IHC analysis described above, T21 protein was localised largely throughout the cytoplasm and in some instances

to the nuclei and cell membrane. Careful observation confirmed no pattern of centrosomal localisation with T21 as would be expected with CEP290 staining. This TMA series demonstrated no statistical significance in the expression of T21 protein between benign (41.3%) and malignant disease (58.7%). Although the staining intensity distribution did not show differences among the strong staining, there were differences in staining distribution between mild and moderately staining intensities; 48.5% of malignant tissues were categorised as moderately positive compared to 28% of benign tissues. The differences between mild and moderate staining which was observed suggests a subtle increase in T21 expression in malignant tissues compared to benign. Further statistical analysis of the prostate cancer TMA series concluded that there are no significance difference with age, survival, PSA, death and Gleason grade. The limited number of patients within groups made it difficult to conduct a full statistical analysis, therefore intensities were grouped and categorised into high and low expression. Perhaps as a consequence, the subtle observations between mild and moderate staining intensities in prostate cancer patients were missed. Further expansion of the TMA series to include more patients should be performed, in addition to cell localisation analysis and in parallel with prostate specific disease marker expression (PSA, PAP, PSMA and alpha-methyl-CoA-Racemase), loss of tumour suppressor expression (p53, p63, PTEN, BRCA genes), overexpression of oncogenic proteins (TMPRESS2:EGFR fusion, PI3K), growth factors and their receptors (TGFβ1 and EGFR) and mitotic markers (Ki67 and PCNA) (Velonas *et al.*, 2013).

The opportunity to screen for mRNA expression derived from various cancer cell lines showed that many cancer cell lines from breast, melanoma and head and neck demonstrated, to varying degrees, T21 expression. Interestingly, normal (immortalised) prostate cell lines (PNTα and PNT2) although positive for T21, showed lower expression compared to prostate cancer derived cell lines (PC3, DU145 and LNCaP). As the latter are derived from secondary tumour sites, a primary prostate cancer cell line (OPCT-1) was also assessed, revealing expression levels similar to that of the normal prostate cell lines. This suggests that T21 expression may preferentially be associated with metastatic disease although further experimental studies would be required to support this hypothesis, for example associating with *in vitro* migration and the drug resistance.

An important question that until now has remained unanswered was the nature of the relationship between T21 and CEP290. Evidence at the mRNA level failed to distinguish any associations in expression and so far western blot analysis concluded a seemingly lower expression of T21 compared to CEP290 in prostate and breast cancer cell lines. A consequence of this finding suggests that previous T21 protein analysis using the anti-T21/CEP290 antibody may have been more likely to show the detection of CEP290 and its other molecule weight variants, overshadowing the rather more modest expression of T21. In order to distinguish the two proteins T21 was further analysed using the T21 positive prostate cancer derived cell line, PC3 by dual immunofluorescence staining. Interestingly, staining with anti-T21u antibody and anti-CEP290 antibody (Everest Biotech) revealed that T21 does not share a localised pattern of cellular expression, moreover no centrosomal localisation with CEP290 was observed. This observation was further corroborated using pericentrin, a centrosomal protein confirming that T21 does not share a functional role with CEP290 at the centrosomal bodies during cell division.

4.3.2 Functional investigation of T21

The second objective involved conducting a series of experiments using immunofluorescence protein detection and gene silencing techniques designed to determine whether T21 and CEP290 share functional characteristics and attempted to give an insight into their relationship and T21 involvement in cancer progression. Firstly, three siRNA oligonucleotides designed within the unique region of *T21* were synthesised and transfected into PC3. All three siRNA led to the knockdown of *T21* with *T21-3* achieving the most significant knockdown in expression of both mRNA and protein between 24 hour and 72 hours post-transfection. Transfection using RNA interference can be subject to off-target stimulation of interferon response-genes (Bridge *et al.,* 2003), therefore mRNA levels of two interferon-response genes (*OAS1* and *STAT1*) were measured to ensure the responses observed were in fact gene-specific, which proved to be the case (data not shown). No noticeable changes occurred in cell morphology; however cells treated with *T21* siRNA were seemingly hindered in their capacity to proliferate. Further proliferation assays confirmed this observation. Later, siRNA transfection experiments coupled with cell cycle analysis revealed that although cells do not seem to be inhibited at S phase, a larger percentage of cells were arrested at the G_2/M phase transition compared to the

equivalent control cells. These findings go some way to explain why PC3 cells stained with anti-T21u antibody and Ki67 failed to selectively co-express during cell division or the centrosome cycle. As with CEP290, Ki67 is expressed throughout all active stages of the cell cycle whereas T21 may in fact only be highly expressed and/or activated during a short phase of the cycle. Further experiments using cell cycle markers for G_1/S phase (cyclins A and D) and G_2/M phase (cdc2-cyclin B and phosphohistone H3) transitions would be needed to further elucidate the role that T21 may play in cell proliferation. In addition to knockdown experiments, a cell line which expresses little or no *T21* (such as PCI30) would be important in order to demonstrate the role of T21 in proliferation. Reverse experiments in which transfection of a T21 cDNA into cells should theoretically lead to an increase in cell proliferation. Meanwhile, further knockdown experiments were performed using *T21* specific shRNA transfection, which could be employed to study what long term effects arise from silencing of *T21* expression. Stable silencing of *T21* in cells treated with shRNA do however require further refinement of cell line procedure to achieve a stable knockdown over several passages (work ongoing).

The analysis of selected genes that are either up or down regulated in the presence of *T21* provided yet further evidence that T21 may not share common protein functions with CEP290. The results did however indicate an up regulation of genes that promote tumourogenesis 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. Conversely, genes involved in regulatory pathways were suppressed in the presence of *T21* and included *RAS* family members, cell stress/DNA repair pathways the PI3K-Akt pathway. Although a full and through analysis is essential to elucidate pathways that may involve T21 activity (work ongoing), the data presented here indicate that T21 appears to be involved in promoting tumourogenesis and therefore could represent an attractive target for therapy.

CHAPTER 5

INVESTIGATING THE IMMUNOLOGICAL POTENTIAL OF T21

5.1 Introduction

Since the 18th century, vaccines have been successfully utilised to protect against otherwise lethal diseases and have been responsible for the eradication of small pox reported by Fenner *et al.*, 1988 and more recently, WHO initiatives have reduced annual cases of polio to just over 200 as well as countering other infectious diseases. The application of vaccines against viruses known to be involved in certain cancer have also proved extremely successful, however vaccines used to treat established tumours have remained incurable in this manner. This is partly due to their lack of immunogenic potency of the antigen used or suppression mechanisms elicited by the tumour. Since the first reported human tumour associated-antigen (TAA) (van der Bruggen *et al.*, 1991) coupled with the knowledge that T cells respond through recognition of short peptide sequences bound to MHC molecules, various strategies have been developed to identify further TAAs and assess peptides for their immunogenic potential for their ultimate inclusion in therapeutic vaccines against cancer. Some reported antigen-based vaccination targets such as MAGE-A3, gp100, tyrosinase, carcino-embryonic antigen, NY-ESO and HER2/neu

have been shown to elicit antigen-specific immune responses with encouraging clinical outcomes (as outlined in Chapter 1).

Current techniques used to identify immunogenic TAA-derived epitopes can be broadly classified as belonging to either "direct" or "reverse" immunological approaches. Techniques that rely on isolating epitopes directly using genetic or biochemical approaches are termed direct immunology procedures. An early example, involved transfection of DNA or cDNA libraries derived from patient tumour cells into cell lines expressing matching MHC molecules and isolating reactive CTL clones generated following *in vitro* screening using patient TILs. The technique was used by Boon and colleagues to identify MAGE-1 (van der Bruggen *et al.,* 1991). The second subgroup of direct techniques relies on acid elution of antigenic peptides either from whole cellular extracts or from those bound to MHC class I complexes on tumour cells. Subsequent tumour derived peptides are purified and fractionated using reverse phase chromatography and assessed for CTL reactivity. Those conferring tumour lysis are sequenced using mass spectrometric techniques (van Bleek & Nathenson, 1990; Falk *et al.,* 1991). Although successful in the discovery of a number of epitopes, these direct immunology approaches could not compensate for the techniques being time consuming and the cellular resources required were too demanding to be considered suitable to employ on a routine basis.

In contrast to the direct approaches, reverse immunology attempts to streamline epitope discovery by screening peptide sequences taken from TAAs identified using either bioinformatics based data mining, serological expression in cancer patients or restricted protein expression in solid tumours. Briefly, candidate proteins are identified as TAAs and proposed as suitable targets for immunotherapy. Then, using predictive *in silico* computer-based algorithms, putative antigenic peptides are selected based on scoring of their high affinity binding to predefined MHC alleles. Following predictive modelling, candidate sequences can be validated for immunological responses experimentally using synthetic peptides. This can include *in vitro* peptide stimulation of PBMCs from tumour patients or healthy donors of the appropriate MHC haplotype in order to expand reactive T cell clones that are capable of recognition and lysis of tumour cells (Viatte *et al.,* 2006). A number of methods for *in vitro* expansion of specific T cells have been employed including multiple simulation of whole PBMC populations using peptide and cytokines, used to ensure survival and proliferation of reactive T cells (Montes *et al.,* 2005) or using peptide-pulsed

PBMC subpopulations (such as APCs) cultured together with either/both $CD8^+$ and $CD4^+$ T cells (Salio *et al.,* 2001) and finally towards overcome the issue of limited patient material, PBMCs can be stimulated with artificial APCs (Dupont *et al.,* 2005). A major disadvantage concerning *in vitro* stimulation of PBMCs cultures, is that in the majority cases, T cells are non responsive or elicit extremely weak responses to tumour antigens most likely due to many antigens identified as belonging to self and are unable to overcome tolerance mechanisms. In addition, difficulties in expanding reactive T cells to the numbers required for assessment and the availability of patient material when screening for multiple peptides can also hinder epitope identification.

An alternative approach which utilises *in vivo* models can be used by engineering mice to express human MHC class I and/or II molecules either with or without "knockout" of their own corresponding MHC molecules. These "humanised" transgenic mice models have demonstrated a capacity to identify both class I and II human HLA restricted viral peptides (Mann *et al.,* 1995; Ressing *et al.,* 1995) and human TAAs (Visseren *et al.,* 1997; Rojas *et al.,* 2005) however, differences in HLA restricted T cell repertoires between human and HLA transgenic mice have been reported (Wentworth *et al.,* 1996) and therefore it should not be simply assume that conserved antigen processing and presentation between the two species always exists (Street *et al.,* 2002). For this reason, newly identified peptide epitopes should be extensively assessed using human T cells from healthy and diseased donors prior to advancing to human trials. Due to the reported high global population frequency of the HLA.A2 (up to 54.5%) and DR1 (up to 24%, depending on ethnic background) (Gonzalez-Galarza *et al.,* 2011), these alleles have been most frequently selected for double transgenic study (Pajot *et al.,* 2004).

The advancement of *in silico* epitope prediction tools combined with both *in vitro* and *in vivo* approaches to determine the immunogenicity of peptide epitopes have been used to identify both MHC class I and II defined peptides from numerous TAAs such as TRP2, gp100, HER2/neu and p53 (Parkhurst *et al.,* 1998; Tsai *et al.,* 1997; Kawashima *et al.,* 1998; Rojas *et al.,* 2005). Despite the continued refinement of predictive algorithm programs, high affinity binding scores do not necessarily translate into highly immunogenic peptides. Another consideration is that T cells with TCRs displaying high affinity binding toward self antigens may indeed have been deleted during early T cell selection to avoid autoimmunity. Furthermore, identifying peptides that have high affinity

binding may not guarantee high avidity T cell responses (Zhong *et al.,* 2013). There is the possibility that prediction models may exclude low-moderate affinity binding epitopes which may provide suitable targets to generate anti-tumour responses.

Self antigens must overcome several immune safeguard mechanisms in order to maximise their desired effect and owing to the majority of TAAs being derived from inherently expressed normal cellular proteins, inducing unwanted immune tolerance can be problematic. Identification and targeting of single epitopes could lead to specific MHC down regulation and the opportunity of immune escape. Exogenous peptides may also bind directly to MHC on non-professional APCs leading to the induction of tolerance mechanisms. Peptide vaccines can be disadvantageous in that MHC class I restriction limits peptides to particular *HLA* haplotypes and not all *HLA* matched patients will therefore have the same degree of relevance to a particular epitope, however strategies to incorporate multiple peptides could circumvent these restriction issues (Salazar *et al.,* 2003).

Refining the reverse immunology approach to integrate the use of synthetic overlapping long peptides (OLPs) taken from TAAs (ranging from over 12 – 50 amino acids in length) which could incorporate multiple epitopes capable of priming both $CD8^+$ and $CD4^+$ T cells may address the issues raised above. In more recent times, long peptides and OLPs have been assessed in a number of cancer trials demonstrating long-lived detection of reactive T cell responses and evidence of prolonged overall survival using peptides derived from HPV in cervical cancer patients (Welters *et al.,* 2008), p53 in ovarian and metastatic colorectal cancer (Leffers *et al.,* 2009; Speetjens *et al.,* 2009), Her2/neu in multiple cancer types (Disis *et al.,* 2002) and Muc-1 in lung cancer (Sangha & Butts, 2007). Unlike short 8 - 10mer peptides, OLPs are not able to bind directly to cell surface MHC receptors and therefore their presentation for specific cell uptake requires correct processing and presentation to MHC by professional APCs which is perhaps why prolonged responses have been observed and not short lived responses which lead it functional deletion of antigen-specific CTL (Toes *et al.,* 1996a) and so avoid the induction of T cell tolerance (Toes *et al.,* 1996b). The delayed clearance of OLPs from the inflamed lymph nodes draining the vaccination site may also lead to increased epitope presentation and clonal expansion of IFNγ producing effector T cells (Bijker *et al.,* 2008; Perez *et al.,* 2010a). Moreover, long peptides that interact with multiple *HLA* alleles compared to short peptides

have broader T cell responses against multiple epitopes, particularly immunodominant, high avidity T cell inducing epitopes which would assist in reducing opportunities of tumour escape. In addition, the presence of multiple epitopes on the surface of the same APCs may indeed further potentiate antigen presentation and support greater activation of $CD4^+$ and $CD8^+$ T cell responses as observed in vaccinations using viral antigens (Shirai *et al.*, 1994; Hiranuma *et al.*, 1999).

In the present study, the use of OLPs to identify endogenously processed immunogenic peptides using both *in vitro* and *in vivo* stimulation was considered the most appropriate approach for the purpose of this investigation. A peptide library consisting of OLPs was synthesised and screened in pools using HLA.A2/DR1 double transgenic mice assessing for generation of immune responses following a series of *in vitro* restimulation procedures involving cell proliferation and IFNγ release (Figure 5.1). Further importance was placed on the newly identified unique region sequence within T21 and prospective peptides were analysed for their endogenous processing using T21 cDNA immunisation. Finally PBMCs derived from prostate cancer patients were chosen to validate T21 specific T cell reactivity to unique region OLPs.

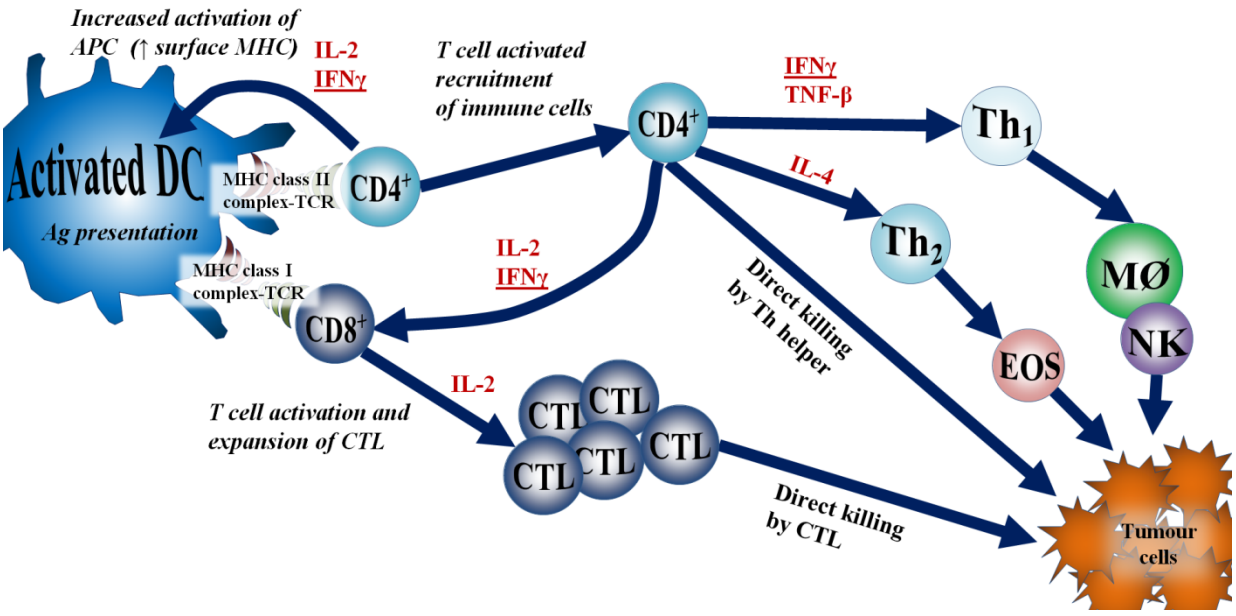


Figure 5.1 – Overview of T cell activation cascade following antigen presentation. *Upon TCR activation, cells initiate signalling pathways resulting in, among other events, clonal expansion of T cells and cytokine secretion which can be measured as T cell proliferation and cytokine release upon in vitro restimulation of T cells using antigen. (MØ=Macrophage, EOS=Eosinophil, NK=Natural Killer cell, CTL=Cytotoxic T Lymphocyte, DC=Dendritic Cell, Th=T helper cells).*

5.2 Results

5.2.1 Identification of immunogenic epitopes from T21 derived peptide library using HLA.A2/DR1 transgenic mice

5.2.1.1 Evaluation of T21 derived peptide immunogenicity using HLA.A2/DR1 transgenic mice

In order to investigate peptide immunogenicity, overlapping long peptides (OLPs) derived from the whole T21/CEP290 protein sequence consisting of 246 peptides, 15mer in amino acid length and overlapping by 10 amino acids were synthesised (Purchased from ProImmune Inc, Florida, USA) and segregated into six groups. The first group (43 peptides) were prioritised for screening for two reasons; primarily, this region was first identified in the discovery of T21 using SEREX (Dr A Miles, PhD Thesis) to elicit IgG antibody responses toward prostate cancer patient sera suggesting that this region would be considered immunoreactive. Secondly, this group of peptides consists of the entire T21 unique region including a CEP290 overlap and therefore provides a logical starting point if the immunogenicity of T21 only were to be investigated (Figure 3.2). These 43 OLPs were randomised and grouped into four pools consisting of 10-11 OLPs (Table 5.1), each group was then separately used to immunise HLA.A2/DR1 double transgenic mice subcutaneously at the base of the tail with a subsequent second round of immunisation with fresh peptide/IFA emulsion after 7 days. A further seven days after the final boost, mice were euthanised and splenocytes harvested and assessed using their corresponding OLP pool for T cell function and cell proliferation.

Following vaccination a number of stimulation assays using *ex vivo* T cells have been utilised to assess T cell mediated cytotoxicity and other immunological responses. The most widely used assay for analysing CTL mediated lysis of is the radioactive chromium (^{51}Cr) release assay developed by Brunner *et al.,* 1968 and has been long since been considered the "gold standard" in measuring CTL mediated killing. More recently, enzyme-linked immunospot assay (EliSpot assay) used to measure secreted cytokines has emerged as a valuable technique of evaluating both CTL frequency and functional responses (Scheibenbogen *et al.,* 2000). The EliSpot assay also has the added benefit over ^{51}Cr release assay of requiring less effector cells whilst delivering higher sensitivity and specificity. These techniques have allowed for the efficient screening of several peptides at

once for their ability to generate peptide specific T cell responses using pre-clinical animal models. Further modifications of current *in vitro* restimulation methodologies would be required when using longer peptides (>10mer). If for instance the internal antigen epitope is unknown, it can become problematic to restimulate target cells using long peptides to assess direct CTL mediated killing. Hence for the purpose of this study, three methods of *in vitro* restimulation were employed to generate T cell responses measured by way of T cell production of cytokine (IFNγ release EliSpot assay) and corresponding T cell proliferation (^{3}H-thymidine incorporation assay) in each instance.

Method 1 - Ex vivo restimulation: Following harvesting of splenocytes from immunised animals, cells were dispensed directly at a density of either $1x10^6$ or $5x10^5$ into nitrocellulose bottomed 96-well plates pre-coated with IFNγ capture antibody. T cells were directly stimulated using 1 µg/ml of peptide(s) and incubated for 48 hours before addition of IFNγ detection antibody and proceeding to spot development. Culture plates for ^{3}H proliferation assay were prepared in parallel under the same conditions with the exception of assay specific growth media. This method allowed for very minimal *in vitro* T cell manipulation relying on inherent APCs present at the time of harvest from the splenic pulp (Swirski *et al.,* 2009).

Method 2 – Peptide-pulsed LPS blast mediated restimulation: Naive syngenic splenocytes were harvested and cultured in the presence of lipopolysaccharide (LPS) to induce LPS blast cells. These cells were then subject to gamma radiation and pulsed with 1 µg/ml peptides for 1 hour to act as stimulators of primed effector T cells when co-cultured alongside splenocytes harvested from immunised animals. Following 6 to 7 days of culture, cells were harvested and dispensed into assay plates at either $1x10^5$ or $5x10^4$ along with cultured DCs pulsed with relevant peptide(s) at a T cell:DC ratio of 10:1. After 24 hour incubation cells were assessed for specific IFNγ release and cell proliferation as described above.

Method 3 – Peptide restimulation: Immunised splenocytes were harvested and cultured alongside peptide(s) at 1 µg/ml and in the presence of IL-2 for two weeks to stimulate T cell expansion and activation before being seeded at either $1x10^4$ or $5x10^3$ into assay plates alongside cultured DCs pulsed with relevant peptide(s) at a T cell:DC ratio of 10:1. Then,

cells were subjected to assay development after 24 hour incubation as described previously to detect peptide-specific T cell function and cell proliferation.

Each IFNγ release assay experiment was performed in triplicate wells and along with media containing T cells and Staphylococcal Entotoxin B (SEB) super-antigen, a potent stimulator of T cell activation which was used as an assay positive control.

Peptide pool A:

Peptide No.	Peptide Sequence
29	H-RNEELRQELRESRKE-OH
11	H-RDLERSRTVIAKFQN-OH
12	H-SRTVIAKFQNKLKEL-OH
17	H-MKEILQAIKEMQKDP-OH
21	H-ETSLIIPSLERLVNA-OH
8	H-AEQNEFLSRELIEKE-OH
42	H-IINSQNEYLIHLLQE-OH
33	H-QQLAKANLKIDHLEK-OH
32	H-AINYSQQLAKANLKI-OH
41	H-PSSASIINSQNEYLI-OH
34	H-ANLKIDHLEKETSLL-OH

Peptide pool B:

Peptide No.	Peptide Sequence
31	H-ESRKEAINYSQQLAK-OH
28	H-DQLTGRNEELRQELR-OH
6	H-DIELEHHRSQAEQNE-OH
7	H-HHRSQAEQNEFLSRE-OH
20	H-DVKGGETSLIIPSLE-OH
40	H-PDGIAPSSASIINSQ-OH
25	H-AEGIFDASLHLKAQV-OH
43	H-NEYLIHLLQELENKE-OH
26	H-DASLHLKAQVDQLTG-OH
24	H-IESKNAEGIFDASLH-OH
18	H-QAIKEMQKDPDVKGG-OH

Peptide pool C:

Peptide No.	Peptide Sequence
14	H-KLKELVEENKQLEEG-OH
13	H-AKFQNKLKELVEENK-OH
5	H-ECRNADIELEHHRSQ-OH
35	H-DHLEKETSLLRQSEG-OH
3	H-IAQEFLIKEAECRNA-OH
1	H-MSEAQSKNEIIAQEF-OH
10	H-LIEKERDLERSRTVI-OH
19	H-MQKDPDVKGGETSLI-OH
9	H-FLSRELIEKERDLER-OH
15	H-VEENKQLEEGMKEIL-OH
23	H-RLVNAIESKNAEGIF-OH

Peptide pool D:

Peptide No.	Peptide sequence
16	H-QLEEGMKEILQAIKE-OH
4	H-LIKEAECRNADIELE-OH
2	H-SKNEIIAQEFLIKEA-OH
30	H-RQELRESRKEAINYS-OH
36	H-ETSLLRQSEGSNVVF-OH
38	H-SNVVFKGIDLPDGIA-OH
39	H-KGIDLPDGIAPSSAS-OH
37	H-RQSEGSNVVFKGIDL-OH
27	H-LKAQVDQLTGRNEEL-OH
22	H-IPSLERLVNAIESKN-OH

Table 5.1 – Overlapping long peptide pool table used in animal immunisations. *Table showing 43 overlapping 15mer peptides derived from the T21 sequence randomly assigned to pools (A - D) and used in animal studies. Peptides 1 -7 lay within the unique region of T21 highlighted in red.*

Positive responses were determined between peptide pool restimulated and non-restimulated cells using Student's *t*-test. The data demonstrated that statistically, peptide pool C generated a 100% positive response score. Pools A and D overall generated positive responses across 67% of assays and peptide pool B generated 58% positive T cell function and cell proliferation responses (Figures 5.2 - 5.4). Taken together the results revealed that all peptide pools generated varying levels of immune responses with peptide pool C triggered T cell responses that were significant in all three restimulation methods (Figures 5.2 – 5.4). It can therefore be theorised that peptide pool C may contain either, single or multiple immunogenic epitopes, which clearly demonstrated the ability to elicit an immune T cell response in 2/2 animals in all three methods used compared to non-restimulated T cells. Moreover, this pool was able to induce T cell proliferation in response to peptide restimulation with results consistent with IFNγ release (Figures 5.2 – 5.4). The remaining peptide pools were also able to generate responses in both IFNγ release and in cell proliferation; however this occurred at reduced significance and with less consistency between mice replicates, restimulation methods and between assays.

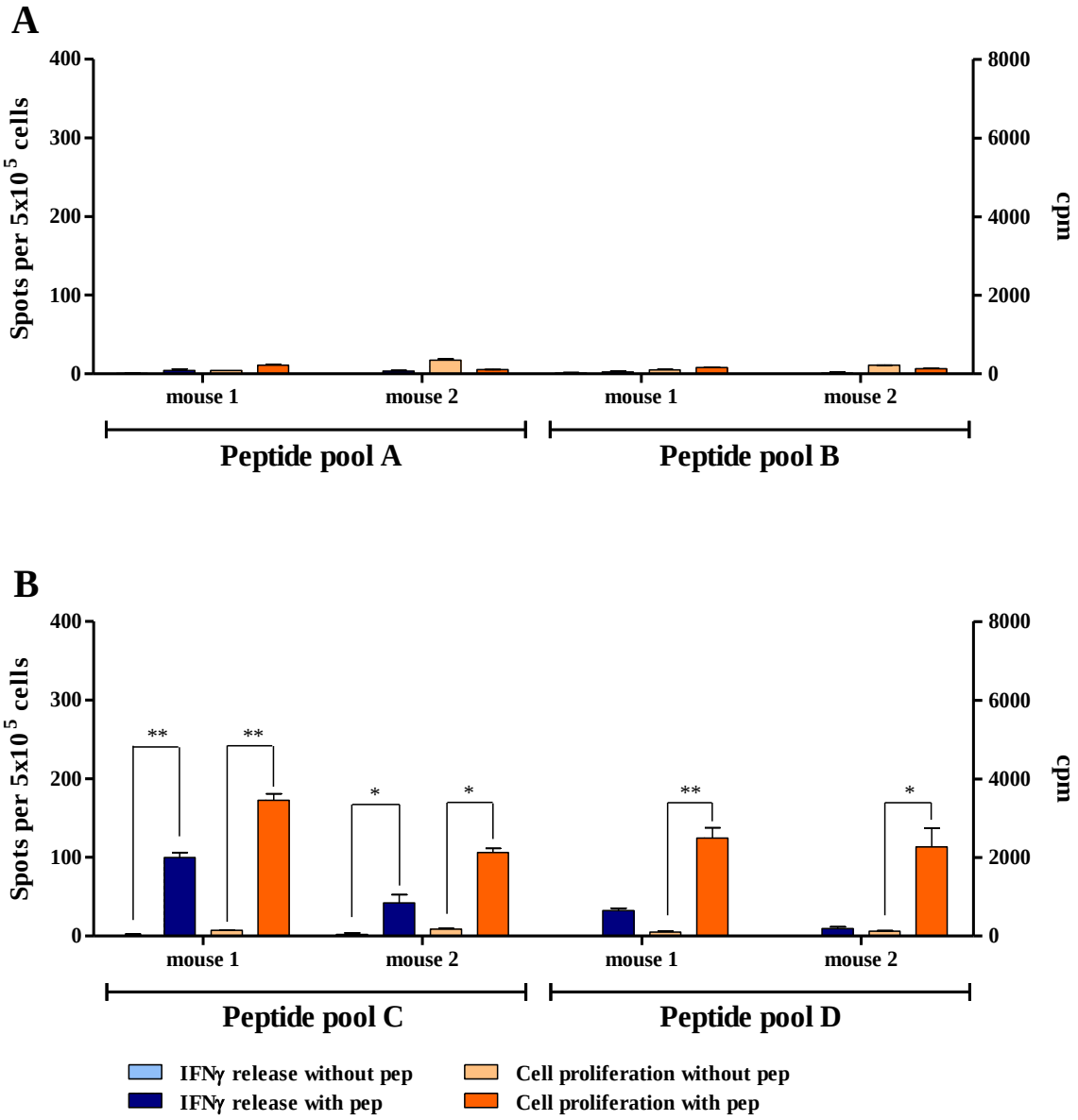


Figure 5.2 – IFNγ release assays and proliferation assays by method 1 using T cells generated from HLA.A2/DR1 transgenic mice immunised with peptide pools A to D (method 1)*. Mice were immunised as previously described and splenocytes harvested for assessment of T cell response to peptide restimulation using IFNγ release EliSpot assay (x-axis) and ^{3}H incorporation cell proliferation assay (right y-axis). Assessments were performed following method 1 of in vitro restimulation of splenocytes using peptide pools with direct restimulation. Experiments were performed once in triplicate represented with standard error of mean between T cells either with or without stimulation using peptide pool, with statistical significance indicated as (*p<0.05 and **p<0.01) as determined by Student's t test.*

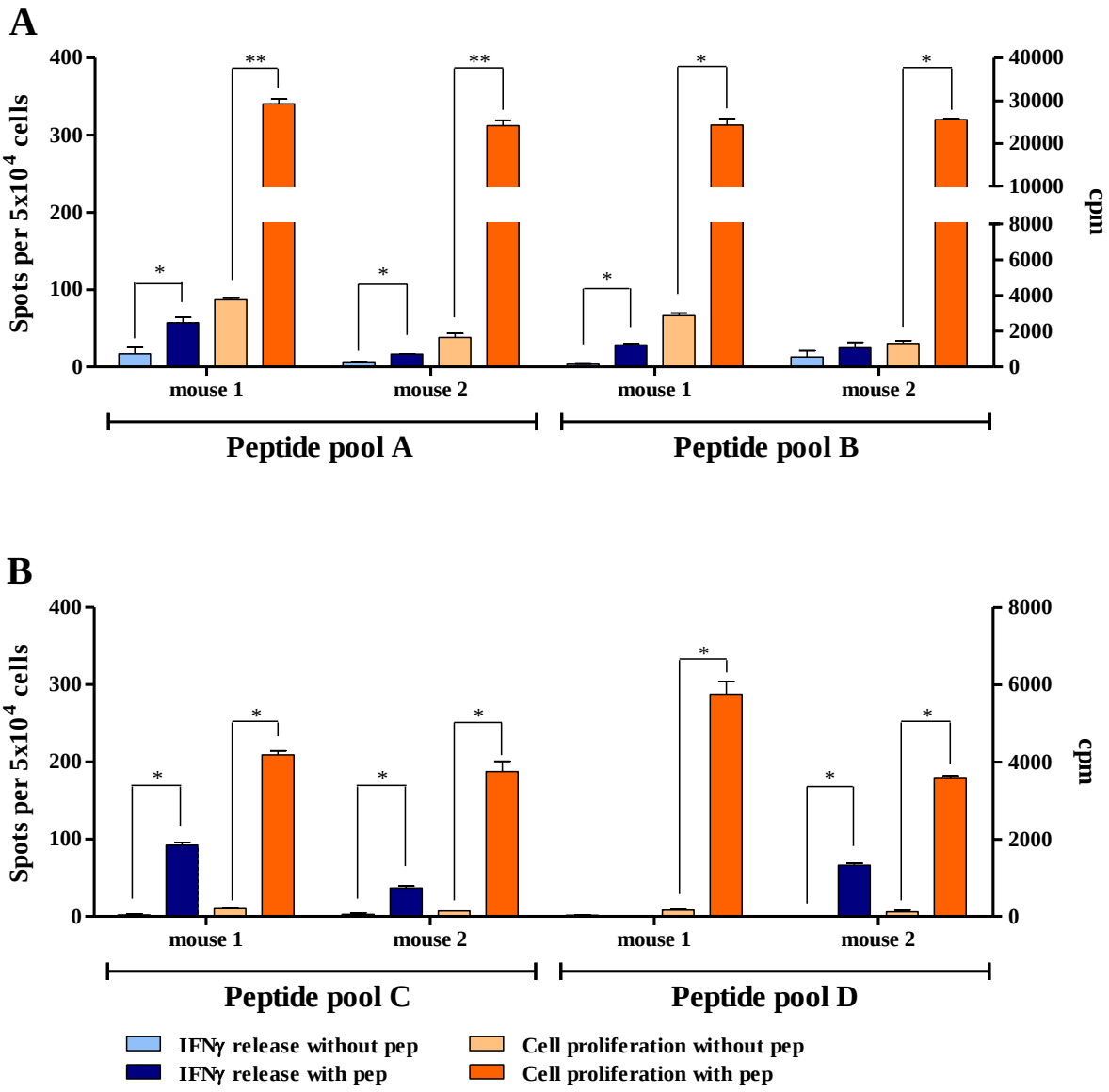


Figure 5.3 – IFNγ release assays and proliferation assays by method 2 using T cells generated from HLA.A2/DR1 transgenic mice immunised with peptide pools A to D (method 2). *Mice were immunised as previously described and splenocytes harvested for assessment of T cell response to peptide restimulation using IFNγ release EliSpot assay (x-axis) and ^{3}H incorporation cell proliferation assay (right y-axis). Assessments were performed following method 2 of in vitro restimulation of splenocytes using irradiated LPS blast and pulsed DCs. Experiments were performed once in triplicate represented with standard error of mean between T cells either with or without stimulation using peptide pool, with statistical significance indicated as (*p<0.05 and **p<0.01) as determined by Student's t test.*

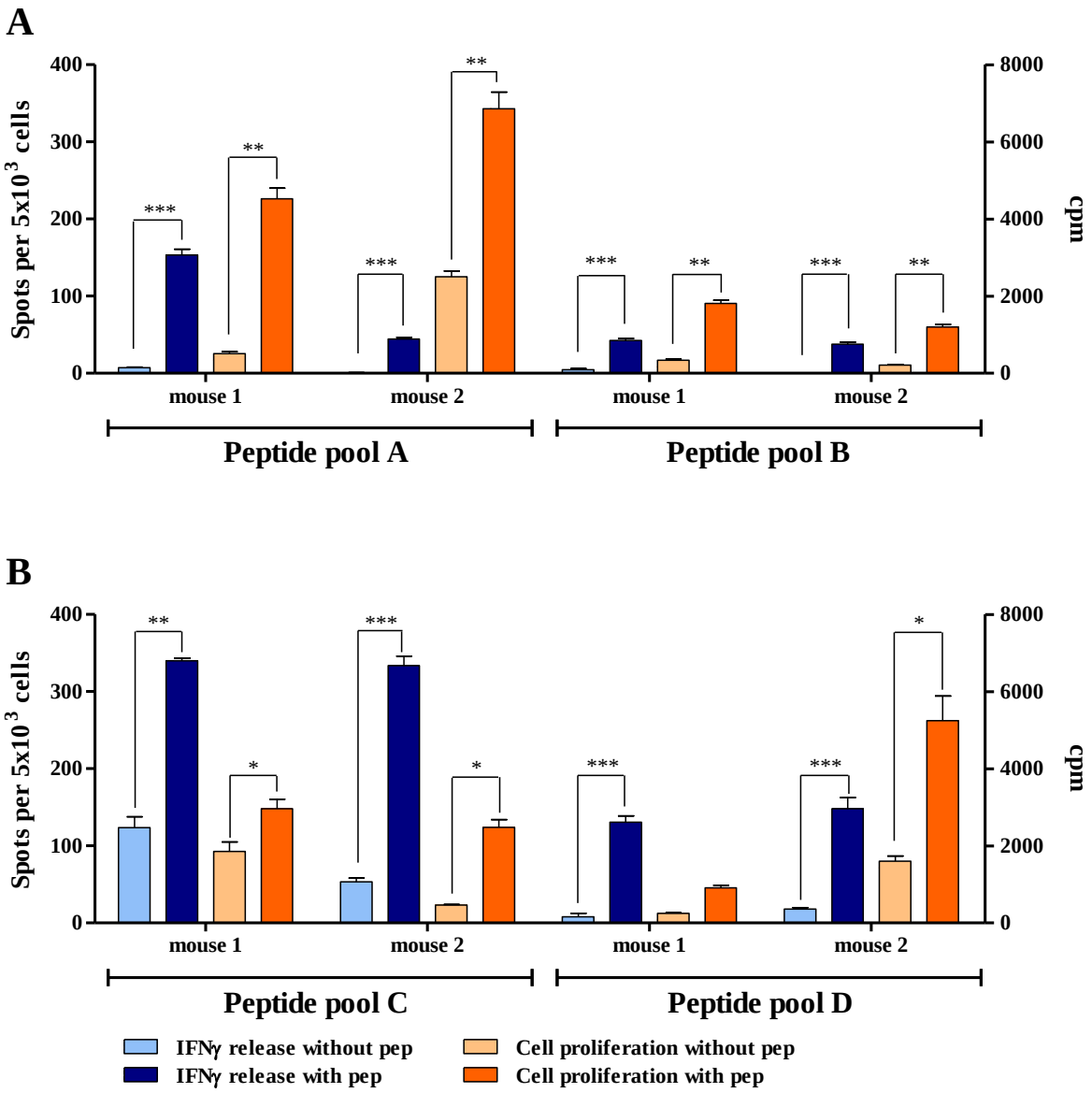


Figure 5.4 – IFNγ release assays and proliferation assays by method 3 using T cells generated from HLA.A2/DR1 transgenic mice immunised with peptide pools A to D (method 3). *Mice were immunised as previously described and splenocytes harvested for assessment of T cell response to peptide restimulation using IFNγ release EliSpot assay (x-axis) and ^{3}H incorporation cell proliferation assay (right y-axis). Assessments were performed following method 3 of in vitro restimulation of splenocytes using peptide pools followed by presentation using pulsed DCs. Experiments were performed once in triplicate represented with standard error of mean between T cells either with or without stimulation using peptide pool, with statistical significance indicated as (*p<0.05, **p<0.01, ***p<0.001) as determined by Student's t test.*

Based on the conclusion of these data, the decision was taken to interrogate peptide pool C further. To this end, peptides derived from pool C were randomly assigned into three subgroups and again assessed for their immunogenicity in HLA.A2/DR1 transgenic mice (n=2) using the three restimulation methods, however this time, using individual peptides to restimulate splenocytes using *in vitro* assays. In these experiments, a 15mer peptide was employed as a control (irrelevant peptide 1 - Seq: SWEKQWYGKAMERAF) to assess positive peptide responses (Table 5.2). To ensure the responses observed were specific to the peptide restimulation, a threshold of 20 spots above the non-restimulated T cells was applied and significance was obtained using Student's t-test measuring T cell responses between pool C specific peptide restimulation and irrelevant peptide 1. Several peptides showed significant responses, elicited using methods two (Figure 5.5A) and three (Figure 5.5B) in the two immunised mice with the strongest and most consistent responses arising from peptide 19 in subgroup C1, peptides 3, 15 and 23 in group C3 and peptide 5 in subgroup C2. On this occasion however, no responses were observed following *ex vivo* restimulation (method one). Further examination of the data by applying a threshold of greater then 2-fold spot change from the control (irrelevant peptide 1) revealed peptides 3, 5, 15, 19, and 23 to generate significant responses to peptide restimulation (Figure 5.6) distinguishing these peptides as being capable of eliciting the most potent responses of all peptides assessed. Positive responses, shown to be statistically significant and demonstrating greater then 2-fold difference over peptide control, are presented in Table 5.2.

It is of particular note that both peptides 3 and 5 are present within the unique region and if determined to be strongly immunogenic, could potentially provide an opportunity as T21-specific targets in immunotherapy (Figure 5.5). The pooled peptide based approach for screening OLPs has demonstrated that T21 specific T cell responses are generated in a HLA.A2.1/DR1 transgenic mouse model. Further confirmation of the immunogenicity of the identified peptides derived from T21 by immunising individual OLPs would be required, however due to animal and time constraints this could not be achieved during the course of this investigation.

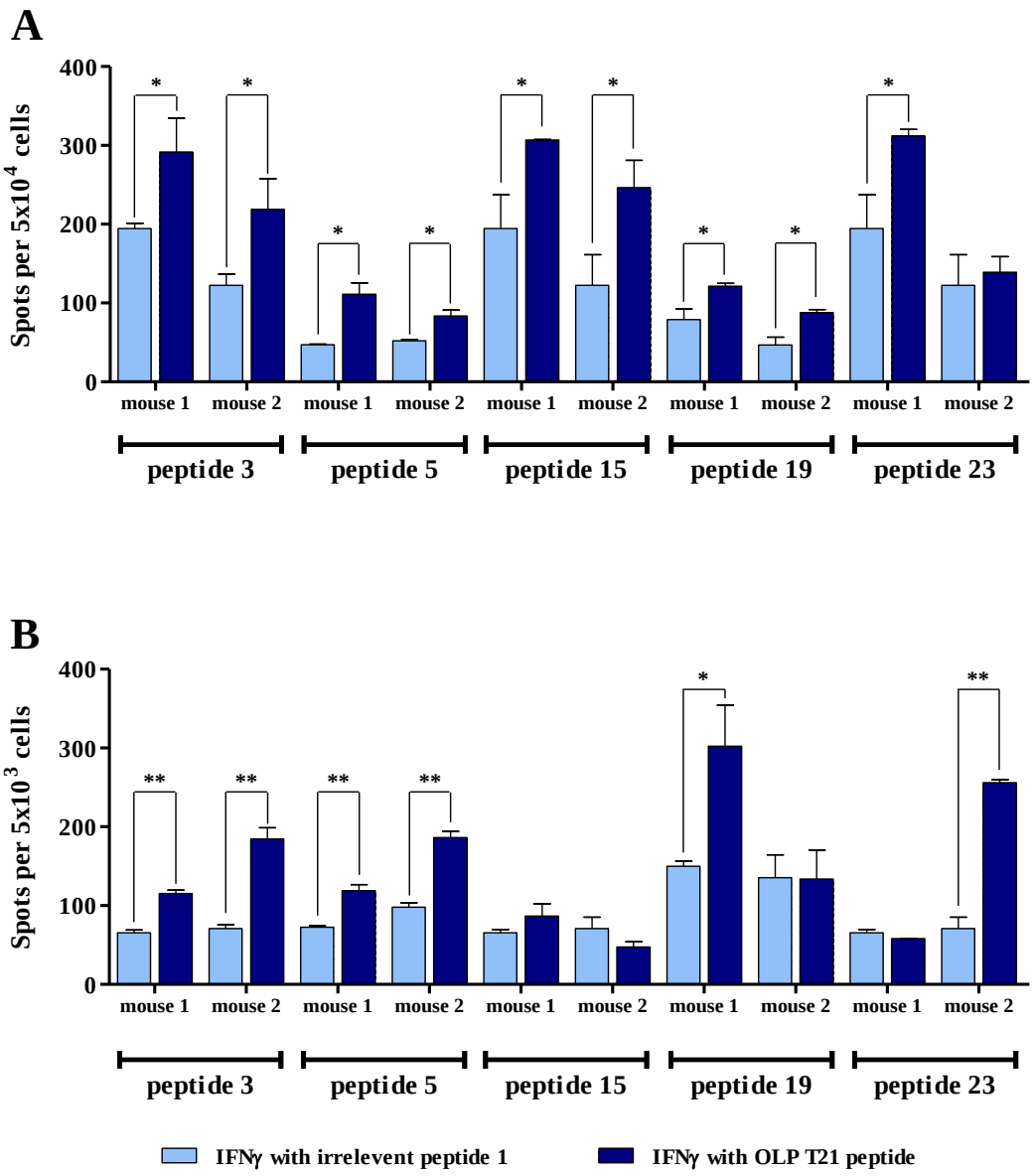


Figure 5.5 – IFNγ release assays and proliferation assays performed using T cells generated from HLA.A2/DR1 transgenic mice immunised with peptide pool C1, 2 and 3 subgroups. *Mice were immunised with one of pool C derived subgroup peptide pools and splenocytes harvested for assessment of T cell response to peptide restimulation using IFNγ release EliSpot assay. Assessments were performed following three methods of in vitro restimulation of splenocytes using individual peptides derived from the immunising pool. Graphs represent (method 2) irradiated LPS blast and pulsed DCs (**A**), and (method 3) restimulation using peptide followed by presentation using pulsed DCs (**B**). Experiments were performed in two mice in triplicate represented with standard error of mean between T cells either with relevant or irrelevant peptide 1, with statistical significance indicated as (*p<0.05 and **p<0.01) as determined by Student's t test.*

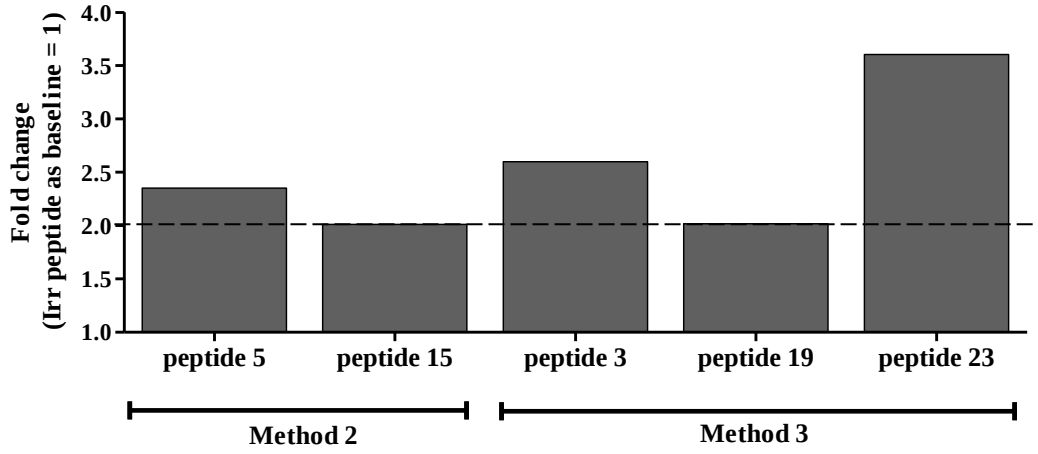


Figure 5.6 – Significant fold-change differences with peptide pool C1, 2 and 3 subgroups. *Mice were immunised with one of pool C derived subgroup peptide pools and splenocytes harvested for assessment of T cell response to peptide restimulation using IFNγ release EliSpot assay. Assessments were performed following three methods of in vitro restimulation of splenocytes using individual peptides derived from the immunising pool. Experiments were performed in two mice in triplicate between T cells either with relevant or irrelevant peptide 1, with statistical significance determined as a minimal 2-fold change difference to irrelevant peptide 1.*

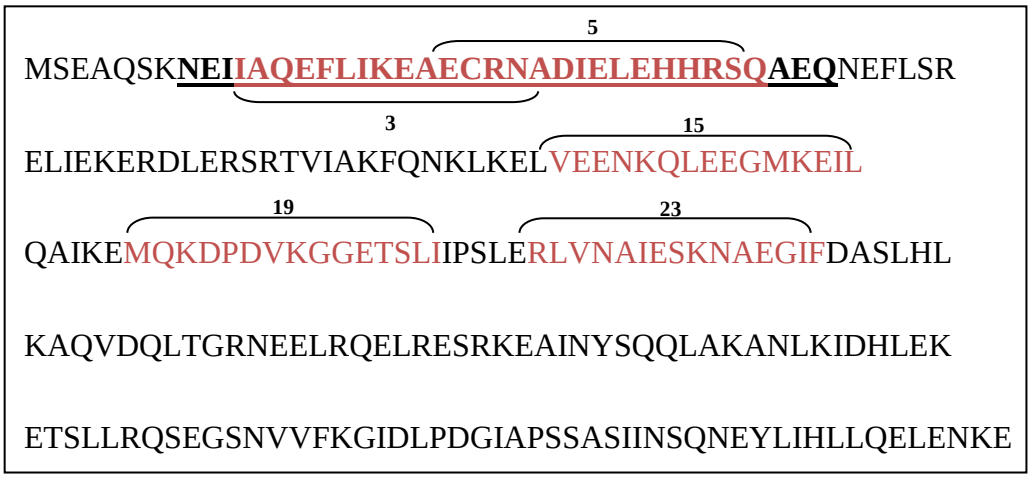


Figure 5.7 – Diagram illustrating location of immunogenic peptides identified in pool C on a section of the T21 protein sequence. *Peptides from pool C identified to have the most immunogenic potential following immunised T cell restimulation with individual peptides highlighted in red with the unique region bold, underlined.*

Pool No.	Peptide assessed	IFNγ release (≥ 2 fold change) (Positive responses / total mice)		
		Method 1	Method 2	Method 3
Pool C1	OLP:1	(0/2)	(0/2)	(0/2)
	OLP:9	(0/2)	(0/2)	(0/2)
	OLP:14	(0/2)	(0/2)	(0/2)
	OLP:19	(0/2)	(0/2)	**(1/2)**
Pool C2	OLP:3	(0/2)	(0/2)	**(1/2)**
	OLP:10	(0/2)	(0/2)	(0/2)
	OLP:15	(0/2)	**(1/2)**	(0/2)
	OLP:23	(0/2)	(0/2)	**(1/2)**
Pool C3	OLP:5	(0/2)	**(1/2)**	(0/2)
	OLP:13	(0/2)	(0/2)	(0/2)
	OLP:35	(0/2)	(0/2)	(0/2)

Table 5.2 – Immunogenic peptides derived from dissection of peptide pool C*. Tabulation of responses determined as minimal 2-fold change compared to irrelevant control peptide 1. Assays were performed on T cells generated from 3 groups of 2 mice immunised with peptide pool C1, 2, or 3 harvested and restimulated in vitro using individual peptides and assessed for T cell function using three methods (described previously). Experiments were performed twice in triplicate between T cells either with relevant or irrelevant peptide 1. (n=a/b) with 'a' being the number of mice that responded to the peptide immunisation and 'b' being the total number of immunised mice.*

5.2.2 Identification of candidate immunotherapeutic targets within the T21 unique region

5.2.2.1 Determination of endogenously processed T21 unique region peptides

One important advantage of studying the immunogenicity of this protein sequence lies in the inclusion of the region unique to T21. To determine whether this 31 amino acid sequence within the protein comprises of immunogenic epitopes would allow for T21 specific vaccination avoiding unwanted targeting against to CEP290 and its associated protein variants. With this considered a closer examination of the unique sequence to establish whether natural endogenously processed epitopes display immunogenic capability would be needed.

To achieve this, previously described *T21* cDNA (Figure 3.2) inserted into pBudCE4.1 expression vector was utilised for gene gun immunisation using HLA.A2/DR1 transgenic mice. This procedure would confirm proteins to be endogenously processed by the proteasome, presented within an MHC context and result in specific priming of T lymphocytes against T21-specific epitopes. Following 7 days subsequent to the final boost, T cells from immunised mice were harvested and assessed by restimulation directly with peptides or through APC presentation of peptide, a positive T cell recognition would elicit IFNγ response. In order to gauge the most effective positive response of T cells activated via peptide stimulation, immunised T cells were assessed *in vitro* using all three procedures as described previously.

Due to the quantity of peptides now required for this study, peptides from the unique region and a 15mer control peptide (irrelevant peptide 2 – Seq: DESFRKYTAFTIPSM) were produced in-house applying a standard solid phase peptide synthesis protocol using a CEM Discover microwave with peptides being synthesised onto a polystyrene resin. The peptides were dissolved in HPLC grade water, HPLC Grade Acetonitrile 99% with 1% TFA and quality assured using mass spectrometry analysis (Dr V Mundell, School of Science and Technology, Nottingham Trent University, UK) (Table 5.3). Positive T cell responses were once again determined by applying a greater then 2-fold difference between the T21 derived peptide to the irrelevant peptide 2. The resulting T cell responses observed across the seven peptides analysed are summarised in Table 5.4 (see appendix VI) with significant responses observed with peptides 1, 4 and 5. Peptide region 3, 4, and 5

once more showed significant responses inviting further investigation of these three peptides as they may share a common immunogenic epitope that is also endogenously processed.

Designated name	Protein origin	Sequence	Top sequence similarity score	
			Homo sapiens	*Mus musculus*
OLP:1	T21, prostate cancer antigen T21, (AAT01278)	MSEAQSKNEIIAQEF	CEP290 (93%)	CEP290 (57%)
OLP:2		SKNEIIAQEFLIKEA	----	----
OLP:3		IAQEFLIKEAECRNA	----	----
OLP:4		LIKEAECRNADIELE	----	----
OLP:5		ECRNADIELEHHRSQ	----	----
OLP:6		DIELEHHRSQAEQNE	----	----
OLP:7		HHRSQAEQNEFLSRE	----	----
Irrelevant peptide 1 (Irr 1)	hCG25653, isoform CRA_b (EAW85903)	SWEKQWYGKAMERAF	hCG25653 (100%)	----
Irrelevant peptide 2 (Irr 2)	Pol protein, Human immunodeficiency virus 1 (ADY92492)	DESFRKYTAFTIPSM	HIV (100%)	----
(----) = No significant matches using online alignment tools (NCBI and Ensembl)				

Table 5.3 – A summary of T21 overlapping peptide alignment*. Table summarising the overlapping peptide sequences spanning the unique region and the irrelevant peptides used during this study and their alignments to the human and murine proteomes.*

Peptide assessed	IFNγ release (≥ 2 fold change) (positive responses / total mice)		
	Method 1	Method 2	Method 3
OLP:1	(0/6)	**(1/6)**	**(3/6)**
OLP:2	(0/6)	**(1/6)**	**(2/6)**
OLP:3	(0/6)	**(1/6)**	**(2/6)**
OLP:4	**(1/6)**	**(1/6)**	**(2/6)**
OLP:5	**(1/6)**	**(1/6)**	**(2/6)**
OLP:6	(0/6)	**(1/6)**	**(2/6)**
OLP:7	(0/6)	(0/6)	**(2/6)**

Table 5.4 – Immunogenic responses of T21 unique region derived peptides. *Tabulation of positive responses determined as minimal 2-fold change compared to irrelevant control peptide 2. Assays were performed on T cells generated from 2 groups of 3 mice immunised with T21 cDNA using gene gun immunisation (previously described) harvested and restimulated in vitro using individual peptides and assessed for T cell function using three restimulation methods (described previously). Experiments were performed between T cells either with relevant or irrelevant peptide 2. (n=a/b) with 'a' being the number of mice that responded to the peptide immunisation and 'b' being the total number of immunised mice.*

5.2.3 Assessment of T21 derived peptide in prostate cancer patient PBMCs

5.2.3.1 Identification of immunogenic T21 specific peptide in patients

Experiments involving murine models to identify antigen derived immunogenic epitope regions remains a valuable and practical tool however, as alluded to previously, a cautious view must be applied when drawing conclusions from novel epitopes identified using pre-clinical models. Natural differences in the cellular processing of endogenous proteins through the proteasome and TAP transport have been reported between the human and mice (Wentworth *et al.,* 1996; Sesma *et al.,* 2003). In regard to T21, the seven unique region peptides evaluated for endogenous processing share no equivalent mouse homologue; therefore it is not surprising that T cell responses were observed. As T21 is a "self-antigen" in humans there is a limitation in assessment of its peptides in mice as there is no need to overcome self tolerance in order to generate immune responses. Hence careful verification of novel epitopes must be sought using *in vitro* culture of healthy donor and cancer patient PBMCs.

The most important question that requires addressing is whether, or if any, inherent T cell mediated immune response can be mounted against the peptide region of interest in patients with cancer. Initial identification of tumour antigen T21 had been derived from immunogenic screening for sera taken from patients with prostate cancer using SEREX technology which relies on high levels of IgG antibody titre in the patient serum towards the reactive cancer antigen. It can therefore be inferred that the production of antibody is supported by both B cells and Th_1/Th_2 cells which implies the presence of a $CD4^+$ T cell response against T21.

In collaboration with Nottingham City Hospital, Urology Centre, prostate cancer patient PBMCs from four patients were assessed for T cell reactivity specific to the seven OLPs derived from the T21 unique region. In this pilot study, confirmed prostate cancer patients were selected with a view to identify any immunogenic peptides, optimise protocol and later to expand screening of identified peptides to a larger study of both cancer and BPH patient cohorts.

Patients selected for this study comprised of, patient-A, a 78 year old man presenting with elevated PSA and diagnosed with prostate cancer with biopsy confirming Gleason 7 (4+3) and metastatic disease. Patient-B, -C and –D all presenting with above age range PSA and diagnosed with localised prostate cancer with biopsy confirming Gleason 7 (3+4) and categorised as intermediate risk. All patients underwent radical prostatectomy and were monitored for disease recurrence. No tumour was available for T21 expression analysis. Patient blood analysis confirmed validity of the experiments (Table 5.5).

		Normal Range	Patient-A	Patient-B	Patient-C	Patient-D
Age			78	58	63	57
Gleason grade			7 (4+3)	7 (3+4)	7 (3+4)	7 (3+4)
PSA (aged >70)*	**(µg/L)**	0 – 5	42.1	4.3	10.5	4.6
Lymphocyte	**($x10^9$/L)**	1.0 – 4.0	2.9	2.5	2.8	1.9
Monocytes	**($x10^9$/L)**	0.1 – 1.5	0.7	0.5	0.7	0.7
Neutrophils	**($x10^9$/L)**	2.0 – 7.5	3.6	2.6	3.2	3
Basophils	**(%WBC)**	0 – 0.15	0	0.1	0	0.1

Table 5.5 – Blood analysis of prostate cancer patients. *Table showing patient age, Gleason grade, PSA level and blood differentials at the time of radical prostatectomy. (*****)Elevated PSA range determined as (age +50=PSA over 3.0) (age +60=PSA over 4.0) and (age +70=PSA over 5.0)*

Due to the limited number of PBMCs retrieved after defrosting, it was decided to adopt a longer stimulation approach that would expand peptide specific reactive T cell populations within the cells.

PBMCs from patients were thawed and pulsed with a pool of seven OLPs originating from the unique region or control peptide (irrelevant peptide 2). This was performed using a relatively low level peptide stimulation (1.4 ng per peptide) equating to a total of 10 ng/µl of total peptide pool per stimulation. After 7 days, wells were again stimulated with the peptide pool and IL-2 and following a further 6 days, cells were gently washed using a centrifuge and seeded into wells with the addition of 1 µg/ml of individual peptides or control added to the appropriate wells. Using this extended stimulation method, no

responses were observed in patient-B, -C and –D (data not shown), however peptide 4 was able to induce a positive IFNγ response in patient-A compared to a healthy donor ($p=<0.001$) (Figure 5.8A). This is perhaps due to this patient having a more aggressive Gleason grading with metastatic disease and considerably elevated PSA levels compared to the other three patients. Despite a positive T cell response demonstrated in one patient, it is premature to suggest that peptide 4 contains a HLA restricted naturally expressed epitope; unfortunately, clinical information in this instance does not extend to assessing patient haplotype.

Although the extended stimulation method provided considerably more reactive T cells to specific peptides, there is a risk of potentially skewing the response due to long term exposure of T cells to an immunostimulative environment following the addition of supplementary cytokines. With this considered, a more representative response was sought using PBMCs from patient-A that underwent immediate stimulation with either peptide pool, peptide 4 or control peptide and underwent assessment after 48 hours of culture, thus avoiding much of the response distortion which may be attributed to long term *in vitro* culturing of T cells.

The resulting experiment confirmed a strong response to stimulation with peptide 4 compared to control peptide or the response of a healthy donor, suggesting that the responses observed previously were indeed genuine and that restimulation in this way can generate reactive T cell responses in patients (Figure 5.8B). Interestingly, on comparison of the responses generated across the two stimulation methods, a stronger T cell response to peptide was observed following extended stimulation, perhaps as a consequence of the immunostimulative phenomenon described above. Indeed the lack of T cell response generated by the direct stimulation method using healthy donor PBMCs in both control and T21 peptide specific wells supports this notion.

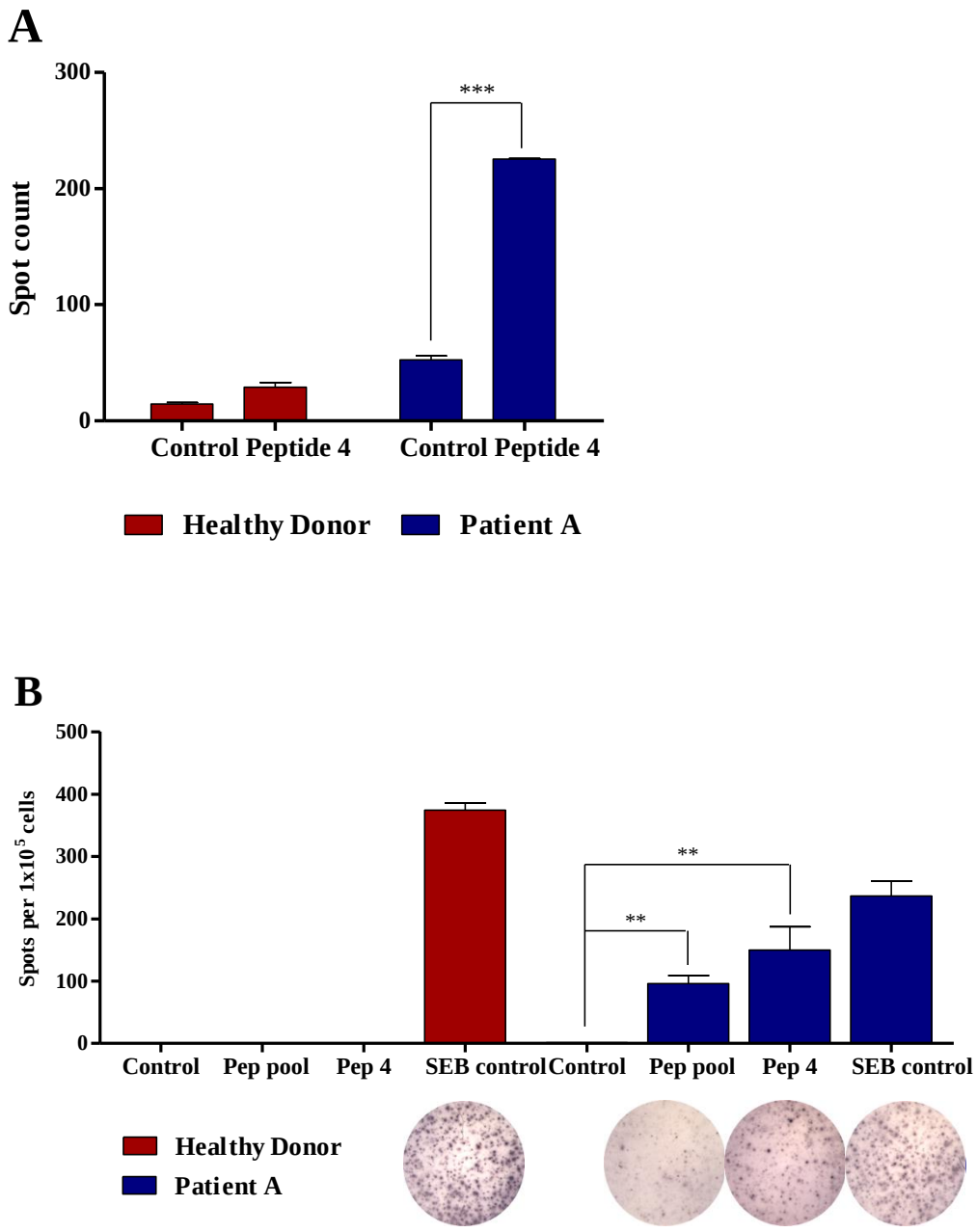


Figure 5.8 – IFNγ release assay evaluation of a prostate cancer patient PBMCs response to peptide 4. *Prostate cancer patient-A PBMCs were stimulated with unique region peptides and assessed using IFNγ release EliSpot assay. Data shown here represent PBMC response to peptide 4 versus control (Irr pep 2) with low levels observed in healthy donor compared to elevated levels in Patient-A (**A**) Further assessment was performed with either peptide 4 stimulation or unique region pooled peptides (**B**). Experiments were performed once in triplicate represented with standard error of mean with statistical significance indicated as (**p<0.01 and ***p<0.001) as determined by Student's t test.*

5.2.3.2 T21 specific T cell reactivity in vaccine trial patients

During the course of this study, and through collaboration with Professor Gustav Gaudernack (Oslo University Hospital, Norway), an opportunity arose to investigate clinical samples from a Phase I/II Norwegian vaccine trial using tumour derived mRNA-transfected autologous dendritic cells to treat metastatic, androgen resistant prostate cancer patients, the findings were later published by Mu *et al.,* (2005). Patient samples collected during the trial offered the chance to assess prostate cancer patient PBMCs for T cell reactive responses specific to T21 derived peptide prior to DC treatment and following completion of the treatment. Crucially, the therapeutic approach centred on immunogenic polyvalent prostate cancer antigen generated from three classical prostate cancer cell lines, LNCaP, DU145 and PC3. As previously confirmed in chapter 4, these cell lines express *T21* mRNA and it is perhaps reasonable to consider that the therapeutic strategy used may indeed lead to the expression of T21 derived epitopes in the context of MHC on patient DCs (Figure 5.9).

Briefly, the trial employed a vaccination method using DC's transfected with mRNA from allogeneic prostate cancer cell lines with each patient receiving a minimum of four weekly injections administered either intranodally or intradermally, with $20x10^6$ transfected DCs. In the procedure, PBMCs were obtained by leukopheresis and enriched for monocytes by magnetic depletion of B cells and T cells. Immature DCs were generated following 5 days of culture in serum free media containing GM-CSF and IL-4 (Mu *et al.,* 2003). Subsequently, bulk tumour mRNA extracted from DU145, LNCaP and PC3 cells (equating to $50x10^6$ of each cell line per vaccine) was prepared and mixed prior to transfection of immature DCs using a square-wave electroporation procedure. At 48 hours post-transfection, cells underwent maturation in media containing IL-1β, IL-6 and TNF-α and PGE_2. Mature DCs were then stored frozen and thawed as required.

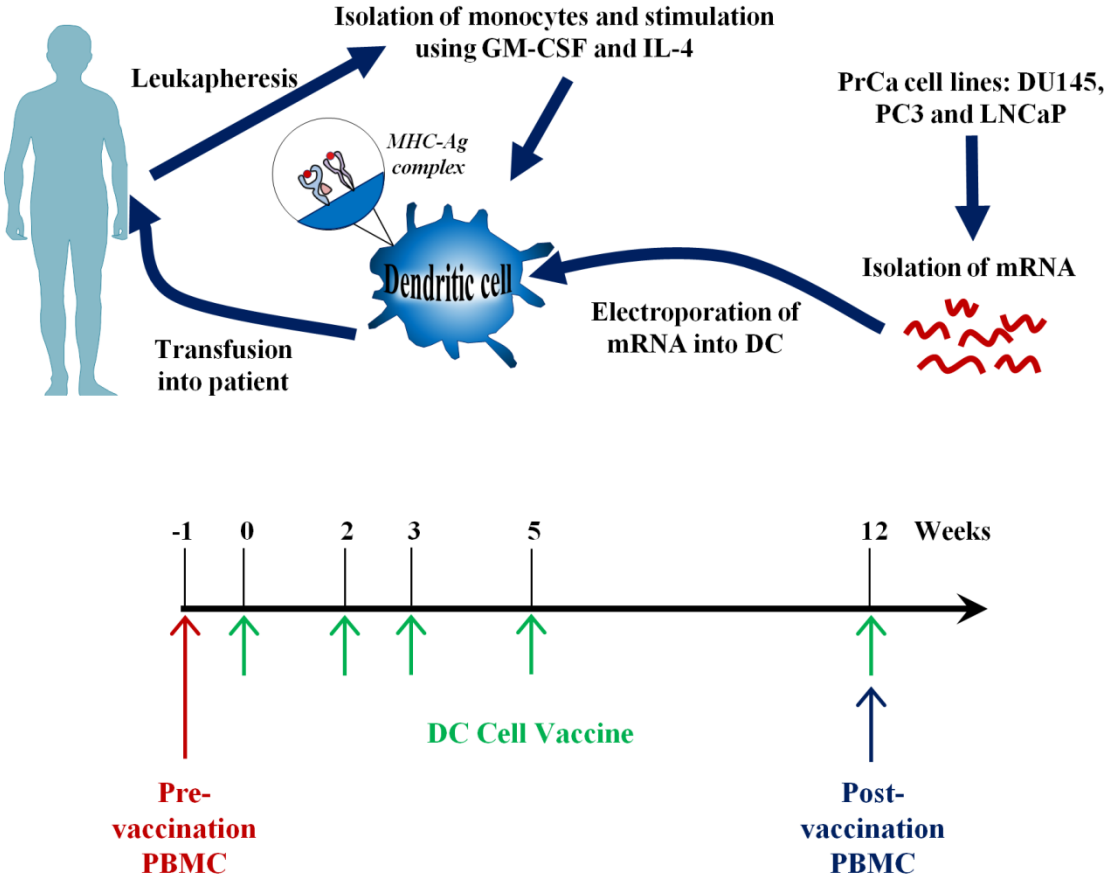


Figure 5.9 – Diagram illustrating trial experimental procedure, vaccination schedule and acquisition of PBMCs used in this study. *Monocytes isolated and cultured in vitro from the patient undergo transfection of mRNA isolated from allogenic prostate cell lines and re-transfused into the patient.*

For entry into the trial, patients were required to have a verified adenocarcinoma of the prostate with evidence of disease progression considered as increasing PSA levels in three subsequent analyses. Patients also had an above minimal white blood, neutrophils and platelets with adequate haematological renal and hepatic function. In all, 20 patients were selected and of which four patients were diagnosed at stage T2 disease and the remaining 15 patients at stage T3 (one patient did not complete the study).

Patients were monitored and evaluated for clinical signs of disease progression defined as the appearance of new lesions of malignant disease, or development of symptoms consistent with metastatic disease. In addition, PSA levels in serum were assessed before therapy (baseline) at week 5 and 3 months after the first round of vaccination. Disease status was defined as partial PSA response (50% ≤ than baseline), stable response (>50% reduction or <50% increase to baseline) and progressive disease (50% ≥ than baseline).

To evaluate the immune response generated peripheral blood monocytes (PBMCs) were taken pre-vaccination (and used a baseline), at 5 weeks and after the final round of therapy at 3 months (post-vaccination). First, responses of T cells derived from the PBMCs were assessed after 5 days of co-culture with tumour mRNA-transfected stimulator cells using ^{3}H-thymidine incorporation assay and IFNγ EliSpot assay. T cells from PBMCs that demonstrated proliferative cell responses were co-cultured with irradiated allogeneic PBMCs and IL-2 and tested for the generation of T cell clones using cell proliferation and for CTL specific killing of target tumour mRNA-transfected cells and prostate cell lines (where appropriate) using ^{51}Cr release assay.

The trial concluded that clinical outcome was significantly related to immune response with 9 out of 11 patients with stable disease demonstrating both a positive immune response and decreasing PSA. Conversely, of the 9 patients that showed progressive disease, 44% demonstrated no immune response and no improvement in PSA.

In the current study, cryo-preserved PBMCs that had been collected at two time points from 12 patients who all underwent therapy, referred to as before therapy (pre-vaccination) and 12 weeks after the first vaccination (post-vaccination) as indicated on Figure 5.9. To begin this investigation, PBMCs from one patient were rapidly thawed and underwent analysis using flow cytometry using combinations of CD markers to confirm that leukocyte populations had viable phenotypes compared to healthy levels (Table 5.6).

	Normal Range	Pre-vaccination	Post-vaccination
Class and (associated CD markers)	*%*	*%*	*%*
Lymphocyte (CD45$^+$, CD3$^+$)	20 – 60	58.33	53.64
CD8$^+$ T cell (CD45$^+$, CD3$^+$, CD8$^+$)	10 – 32	9.67	8.29
CD4$^+$ T cell (CD45$^+$, CD3$^+$, CD4$^+$)	28 – 59	45.91	42.51
Monocyte (CD45$^+$, CD14$^+$)	7 – 10	12.53	9.57

Table 5.6 – Phenotypic analysis of therapy trial patient. *Normal ranges of healthy white blood cells (%) and assessment of one representative patient pre- and post-vaccination PBMCs from frozen using flow cytometry. CD8$^+$ and CD4$^+$ T cell populations derived from gated lymphocyte population. Monocyte population derived from whole cell population.*

Patient PBMCs were thawed and incubated at 37°C to stabilise cell recovery and to reduce non-specific background during the assay. After 30 minutes incubation and gentle washing, cells were pulsed with either T21 peptide 4 or irrelevant control peptide 2 and seeded onto EliSpot plates pre-coated for with IFNγ capture-antibody. After 48 hours of incubation, cells were removed and EliSpot detection was performed and spots counted.

During the trial, after completion of the course of vaccinations, patients were grouped into either "responder patients" described as those who demonstrated T cell responses to *in vitro* restimulation to mRNA loaded DCs and had stable or improved PSA levels following therapy or those that did not respond to therapy showing no improvement throughout the course, referred to as "non-responder" patients. Using this information, the results here demonstrated that 6 out of 8 responder patients were able to generate positive responses following peptide 4 stimulation when compared to control. It is also important to note that four out of eight patients demonstrated responses to peptide 4 in pre-vaccination PBMCs with four out of eight patients showing response after therapy (Figure 5.10 A1 and A2) which may perhaps point toward the existence of an intrinsic T21 peptide 4 specific T cell response. Interestingly, responder patients p12, p15 and p19 all exhibited stable disease following treatment and not only generated significant vaccination responses to T21 peptide compared to control, but also showed significantly increased T cell responses between pre- and post-vaccination (Figure 5.10).

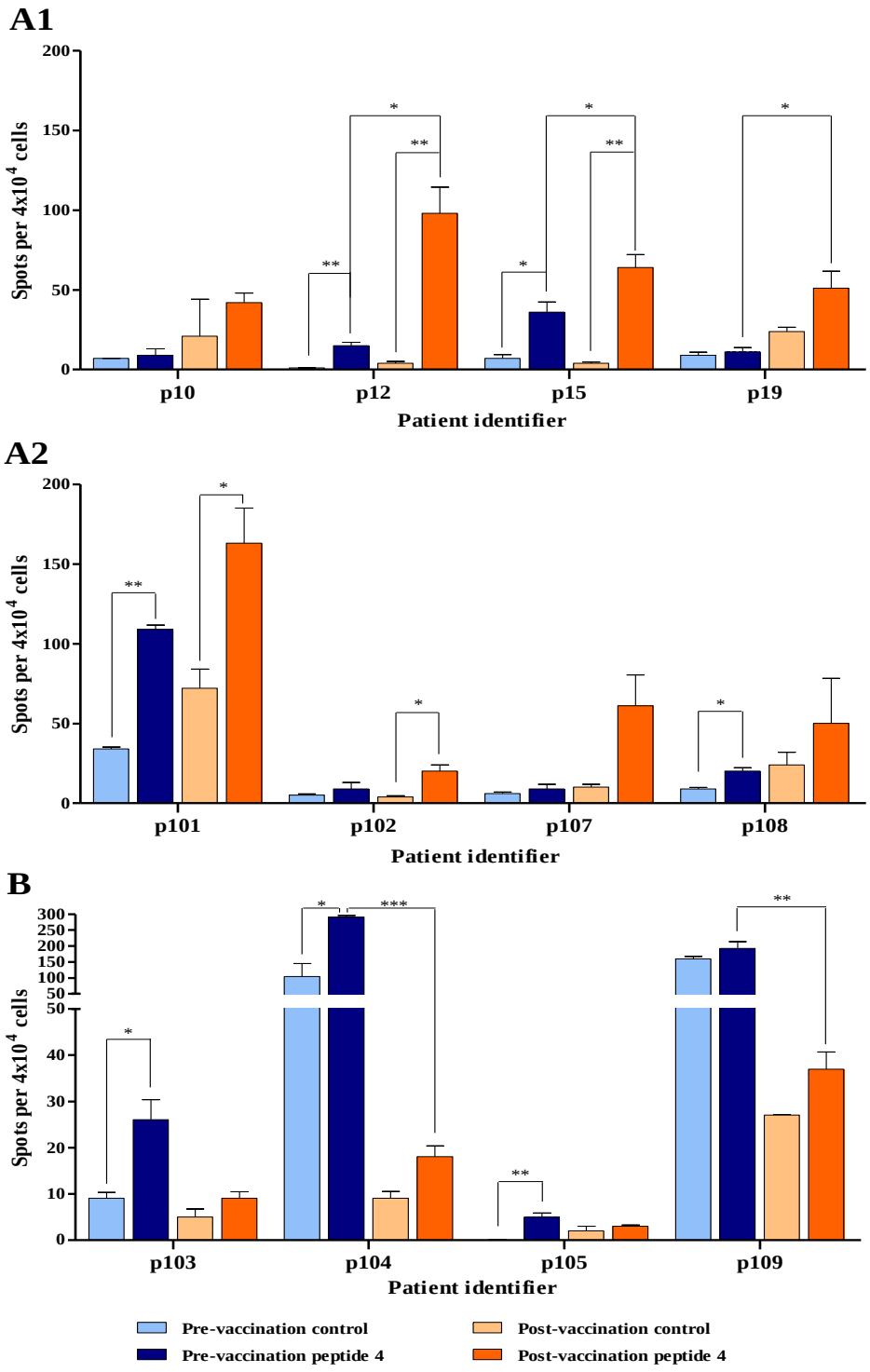


Figure 5.10 –IFNγ release assay performed on prostate cancer patient derived PBMCs. *Patient PBMCs were stimulated with either peptide 4 or control (irrelevant peptide 2) and incubated for 24 hours before being assessed for T cell IFNγ release. 8 patients represented in (**A1+A2**) were classed as T cell responders to therapy whereas 4 patients (**B**) showed no T cell response to therapy. The experiment was performed once in triplicate wells represented with standard error of mean with statistical significance indicated as (*p<0.05, **p<0.01, ***p<0.001) as determined by Student's t test.*

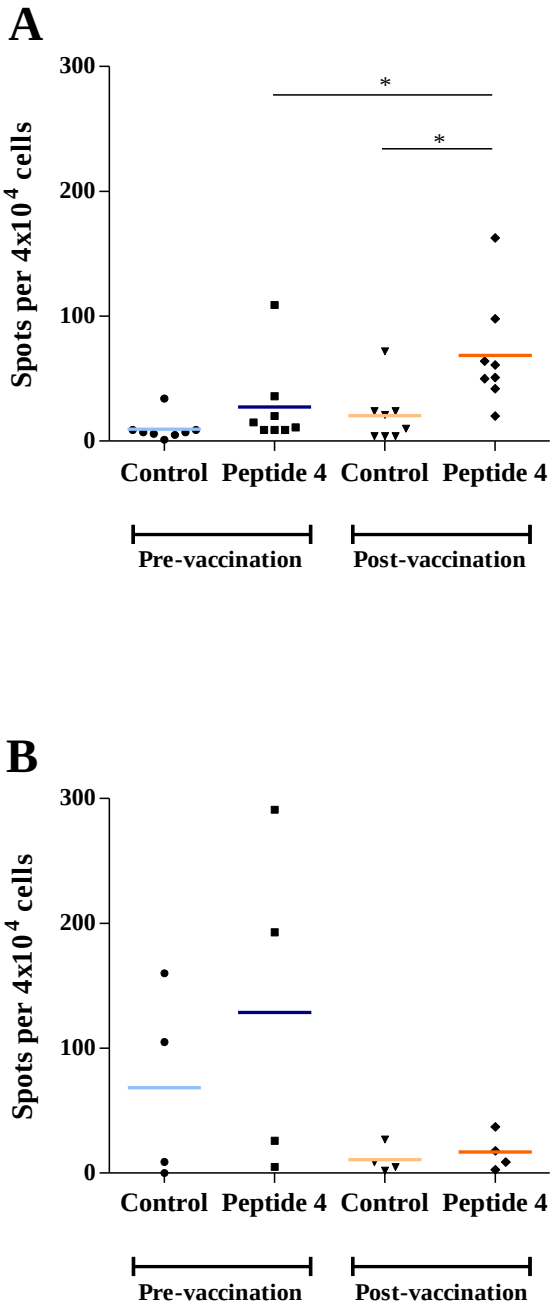


Figure 5.11 – IFNγ spot count distribution in T cell responder and non-responder patient groups. *Graph (**A**) showing 8 therapy responder patients pre- and post-vaccination spot count distribution when stimulated with either peptide 4 or control (irrelevant peptide 2). Post-vaccination responses are statistically elevated when compared with control (*p<0.05) and when compared to pre-vaccination levels (**p<0.01). Graph (**B**) representing 4 non-responder patients determining statistical significance by paired Student's t test.*

In contrast, none of the four non-responder patients generated any significant responses post-vaccination. Within this non-responder cohort, three out of the four patients showed T cell responses in pre-vaccination PBMCs which seemly induced very strong responses compared to the pre-vaccination responses observed in the responders (Figure 5.10B), however strong T cell responses were also observed in the control wells (both in non-stimulated and using control peptide) indicating that the responses were not a consequence of peptide stimulation. In addition to this observation, the post-vaccination stimulation in non-responders failed to generate similar responses to their respective pre-vaccination responses. Overall, no significant differences were observed in this group (Figure 5.11).

It was also possible to differentiate between these groups by depicting the response fold-change from the responder and non-responder groups as the latter consistently failed to generate post-vaccination responses that were stronger than the pre-vaccination responses (Figure 5.12A). Collectively, these data show that a fold-change between pre- and post-vaccination IFNγ responses following stimulation using peptide 4 significantly discriminates between responder and non-responder patients ($p<0.00195$) (Figure 5.12B).

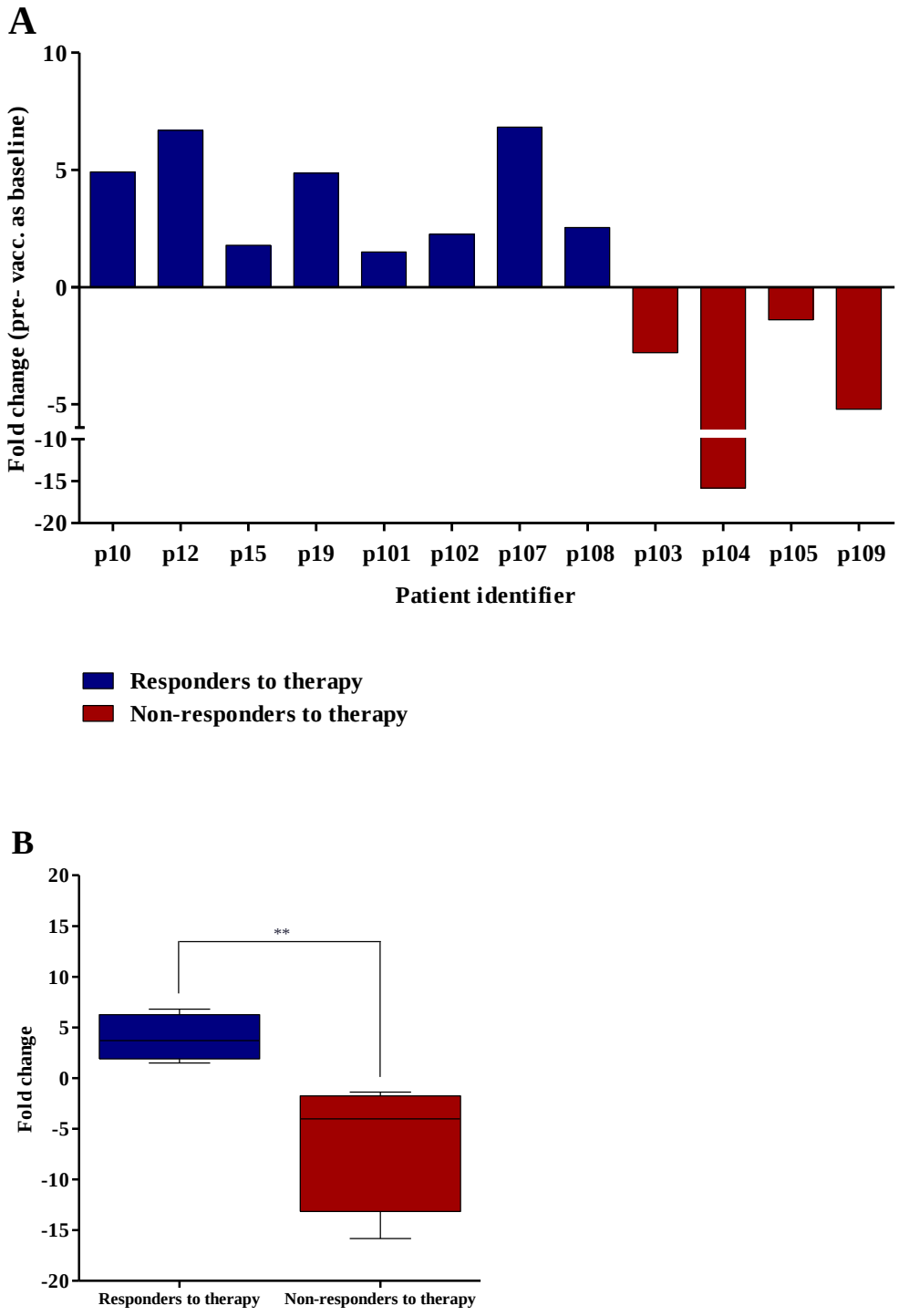


Figure 5.12 – Fold change in T cell responder and non-responder patient groups. *Graph (**A**) showing responder and non-responder fold change of post-vaccination PBMCs with peptide 4 using pre-vaccination level response to peptide 4 as baseline (0) Graph (**B**) representing responder and non-responder fold change distribution significance determined at (**p<0.01) by Student's t test.*

5.3 Discussion

The discovery of T21 using the SEREX approach and importantly, the identification of the uniquely encoded region in chapter 3, has led to the opportunity to trigger tumour-killing mediated by T21 primed T cells. Once identified, immunogenic peptide sequences could have a number of immunotherapeutic applications in cancer. Previous investigation using anti-T21/CEP290 antibody demonstrated restricted expression in normal tissues (Miles *et al.,* 2007). During the current investigation it has been established that the antibody used for previous studies would potentially identify both T21 and CEP290 proteins. The published study also demonstrated T21 reactivity in 5 out of 10 prostate cancer patient sera with no reactivity observed in normal donor sera (0 out of 10). Taken together, it could be concluded from these findings that the mRNA sequence identified using SEREX *i)* holds the ability to evoke pre-existing and detectable humoral immune response suggesting there may be an epitope(s) target which can be exploited for immunotherapy *ii)* the response observed is specific to tumour regardless of it being T21 or CEP290 positive and finally *iii)* the tissue-restricted expression of CEP290 would avoid the induction of an unwanted autoimmune response against healthy normal cells if triggered during immunotherapy. On this premise, the current study of T21 sought to identify immunogenic peptide sequences which were capable of generating immune responses using an *in vivo* double transgenic mouse model (HLA.A2/DR1) and *in vitro* studies using human PBMCs derived from prostate cancer patients.

To begin, peptides were screened for their immunogenic reactivity using a library of OLPs synthesised from the T21 sequence focusing specifically on 43 OLPs derived from the translated mRNA sequence initially identified using SEREX. It was decided that pooling OLPs into four groups and immunisation using HLA.A2/DR1 transgenic mice would be most suitable to determine the presence of immunogenic peptides. The most reactive pool (pool C) was further segregated into sub-pools with three to four OLPs in each which were then individually used to restimulate immunised splenocytes *in vitro*. Interestingly, T21 unique region specific OLPs 3 and 5 generated consistent and statistically significant responses over two of the IFNγ restimulation methods used.

In the light of these results and due to the inclusion of the unique region in T21 and not CEP290, further evidence of endogenous processing of the unique region epitopes was sought. Resulting gene gun assisted *T21* cDNA immunisations demonstrated significant responses with 1, 4 and 5 OLPs. The conclusion from these early studies of immunogenicity in transgenic mice highlighted OLP region 3, 4, and 5 as three peptides which may potentially share common immunogenic epitope(s) and may also be endogenously processed. One major limiting factor experienced throughout this investigation was the availability of transgenic mice due to unforeseen events, therefore confirmation of these findings would be required in repeat experiments giving more thorough statistical analysis before commencing further studies.

As T21 is a "self-antigen" in humans, it could be argued that assessment of the true immunogenicity of T21 derived peptides in mice is compromised as the absence of the unique region in mice suggest that there is no need to overcome self tolerance in order to generate immune responses. Therefore verification of peptides were sought using *in vitro* culture of healthy donors and prostate cancer patient PBMCs. In the first of two independent studies, prostate cancer patient PBMCs were compared against healthy controls using restimulation with OLPs derived from the unique region of T21. Results demonstrated little responses in healthy normal PBMCs however peptide 4: (LIKEAECRNADIELE) demonstrated strong IFNγ release responses indicating that this candidate peptide may indeed merit further investigation for immunotherapy. A second study involving a shorter *in vitro* restimulation procedure confirmed these findings. It is of note that following *in vitro* assessment of splenocytes immunised using pools of 10-11 peptides, pool D which demonstrated consistent responses contained OLP 4. From this it could be hypothesised that pool C containing OLPs 3 and 5 (which span the length of peptide 4) may have together been responsible for generating the most consistently significant responses compared to the other three pools.

Evidence of an immunological response generated following stimulation using peptide 4 in a prostate cancer patient (confirmed Gleason 7 (4+3)), prompted the study of this peptide in a prostate cancer patient cohort. An *in vitro* peptide 4 stimulation procedure was first optimised and performed on PBMCs derived from patients prior to, and proceeding an adoptive cell transfer therapy using autologous DCs pulsed with allogenic mRNA derived from three classical prostate cell lines (PC3, LNCaP and DU145); all of which

express *T21* mRNA (chapter 4). The PBMCs used during this study formed part of a larger phase I/II clinical trial in which patients with metastatic, castrate resistant prostate cancer underwent four doses of DC-therapy (Mu *et al.*, 2005). The trial resulted in 13 out of 19 treated patients demonstrating decreased rate of PSA which was enhanced further with booster vaccinations. Furthermore, PSA reduction and development of specific immune responses were also observed and appeared to share a correlation (12 out of 19 treated patients). The trial established that the vaccine was well tolerated with no toxicity observed and that the clinical outcome is significantly related to T cell response. Since then, two more phase I/II trials using autologous instead of allogenic tumour-mRNA in prostate (ClinicalTrials.gov Identifier: NCT01197625/completion 2021) and in advanced metastatic malignant melanoma (Kyte *et al.*, 2006) have been conducted by the same group.

During the current investigation, the patient cohort used consisted of eight patients responders to therapy and four non-responders to therapy. An association between treatment responses and *in vitro* stimulation with T21 peptide 4 was observed in all clinical responders that were able to generate higher post vaccination responses following stimulation, four out of eight of responder patients generated significant levels of IFNγ post treatment. It is of note that responder patient's p12, p15 and p19 all demonstrated stable disease following treatment and all showed a significantly increased T cell response to stimulation with peptide 4. Conversely, the four clinical non-responder patients seemly generated responses pre-vaccination but failed to generate responses post-vaccination. In this way simulation using T21 peptide 4 allowed for significant discrimination between these two subgroups ($p<0.00195$). Although limited in the patient numbers in each subgroup, stimulation using peptide 4 suggests the following; firstly, human T cells may evoke a pre-existing response to T21 peptide 4 therefore breaking immune tolerance may be less difficult than first considered. Secondly, a clear discrimination between the subgroups following stimulation suggests T21 may contribute in some way to an anti-tumour response against prostate cancer. Therein, targeting T21 peptide 4 could potentially generate immunoreactive responses offering potential in a vaccine setting.

In drawing parallels between the *in vivo* transgenic studies and the *in vitro* PBMC studies, the evidence suggests that the focus of future immunogenic studies with T21 for translation into immunotherapy may lie in examining the immunogenic properties of

peptide 4. Investigations must first determine the immunoreactivity of peptide 4 that exists in cancer patients. Furthermore, patients who demonstrate reactive responses to peptide 4 may benefit from T21 directed therapy.

Although mice lack natural expression of the T21 unique region further evaluation of T21 peptide 4 using pre-clinical models would be useful in demonstrating whether T21 would generate T21 specific CTL activity. One approach would be to develop a tumour model using murine tumour cell lines transfected to express T21 which could be used to assess prophylactic and therapeutic peptide and DNA vaccination strategies. Initial attempts using a murine lymphoma cell line (ALC) transfected with both human HLA.A2 and T21 containing constructs and subsequently injected subcutaneously into the right side flank of six HLA.A2/DR1 transgenic mice and monitored for tumour growth. Four out of the six mice had measureable tumours after 22 days, the remaining two mice failed to generate any tumour during the course of the experiment (data not shown). An alternative to transfection would be to use a HLA.A2^{+} human cell line that naturally expresses T21 such as the prostate cancer cell line, LNCaP or breast cancer cells MCF-7 and MDA-231. T21 expression studies detailed in chapter 4 indicate that LNCaP and MCF-7 may be useful for adoptive transfer of T cells derived from HLA.A2/DR1 transgenic mice immunised with T21 specific peptide into immunocompromised mice bearing human tumours to assert direct tumour killing and determine whether either/both CD8^{+} and CD4^{+} T cell recognition occurs during anti-tumour responses.

Entry of T21 protein sequence into the *in silico* prediction algorithm (http://www.syfpeithi.de/) using either *HLA.A*0201* or *HLA.DRB1*0101* MHC types failed to rank any unique region peptide over a score of 20 suggesting that they may be considered low-moderate affinity binding peptides (data not shown). One explanation of the apparent immunogenicity of peptide 4 may be due to specific CTL, T helper or B cell activity or to a combination of two or all of these responses at a low level supported by the presence of auto-antibodies (indicative of a humoral response) during initial serological identification of T21 (Jäger *et al.*, 2000). If indeed a T21 peptide directed anti-tumour response occurs *in vivo* in man, this may be due to an underling low level self autoimmunity response to the T21 unique region. The appeal of targeting intron retention sequences of self antigens such as the unique region of T21 is that TCR's recognising native sequences, in this instance CEP290, may be preferentially selected over T21 for

deletion during thymic and peripheral selection due to high affinity TCRs recognising self-antigen (CEP290) leaving low affinity (T21 unique specific) TCRs to avoid termination. Other investigators have reported the presence of anti-CEP290 antibodies in the sera of patients from various cancers (Eichmuller *et al.,* 2001; Chen & Shou, 2001) which may suggest functional tolerance and loss of high avidity immunodominant antigen-recognising CTL's whilst retaining those with lower antigen binding affinity which may achieve only weak immune responses (Theobald *et al.,* 1997). Functional characteristics of these low-moderate affinity T cells are still not clearly understood. A recent study in mice demonstrated the fully functional capability of auto-reactive low avidity T cells to self-antigen during infection (Enouz *et al.,* 2012). Therein, the potential of targeting T21 and avoiding CEP290 may indeed be viable.

In some cases cancer-specific alternative transcripts have been functionally attributed toward tumorogenesis and represent neo-antigens that could be utilised for immunotherapy (Kalnina *et al.,* 2005). In mice, targeting of a CD44 variant using DNA encoded vaccination demonstrated a reduction in mammary tumour progression (Wallach-Dayan *et al.,* 2008). In humans, a number of retained intron sequences have been identified such as melanoma ubiquitous mutated-1 (Coulie *et al.,* 1995), GnT-V (Guilloux *et al.,* 1996), TRP-2-INT2 (Lupetti *et al.,* 1998) and gp100-in4 (Robbins *et al.,* 1997), all of which are capable of *in vitro* HLA-restricted tumour cell killing. In human trials a phase I peptide-based study in advanced oral cancer using survivin-2B, found to contain partial retention of intron 2 as a cryptic exon (Mahotka *et al.,* 1999), has so far demonstrated increased peptide-specific CTL in six out of eight patients with no adverse toxicity observed (Miyazaki *et al.,* 2011). The study however failed to prevent patients developing progressive disease, a failure on the part of vaccination strategy rather than the antigen used, which recent studies demonstrated an improved clinical response following peptide vaccination combination of incomplete Freund's adjuvant (IFA) and IFNα (Kameshima *et al.,* 2013).

Although monitoring T cell proliferation kinetics and the secretion of IFNγ captures a broad overview of an immunological response following antigenic stimulation, they are considerably limited in determining the specific T cell population(s) responsible for such responses; hence the experiments presented should be considered preliminary. Employing three distinct *in vitro* peptide restimulation procedures during the animal studies attempted

to determine which assay was most suited to assessing OLPs. Direct restimulation (method 1) failed to generate responses comparable to the other two extended culture methods, possibly as a consequence of inadequate antigen presentation to immunised splenocytes by resident APCs or that the responses generated were weak, amplified only when using extended restimulation procedures. Empirically determined optimal peptide doses for *in vitro* restimulation is important in determining the efficiency of T cell responses. Whilst low levels of peptide may not result in adequate T cell activation, high concentrations may result in overstimulation and T cell apoptosis. Further optimisation of the minimal peptide dosage required for T cell responses would therefore be critical in both human and animal studies.

By extension, considerations of *in vivo* peptide vaccination strategies to activate rather than tolerise T cells should also include *i)* Optimisation of peptide vaccine dosage: to avoid unwanted tolerising effects induced by high levels of peptide at the site of injection *ii)* Administration route and inclusion of co-stimulatory modulators: aiding the recruitment of APCs and correct stimulation of the vaccine-site microenvironment to promote TAA processing and presentation *iii)* The use of immunological adjuvants: as TAA peptides are poorly immunogenic, adjuvants serve to induce inflammatory infiltrates such as T cells and APCs (Schaed *et al.,* 2002: Dredge *et al.,* 2002). For example, the use of water in oil emulsions such as Montanide or IFA in short peptide vaccines can elicit $CD8^+$ T cell responses, but not necessarily anti-tumour responses in melanoma patients (Rosenberg *et al.,* 1998; Smith *et al.,* 2003; Walker *et al.,* 2004). This may be due to the dysfunction and deletion of $CD8^+$ T cells at the administration site (Salerno *et al.,* 2013), although surprisingly T cell dysfunction is not observed using longer peptides/IFA vaccinations (Bijker *et al.,* 2007; Hailemichael *et al.,* 2013) observed in clinical studies with HPV peptide/Montanide vaccine (Kenter *et al.,* 2009) and p53 peptide/Montanide administration in ovarian cancer patients (Leffers *et al.,* 2009). This demonstrates the need to thoroughly investigate how vaccine-site microenvironments could be modified to potentiate peptide-based vaccines, not just by the use of adjuvants (such as IFA/ Monotanide/CpG-ODN/Poly-IC), but also immunomodulatory compounds (GM-CSF/IL-2/IFNs), targeting co-stimulatory receptors (OX40/4-1BB), counteracting suppressive immunity by blockade (CTLA-4/PD-1) or depletion (Treg/MDSC) therapy and synergising peptide-based immunotherapy with chemotherapy (Arens *et al.,* 2013).

In conclusion, the presence of a novel immunogenic and naturally processed peptide target in the form of a T21 unique peptide sequence (peptide 4) has been described. Further investigation into the immunogenicity of T21 should now focus primarily on providing evidence of peptide-specific tumour killing using this peptide sequence.

CHAPTER 6

DISCUSSION

6.1 Introduction

There are now many lines of evidence that support the idea first put forward by Burnet and Thomas in 1957 and expanded on by Dunn *et al.,* 2004 that the immune system plays a crucial role in tumour elimination and escape from containment to a state of malignancy. Taking advantage of immune recognition and the targeted destruction of tumours, cancer immunotherapies attempt to harness components of the immune system in order to generate long lasting, anti-tumour responses. The appeal of pursuing immune driven approaches includes the avoidance of debilitating and sometimes life-threatening side effects associated with conventional therapies as well as destruction of tumour cells responsible for refractory disease and fatal metastasis. This said, immunotherapy presents its own specific challenges that must be addressed when developing strategies in order to avoid similar shortcomings associated with conventional therapies. Since the identification of the first tumour antigen (MAGE-1), attempts to evoke tumour-specific cell mediation killing through CTLs in cancer patients. Although clinical trials have demonstrated clinical efficacy for sub-groups of patients, generating responses for all has proved difficult (Rosenberg *et al.,* 1998; Schwartzentruber *et al.,* 2011; Kantoff *et al.,* 2010; Leffers *et al.,* 2009). Issues of tumour immune evasion such as down regulation of MHC class I molecules, induction of T cell anergy and regulation, tolerance, immune

suppression attributed to TME factors and so forth have helped us to conclude that direct tumour targeting with CTL alone is insufficient. The role of $CD4^+$ T helper cells in contributing to the orchestration of immune response have been shown to be pivotal in activating immune cells (DCs, T and B cells) that lend support to CTL and effector cells mediating innate immunity (NK cells and macrophages) to eliminate tumours (Ossendorp *et al.,* 1998). Therefore the use of TAAs that are capable of engaging both CTL and T helper responses may result in generating more potent and long lasting immune responses. Indeed some of the more promising TAAs identified have demonstrated that $CD4^+$ and $CD8^+$ T cells and B cells form an integrate immune response against tumour and have been defined using serological analysis of cDNA expression (SEREX) (Sahin *et al.,* 1997). In principle, the screening of antibody libraries relies on the specificity of B cell responses whereby the induction of high-titre IgG antibody is dependent on cognate $CD4^+$ T cell help which suggests a capability of evoking both the adaptive and humoral responses.

6.1.1 T21 and the centrosomal protein CEP290

In the early 2000's, T21 (testis clone 21) was identified as a candidate TAA as a result of modified SEREX screening of a normal testicular cDNA library expressed in *E.coli* and probed with allogeneic prostate cancer patient serum (Miles *et al.*, 2007). Subsequent studies of T21 mRNA and protein expression indicated towards its restriction to normal testis and cancer tissues, making its application as a biomarker of disease and potential immunotherapeutic target a tangible prospect. Protein expression had been extensively studied in prostate cancer reporting 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). However later *in silico* analysis revealed that *T21* shared significant sequence similarity with a centrosomal protein called CEP290, present in the centrosomes of dividing cells and shown to play a role in tissue specific cillogenesis (Coppieters *et al.,* 2010). To date, mutations of CEP290 have mostly been studied in the context of cilia related disorders. In 2001, two separate studies identified CEP290 as a potential TAA, the first study used SEREX by probing a testicular cDNA library with sera taken from CTCL patients (termed Se2.2) (Eichmuller *et al.,* 2001), the other used an anti-CEP290 mAb (3H11Ag) to probe a cDNA library derived from patients with gastric cancer (Chen & Shou, 2001).

6.2 Identification of a region unique to T21 and its implications

Since the finding that *T21* shared sequence similarly with *CEP290*, it was paramount to establish the extent of this similarly and determine whether T21 was in fact *CEP290*, a variant of *CEP290* or a separate gene within its own right. It was quickly established that T21 PCR primers designed and the custom anti-T21 Ab (referred to throughout this project as anti-T21/CEP290 Ab) used in earlier studies would have been common to both T21 and CEP290 suggesting that either or both could be detected in PCR and protein-based studies.

Chapter 3 details how working with the sequences submitted to NCBI, *T21* mRNA (Accession No. AY590151.1) and *CEP290* gene (Gene ID: 80184) were scrutinised in order to determine the similarity between the two. *CEP290* alignment to the *T21* mRNA transcript confirmed a 31% alignment coverage similarity between *T21* with *CEP290* of which 96.5% similarity between *T21* and *CEP290* mRNA was observed. Two conclusions were drawn from this initial alignment. The first identified that the *T21* mRNA transcript begins 835bp downstream of the *CEP290* ATG start sequence (within exon 10 of *CEP290*) with the coding sequence commencing at position 1802bp (within exon 10 of *CEP290*) up to position 3401bp (ending at exon 28 of *CEP290*). Further, the 96.7% similarity between T21 and CEP290 protein sequences demonstrated that T21 shares in-frame nucleotide-to-protein translation with CEP290, suggesting that the Ab used in previous investigations would indeed recognise peptide binding sites of both CEP290 and T21. The second observation revealed a 3.5% sequence discrepancy between *T21* and *CEP290*. Further analysis of this region on T21 uncovered a 93bp insertion proceeding exon 18 of *CEP290* from *CEP290* intron 18/19. This subsequently encodes for the incorporation of a 31 amino acid sequence which is specific to T21 and not to CEP290 and was thereafter referred to as "unique region" as it was an exclusive protein encoding region of T21. Furthermore, no significant homology of the *T21* unique region to any other organism in the Ensembl and NCBI databases was observed. It can therefore be considered that *T21* is a transcript of the *CEP290* gene located on chromosome 12q21.32 and also T21 protein prematurely terminates after exon 28 of *CEP290*. This was determined to be a consequence of an alternative splicing event occurring after exon 28 of *CEP290* resulting in the uninterrupted transcription into the intronic sequence. As a consequence, an immediate sequence termination signal gives rise to a truncated T21 protein sequence. These findings were

confirmed experimentally using PCR and northern blotting, giving evidence of a novel, previously unreported CEP290 protein variant containing an alternatively spliced exon. As mentioned, previous reports of disease-related *CEP290* mutations deal primarily with cilliopathies and of the other two studies purporting CEP290 as a TAA (Eichmuller *et al.*, 2001; Chen and Shou, 2001), both instances were shown to be independent of T21, either due to the location of the identified antigen (Se2-2) or the exclusion of the unique 93bp sequence indicating an alternative variant being identified and assessed (3H11Ag).

Although these findings confirmed that previous studies conducted did not discriminate between CEP290 and T21, the identification of the unique region and termination sequence now allowed both transcripts to be analysed independently from each other. These sequence differences allowed the identification of an additional adenine residue present in *CEP290* which was previously omitted from the published *T21* sequence via PCR and sequencing data. Consequently, this bp addition into *T21* drew questions as to the validity of the initiation signal of the encoding region first identified. Evidence suggested that the *T21* sequence started at the ATG start of *CEP290*, indicating a possible shared promotor region. This study failed to fully confirm the upstream sequence using 5' RACE PCR. The intention was to insert cDNA obtained from PCR experiments into a cloning vector to enable DNA sequencing in order to elucidate the entire sequence of *T21* and determine the encoding start sequence. Despite *CEP290* being a well characterised gene, the promotor region remains unknown. Further experimental investigation involving specific promotor silencing is necessary to determine whether *T21* and *CEP290* share the same promotor or are indeed independently regulated.

Using a custom mono-specific polyclonal anti-T21u antibody specific to T21 (targeting the unique sequence), western blot analysis performed on various cancer and normal cell line lysates identified two distinct bands at approximately 90 kDa and 70 kDa. The former was identified to possess the correct conformational structure to bind the antibody and was confirmed to be specific to T21 by both gene silencing and immunoprecipitation of cell lysates followed by western blotting analysis. Attempts to verify the protein sequence of T21 using mass spectrometry was not achieved during this study, however pursuing this line of investigation further would help to confirm the sequence of T21 and provide insight into its interactions with other proteins and reveal any functional roles.

6.3 The emerging role of T21 in tumourogenesis and candidacy as an immunotherapeutic target

Due to the inadvertent inclusion of CEP290 during studies of T21, the functional processes of T21 protein had yet to be clearly determined. As the CEP290 protein had been found to be involved in structural and centrosomal functions, it could naturally be concluded that its presence would be in some way linked to cell proliferation. Uncontrolled cell proliferation is a defining feature of tumours and has been reflected in previous findings using the anti-T21/CEP290 antibody and oligonucleotides. Chapter 4 outlines the specific targeting of T21 during tumourogenesis experiments performed in order to gain insight as to its activity and degree of protein expression for possible application as a target for immunotherapy.

In vitro studies using transient and stable gene silencing of T21 in a $T21^{+}$ prostate cancer cell line demonstrated a significant decrease in cell proliferation. Furthermore, cells treated with interfering agents were also assessed using immunofluorescence and cell cycle analysis revealed a role in promoting cell division, despite showing no strong centrosome localisation during any phase of the cell cycle. Otherwise the expression of T21 protein seemed to be dispersed largely throughout the cytoplasm. These observations require confirming along with additional experiments using cell cycle markers and other cell lines. Further "knock-in" experiments using cell lines that express little or no T21 would also provide can give further evidence of the effect of T21 on cell proliferation and possibly reveal the mechanism(s) behind this observation.

Following successful knockdown (gene silencing) of T21, 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 not share functional activity with CEP290. The up regulation of genes that would promote tumourogenesis through signalling pathways involved in cell proliferation and cell cycle control (*MAPK6*, *PKIA*, *UHMK1*, *CDKN2B*) and survival (*HIPK2*, *MDM2*, *RBBP8*), centrosome and structural rearrangement (*SYCP3*, *CALM3*, *MFAP2* and *RAB3IP*) and immune modulation (*CISH*, *PLAU*, *IL6R*) were upregulated in the presence of T21. In contrast, genes involved

in the regulation of these signalling pathways were suppressed in the presence of T21 through Ras family (*DOK1* and 3 *RAB6B*, *FRS3*), cell stress/DNA repair pathways (*PPM1E, CHD8*, *XRCC3*) or the PI3K-Akt pathway (*PI3KIP1*). In this investigation, it was not possible to conduct an exhaustive post-analysis that would be required to uncover the extent of pathways potentially involved in T21 function. However, the data indicated that T21 activity is independent of CEP290 activity and may have a role in promoting tumourogenesis, thus making it an attractive target for therapy.

To demonstrate T21 as a viable marker of disease and a possible immunological target, mRNA and protein expression was assessed in normal healthy and cancer tissues using *T21*-specfic primers for PCR and by IHC staining using anti-T21u antibody. It was demonstrated that *T21* mRNA expression was high in normal testis, prostate, stomach and spinal cord tissues. In addition, protein expression in normal healthy tissues demonstrated an overall absence or relatively low expression with except to some tissues (kidney, prostate and testis). Expression using commercially available TMAs using malignant tissues demonstrated an overall increase in expression of T21 compared to normal healthy tissues. Further assessment of T21 was performed using a prostate cancer patient TMA series. However, this study failed to demonstrate a significant correlation of T21 protein expression with malignant disease or other clinicopathological variables assessed. One criticism of this study is the limited number of patients available within variable subgroups making it difficult to conduct a full statistical analysis. Expansion of the current TMA or staining of larger numbers of tissue samples may enable further evaluation of T21 expression and its relation to cancer can be determined.

Altered self-antigens are a result of point mutations and chromosomal rearrangement, differential expression and in the case of T21, alternative splicing events leading to intron retentions are a somewhat undervalued source of potential antigens. The advantages of targeting self tumour antigens for immunotherapy are that they may be expressed in many patients and be common to many malignancies. Alternatively, self antigens may only generate low level anti-tumour responses due to censorship during T cell selection (Theobald *et al.*, 1997). Altered self tumour antigens that are recognised by T cells may avoid such selection in the thymus (Houghton, 1994; Nickleson & Kuchroo, 1997).

6.4 Identification of immunogenic T21 specific peptides

The premise of screening T21 for immunogenic peptides was based on a number of factors that indicated T21 to be a good prospective candidate tumour antigen. First, T21 was discovered using SEREX screening implying a pre-existing existing humoral response which could be exploited to evoke an adaptive T cell mediated immune response. Second, the identification of a 31 amino acid residue sequence that is unique to T21 arising from a partial intron sequence and the identification of this region in particular during its discovery indicated the potential immune reactivity towards this region. Finally, prior to this project, T21 was indicated to have a restricted protein expression in normal testis and cancer tissues using the anti-T21/CEP290 Ab (Miles *et al.,* 2007). Tissue restricted expression of T21/CEP290 would avoid trigging auto-immunity against healthy normal cells during immunotherapy. Although re-evaluation of T21 protein expression using anti-T21u antibody (detailed in chapter 4) indicated mild to little expression in normal tissues with elevated expression in only some cancer tissues, work was completed following of immunological studies presented here. Interestingly, CEP290 expression has been shown to be ubiquitously expressed throughout normal tissues however auto-antibodies in cancer patients have been detected (Eichmuller *et al.,* 2001).

Here, the immunogenicity of T21 derived peptides sequences were assessed using a synthetic library of 15mer peptides which overlapped by 10 amino acid residues. Peptide responses were generated using an *in vivo* double transgenic mouse model (HLA.A2/DR1) and *in vitro* studies using human PBMCs derived from prostate cancer patients. Emphasis was placed on the peptides that spanned the unique region in order to identify endogenously processed "immune" sequences capable of generating T21 T cell specific responses. The conclusion from these early studies of immunogenicity in mice highlighted significant responses in OLP 3, 4 and 5, which potentially share common immunogenic endogenously processed epitope(s). As these peptides share sequence similarity, it could be presumed that they represent single/multiple common immunogenic epitopes.

Upon critical assessment of the transgenic studies it is clear from the limited number of repeat experiments performed render these findings preliminary, and further confirmation studies in animals should be undertaken. When assessing T cell reactivity against the panel

of peptides, the number of cells available from *in vitro* culture proved to be a limiting factor. The release of IFNγ to assess responses following peptide stimulation captured an overall immune "snap-shot" of anti-tumour immune activation and differentiation (Schoenborn & Wilson, 2007). Further assessment of other cytokines such as IL-2 and TNFα, T cell activation markers (Caruso *et al.,* 1997) and cytotoxicity assays would strengthen the existing data and confirm which sub-populations of cells respond to T21 peptides. Given that the T21 unique region is not naturally presented and processed in mice, a T cell response against peptides of this region may not reflect the challenges of breaking tolerance, which is associated with other tumour antigens in the human host. Assessment of peptides of the unique region using human PBMCs identified OLP 4 as a potentially immunogenic peptide. In a study using peptide 4 a strong immunogenic response in patients responding to DC vaccine therapy (phase I/II trial) was found (Mu *et al.,* 2005). The vaccine itself consisted of autologous DCs transfected with allogeneic mRNA derived from three prostate cancer cell lines. The cell lines used during this trial were shown to express *T21* mRNA therefore it could be inferred that patients may have been exposed to T21 derived antigens during vaccination and respond to T21.

Interestingly, the peptide 4 generated a T cell response was demonstrated in patients that responded (no new lesions of malignant disease, or development of symptoms consistent with metastatic disease in addition to decrease or stable PSA over baseline) to DC therapy during the trial. However, the patient HLA type was not available during this trial to confirm whether the peptide 4 response was HLA-restricted. One of the major advantages of using long peptides, were epitopes would require processing and editing by the host immunoproteasome, is the potential to induce long-lived T cell responses and extend overall patient survival (Welters *et al.,* 2008; Leffers *et al.,* 2009; Speetjens *et al.,* 2009). As alluded to in chapter 1.4, once identified tumour specific epitopes capable of directing CTL and T helper responses can be employed in many immunotherapeutic scenarios, including peptide vaccines, peptide stimulated cellular therapies and in TCR engineering approaches among others (Bijker *et al.,* 2008; Murphy *et al.,* 1999; Powell *et al.,* 2006). The limited, yet encouraging results, demonstrated here may suggest that human T cells are able to evoke T21 specific immune responses. The studies presented here using HLA.A2/DR1 transgenic mouse model together with *in vitro* re-stimulation of patient PBMCs, provides preliminary evidence for the focus of future immunogenic study of T21, suggesting a route to transitional clinical immunotherapy.

6.5 Conclusion and future work

Immunotherapy represents a promising means for targeting cancer. The identification and assessment of candidate tumour associated antigens are crucial in forming the basis for such targeted therapies. The evaluation of T21 as a tumour antigen and as a potential immunotherapeutic target has been performed and presented here.

From the data, there is strong evidence to suggest that T21 is in fact a transcript variant of the centrosomal gene CEP290. Detailed, *in silico* analysis and experimental confirmation suggests that T21 contains an alternative splicing event resulting in the retention of a 93bp sequence derived from CEP290 intronic region which gives rise to protein, thus making this sequence unique to T21 in humans. Northern blotting analysis suggests that the T21 termination region is the same as identified previously, however during this investigation it was demonstrated that the T21 mRNA sequence may extend further upstream than previously reported. Full confirmation of this will be achieved through T21 product amplification and sequencing.

Using this T21 "unique region" sequence, T21 mRNA and protein was shown to be expressed in some normal tissues including prostate, stomach and spinal cord when compared to testis therefore T21 cannot be considered as a cancer testis antigen as previously suggested. The high level of expression of T21 in normal prostate tissues at both the mRNA and protein level suggests that T21 could instead be classified as a tissue specific antigen. However a histological investigation of T21 protein expression failed to demonstrate significant discrimination between benign and prostate cancer patients or association with other clinicopathological variables. This was a limited study and further investigations using a more extensive TMA series are currently being undertaken, therefore re-analysis of T21 expression in the future may shed new light on its potential as a cancer marker of disease status.

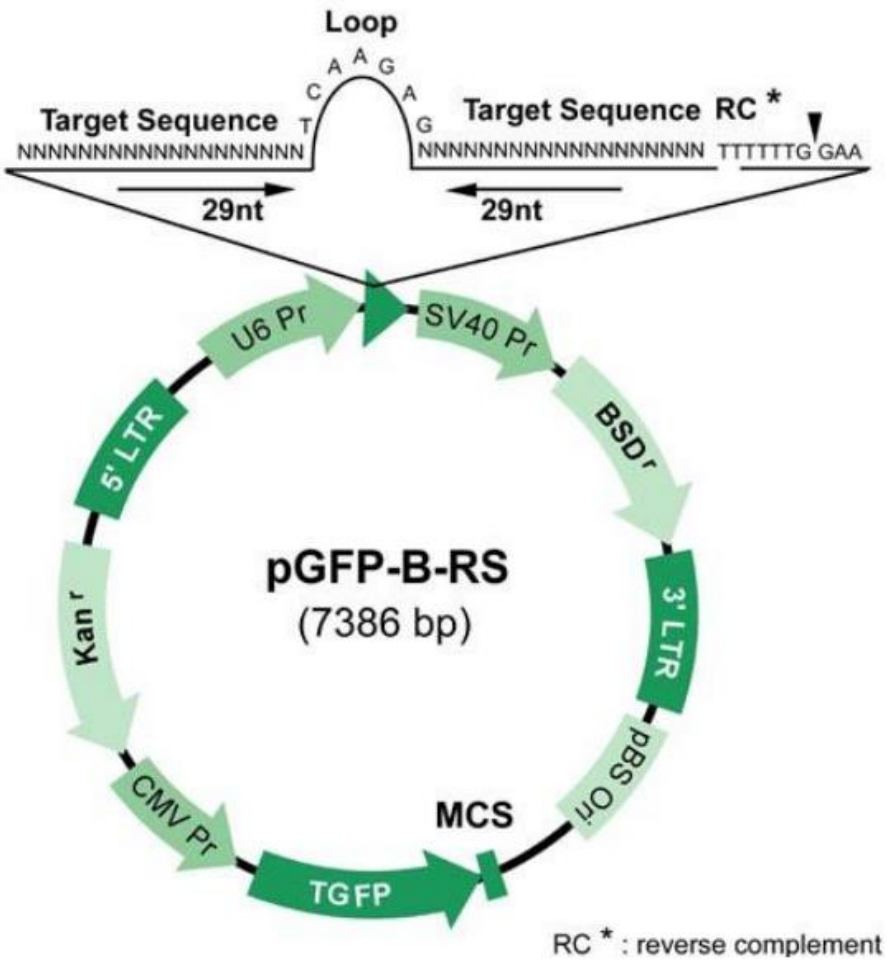
There is also evidence to suggest that T21 activity is independent of that of CEP290. Data collected using immunofluorescence indicated that T21 was not localised to the centrosome, in addition the expression profiling data obtained from Next Generation Sequencing suggest no interactions occur between any known partners of CEP290.

Assessment of cell proliferation following *T21* gene silencing indicated an association between T21 and increased tumour cell proliferation, although further verification is required in order to determine the pathways that are associated with this process. Future analysis and experimental verification of the NGS data described here will be required in order to validate for conclusions relating to T21 activity. The data presented suggests a role in cell proliferation and survival, modulation of immune responses and suppression of regulatory processes involved in tumourogenesis.

Finally, this study identified a novel immunogenic and naturally processed peptide target using a transgenic murine model and further verified this peptide sequence to be of significance using human protein cancer patient PBMCs. Future screening of healthy and confirmed prostate patient PBMCs may validate T21 derived peptides as candidate targets for immunotherapy. In parallel, further experimental investigations are required to determine the nature of the immune response and T cell subsets stimulated by T21 derived peptides and whether these can be used to induce tumour killing *in vivo*.

In summary, this study set out to characterise SEREX-defined antigen T21 in order to ascertain its value as a diagnostic marker through molecular expression analysis and functional studies. A final objective was to determine its candidacy as an immunological target for immunotherapy. The results presented here indicate that T21 is associated with tumour and may therefore be classified as a differential tumour antigen and that immunogenic peptide regions specific to T21 identified here can be taken forward for potential study for the incorporation into a cancer vaccine.

Appendix I



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

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

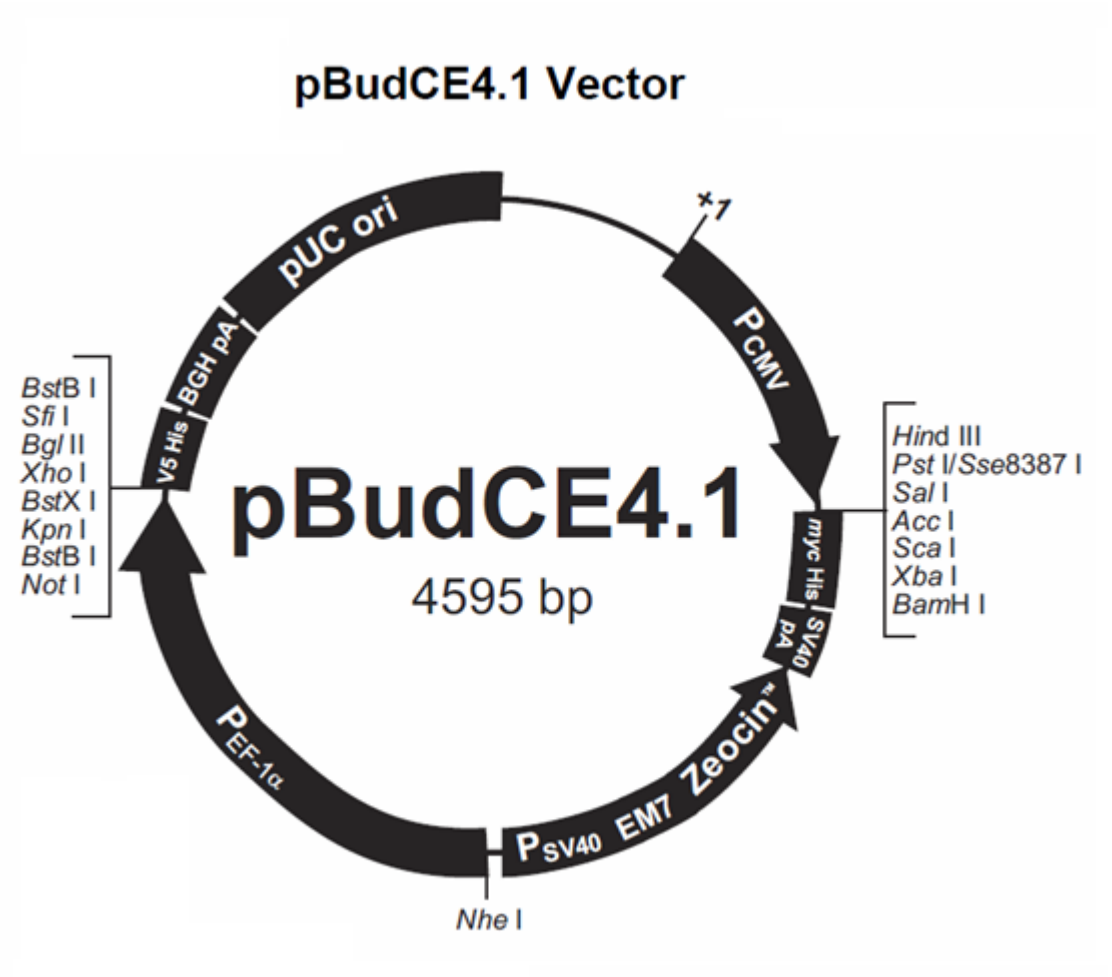


Figure Ib - pBudCE4.1 mammalian expression vector (Invitrogen). *Insertion of T21 CDS was performed previously by double digestion using HindIII and Xba1 restriction enzymes and ligation using T4 DNA ligase (Promega). Vector contains a Zeocin resistance gene for antibiotic selection following cell transfection.*

Appendix II

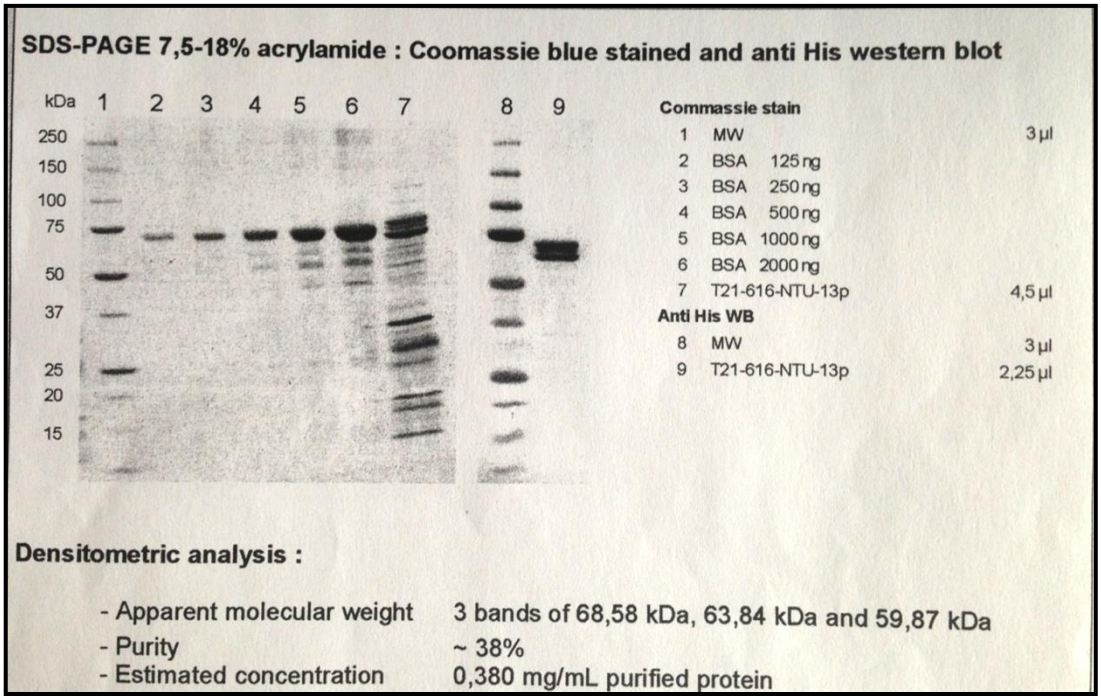


Figure II – Custom recombinant protein SDS PAGE (purchased from GTP Technology). *Accompanying datasheet showing coomassie blue stained T21 elution (lane 7) and western blotting analysis using anti-His (lane 9). Apparent molecular weight between 68.58 - 59.87 kDa.*

Appendix III

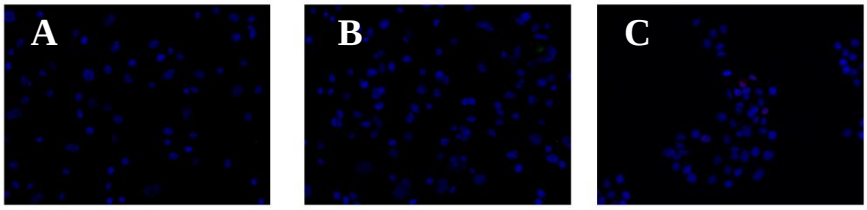


Figure IIIa – Immunofluorescence images showing assay control wells. *PC3 cells were stained using primary anti-T21Ab only (**A**), primary anti-IgG Rabbit antibody + secondary antibody (**B**) or secondary antibody alone (**C**) to ensure fluorescence observed was antibody specific. On each occasion, appropriate control experiments such as these were performed alongside all antibody staining with each cell line. Images acquired using fluorescence microscope (Olympus) - magnification x10.*

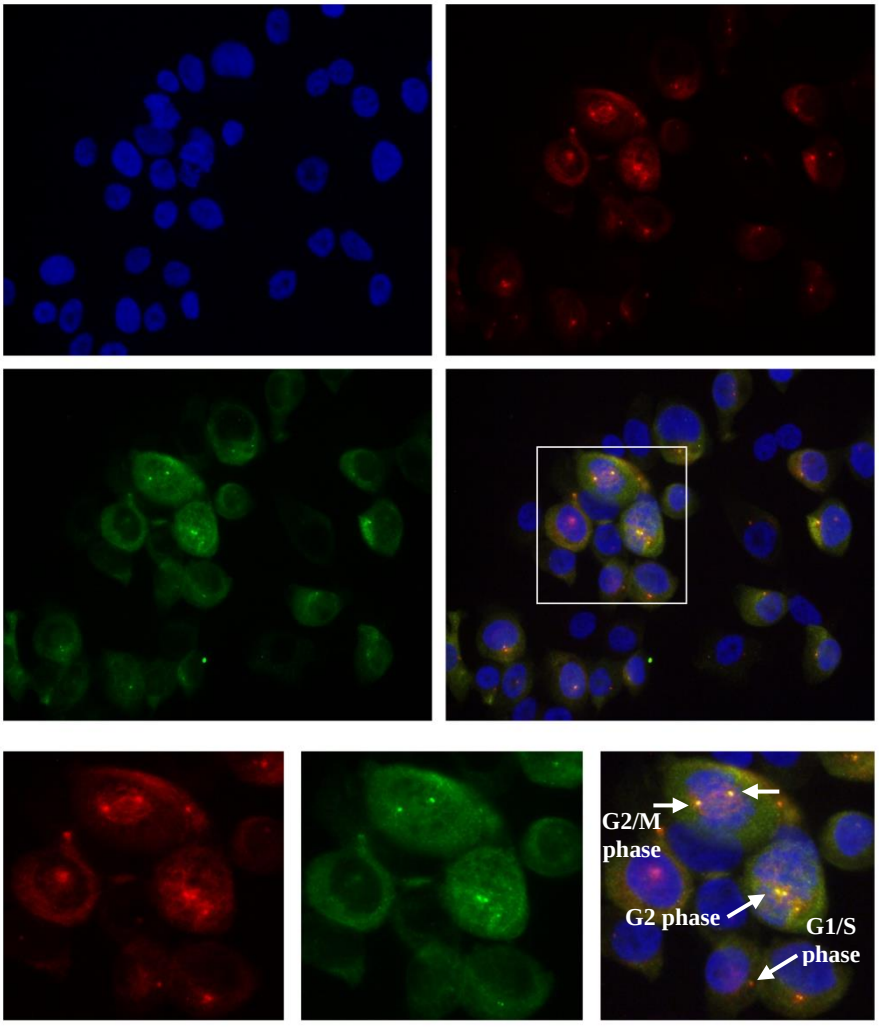


Figure IIIb – Immunofluorescence analysis showing localisation of T21/CEP290 in OPCT-1 cell line. *OPCT-1 cells were stained using anti-T21/CEP290 antibody (green) in order to confirm its protein localisation to the centrosomes using anti-pericentrin antibody (red) and nuclear staining using DAPI (blue). Images acquired using fluorescence microscope (Olympus) Magnification x20 and x40 respectively (n=3).*

Appendix IV

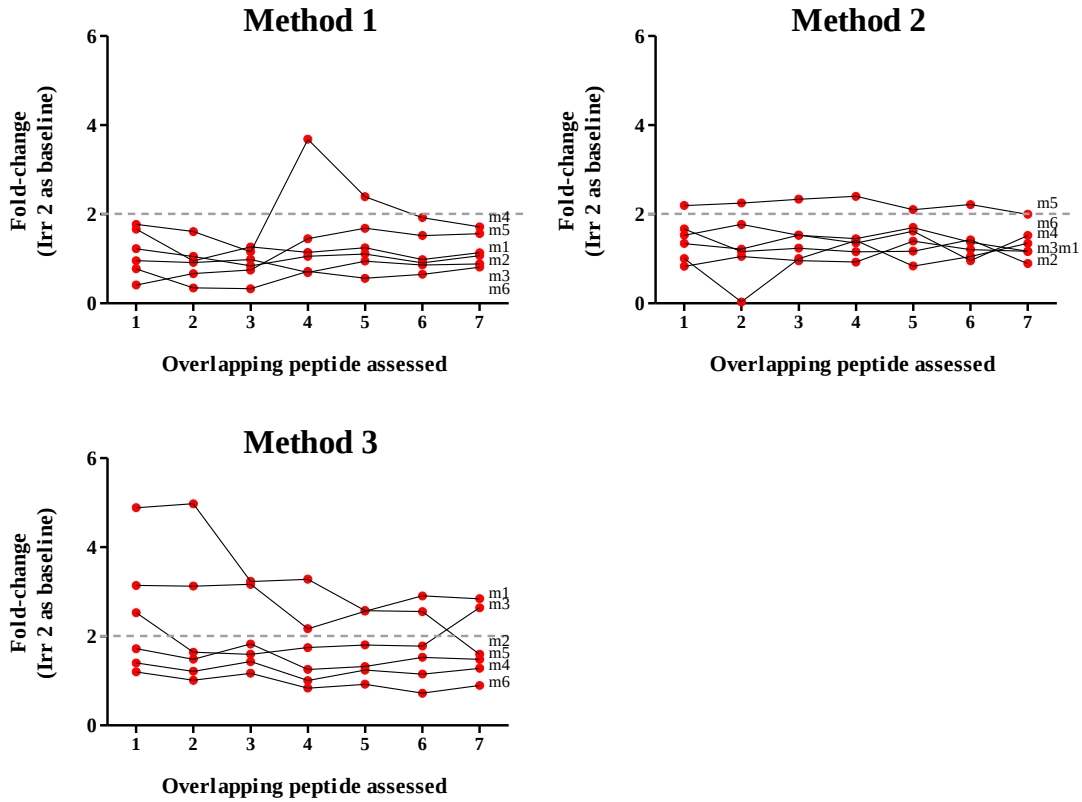


Figure VI – Immunogenic responses of T21 unique region peptides. *Positive responses determined as minimal 2-fold change compared to irrelevant 2 used as baseline. Assays were performed on T cells generated from 6 animals immunised with T21 cDNA using gene gun immunisation (previously described) harvested and restimulated in vitro using individual peptides and assessed for T cell function using 3 restimulation methods (described previously). Graphs annotated to represent individual animal responses to peptides represented as mouse 1 (m1), mouse 2 (m2) and so on to mouse 6 (m6).*

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All sequence and *in silico* BLAST searches correct as of 23 MAY 2013