# Functional identification and mapping of a gene that represses telomerase *hTERT* transcription in prostate cancer cells

**A thesis submitted for the degree of Doctor of Philosophy**

**By**

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# Abstract

Telomerase is present in over 90% of tumour tissues and immortalized cells and is tightly regulated in most normal somatic cells. This suggests the existence of regulatory mechanisms repressing telomerase in normal cells that somehow have become inactive during cancer development. In this project, I used genetic complementation in the form of microcell-mediated monochromosome transfer (MMCT) to search for chromosomes that repress telomerase activity in a prostate cancer cell line, PC-3. Microcell hybrids generated by introducing normal human chromosome 11 strongly inhibited telomerase. Telomerase is regulated primarily at the level of *hTERT* transcription, its catalytic subunit. Consequently, *endogenous hTERT* mRNA levels were measured by quantitative RT-PCR in microcell hybrids generated by transferring normal human chromosomes into a PC-3 sub-clone (PC-3/hTERT) ectopically expressing *hTERT* cDNA to prevent senescence. Only hybrids constructed with transferred chromosome 11 showed strong transcriptional repression of *hTERT*. Next, hybrids were constructed by the MMCT transfer of chromosome 11 fragments (X-ray-induced). FISH analysis of clones with completely silenced *endogenous hTERT* transcription revealed in all cases a discrete chromosome 11 fragment with both the p-arm and q-arm material. A randomly selected *hTERT*-repressed clone was treated with ganciclovir to select against the HyTK marker and reverse the phenotype. *hTERT* expression in majority of GCV-resistant clones returned to levels comparable to the parent PC-3/hTERT cells. Collectively, these results provide strong functional evidence for the presence of a powerful telomerase repressor sequence on the fragment. Transfer of one repressive fragment back into mouse A9 cells was then carried out to facilitate fine-structure

mapping of its sequence content. High density STS mapping of the fragment in each of the clones revealed a considerable DNA content heterogeneity across the panel. These content maps, together with a further round of MMCT to confirm *hTERT*-repressive activity, enabled me to identify three candidate regions on the q-arm of chromosome 11 where the repressor sequence may be located: the first region lies between map positions 64.70Mb to 65.42Mb and the other two regions each flank a single positive STS marker at 69.71Mb and 127.32Mb. *KAT5*, a histone modifying gene has been identified as a potential candidate for repressing *hTERT*.

## Declaration

I hereby declare that this thesis and the work presented in it are my own and have been generated by me as the result of my own original research. All work carried out in collaboration has been acknowledged.

Signature……..

Date……..

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# Abbreviations

| | |
|---|---|
| ATL | Alternative telomere lengthening |
| BICGP | Brunel Institute of Cancer Genetics and Pharmacogenomics |
| BPH | Benign Prostatic Hyperplasia |
| CaP | Prostate cancer |
| cDNA | Complementary DNA |
| CGH | Comparative Genomic Hybridization |
| Ct | Threshold Cycle. |
| dATP | Deoxyadenine triphosphate |
| dCTP | Deoxycytosine triphosphate |
| DEPC | DiEthyl PyroCarbonate. |
| dGTP | Deoxyguanine triphosphate |
| DMEM | Dulbecco's Modified Eagle's Medium. |
| DMSO | DimethylSulfoxide. |
| DNA | Deoxyribonucleic acid |
| Dnase I | Deoxyribonuclease I. |
| dNTP | deoxynucleotide triphosphate |
| DRE | Digital Rectal Examination |
| DTT | Dithioeithorol |
| dTTP | Deoxythymidine triphosphate |
| EDTA | Ethylene-diamine-tetra-acetic acid |
| EDTA | Ethylenediaminetetraacetic Acid. |
| FBS | Fetal Bovine Serum. |
| FISH | Fluorescence In Situ Hybridization |
| GAPDH | Gultaldehyde phosphate dehydrogenase |
| GCV | Ganciclovir |
| HAT | Histone Acetyltransferase |
| HDAC | Histone Deacetylase |
| HPV | Human papilloma Virus |
| hTERT | human telomerase reverse transcriptase |
| hTR | human telomerase RNA template |
| Hyg B | Hygromycin B |
| HyTK | Hygromycin phosphotransferase-Thymidine Kinase fusion gene. |
| KAT5 | K(lysine) acetyltransferase 5 |

| | |
|---|---|
| Kb | Kilobase. |
| KCl | Potassium Chloride |
| LOH | Loss of Heterozygosity |
| Mb | Megabase. |
| MEN-1 | Multiple Endocrine Neoplasia type 1 |
| MMCT | Microcell-Mediated Chromosome Transfer |
| NCBI | National Center for Biotechnology Information. |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| PEG | Poly Ethylene Glycol |
| PHA-P | Phytohaematogglutinin-P |
| PIA | Proliferative Inflammatory Atrophy |
| PIN | Prostatic Intraepithelial Neoplasia |
| PMSF | PhenylMethylSulphonylFluride |
| PSA | Prostate Specific Antigen |
| Rb | Retinoblastoma gene. |
| RNA | Ribonucleic acid |
| Rpm | Revolutions per minute. |
| RQ | Relative Quantification. |
| RT-PCR | Reverse Transcription Polymerase Chain Reaction. |
| SETD2 | SET Domain Containing protein 2 |
| SFM | Serum Free Medium. |
| STS | Short Tagged Sequences. |
| SV40 | Simian Virus 40 |
| TBE | Tris Borate EDTA |
| TRAP | Telomerase Repeat Amplification Protocol |
| TSG | Tumour Suppressor Gene. |
| XMMCT | Irradiated Microcell Mediated Chromosome Transfer |

## Background and the aim of the project

Telomeres are special nucleoprotein structures consisting of tandem repeats of DNA hexamers at the end of linear human chromosomes (Moyzis et al., 1988). Normal somatic cells progressively lose their telomeric repeats with each cell division due to incomplete DNA replication (Harley et al., 1990). When the telomeres become critically short the cells senesce and this is thought to act as a tumour suppressor mechanism in normal cells (reviewed in Campisi and di Fagagna, 2007). However, immortal and cancer cells overcome the end replication problem by expressing telomerase. Telomerase is a ribonucleoprotein that can add hexameric repeats of nucleotides to the ends of chromosomes during replication and maintain telomere length (Greider and Blackburn., 1985). Telomerase activity is detected in over 90% of all human cancers and it is undetected in normal somatic cells (Kim et al., 1994) except in rapidly self renewing tissue cells such as stem cells, human germ cells, lymphocytes and endometrial cells (reviewed in Kyo and Inoue, 2002). Therefore, telomerase has become a preferential target for anticancer drug development.

In humans, the two main components of telomerase are the RNA template (hTR) and the catalytic subunit which is a reverse transcriptase (hTERT). The human telomerase RNA template is ubiquitously expressed in most cells (Feng et al., 1995) whereas *hTERT* is exclusively expressed in immortal cells and cancerous tissues (Kim et al., 1994). Thus hTERT is the limiting component in controlling telomerase activity. A strong correlation between telomerase activity and *hTERT* transcription exists in numerous cancers (Meyerson et al., 1997). This suggests that *hTERT* repressors are present in normal somatic cells that are inactivated in cancerous

tissues. Human TERT expression is predominantly regulated at the transcriptional level but differential splicing of the *hTERT* transcript and post-translational modification of the hTERT protein also contribute to its regulation (reviewed in Kyo and Inoue, 2002). Since the cloning of the upstream *hTERT* proximal promoter, many transcription factors have been identified that regulate its expression either directly or indirectly (Kilian et al., 1997; Cong et al., 1999). Several groups have identified regions on normal human chromosomes that may harbour *hTERT* repressor sequences in various cancers by using the microcell mediated chromosome transfer (MMCT) technique (Horikawa et al, 1998; Cuthbert et al., 1999; Nishimoto et al., 2001; Steenbergen et al., 2001; Backsch et al., 2001). Our own group has used this methodology to identify a region on chromosome 3p that represses *hTERT* mRNA expression in 21NT, a breast ductal carcinoma cell line (Cuthbert et al., 1999).

In our laboratory, MMCT is a well established technique that allows individual transfer of normal human chromosomes, tagged with the selectable HyTK fusion gene, into any recipient cell line. We have available a complete panel of normal human:mouse monochromosomal hybrids, in which the individual human chromosome exists as an intact stable entity in a mouse background. The introduced chromosome is maintained in the recipient cells by drug selection. The selectable HyTK marker provides a potent tool in the search for novel genes by function analysis, since it can be selected “in” by culturing the cells in hygromycin B or selected “out” by treating the cells with ganciclovir (Cuthbert et al., 1995). Different chromosomes have been implicated in various cancers indicating that telomerase regulation is tissue specific (Horikawa et al, 1998; Cuthbert et al., 1999; Nishimoto

et al., 2001; Steenbergen et al., 2001; Backsch et al., 2001). Chromosome(s) that may carry a gene for repressing telomerase are yet to be identified for prostate cancer. Therefore, the aim of this project was to identify gene(s) on normal human chromosome(s) that repress telomerase activity involved in prostate cancer. In the present study, I made use of the MMCT technique to individually transfer normal human chromosomes of the complete donor panel into PC-3, a prostate cancer cell line. This potentially permitted me to screen the whole genome in search of gene(s) that may repress telomerase activity. Furthermore, use of molecular and cytogenetic techniques allowed me to identify the regions on the transferred normal human chromosome where the telomerase repressor sequence may be located.

# Chapter 1

## General introduction

### 1.1 The prostate gland

The prostate gland is a male muscular organ situated directly below the bladder in front of the rectum. At birth the prostate gland weighs only a few grams and it is roughly the size of a pea. Enlargement to adult size starts at puberty from the effects of androgen hormones and stops at the age of about 20. The approximate size of a normal adult prostate gland is 1.5inches long. The prostate gland consists of approximately 30-50 smaller glands and stroma that are surrounded by a fibrous tissue known as the prostatic capsule. The main function of the prostate gland is to produce secretions that form part of the semen; the rest of the seminal fluid is produced by a pair of glands, attached to the prostate, called seminal vesicles (http://library.med.utah.edu; http://www.upmccancercenters.com).

The prostate gland can cause major problems in men above the age of about 50, enlargement of the gland being the main medical concern. Since the urethra is completely surrounded by the prostate gland, enlargement of the latter restricts the flow of urine as the urethra gets squeezed. Both benign prostatic hyperplasia (BPH) and prostate cancer result in enlarged prostate, often showing similar symptoms. (http://www.upmccancercenters.com).

### 1.1.1 Prostate Cancer (CaP)

There has been an enormous increase in incidence rates of prostate cancer over the past 20 years and this has made CaP the sixth most common cancer in the world and the third most common amongst men. In Europe, North America and some parts of Africa it is the most commonly diagnosed malignant neoplasm and the second leading cause of cancer deaths in USA and Europe (Kumar et al., 2004; reviewed in Gronberg, 2003). The clinical behaviour of CaP is extremely unpredictable. The most typical features of CaP include its very slow growth compared with breast or colon cancers. CaP is highly associated with age, typically diagnosed in men aged over 70 years. Other distinct traits of CaP include heterogeneity in its morphology and genotype, suggesting the involvement of multiple pathways in its development, (reviewed in Bostwick et al., 2004).

#### 1.1.1.i Symptoms, diagnosis, prognosis and treatment

Most early prostate cancers cause no symptoms but progression of the disease can cause changes in urination flow, frequency of urination, pelvic, hip or back pain. Nevertheless these symptoms may also occur in other diseases not related to CaP (Cancer Research UK, website). The two most important tests used in conjunction with each other to diagnose early CaP are the prostate specific antigen (PSA) blood test and the digital rectal examination (DRE). However, the changes observed by these tests are not specific for prostate cancer and, in the event of elevated PSA and/or abnormal DRE, a needle biopsy has to be performed. The prostate specific antigen (PSA) blood test has been used widely for over 25 years to diagnose CaP but it fails as a reliable test because it often leads to false positive and false negative

results. PSA is not a specific test for CaP as elevated levels are also observed in individuals with benign prostatic hyperplasia (BPH) and prostatitis. BPH is the enlargement of the prostate gland, a non-cancerous condition associated with aging and prostatitis is the inflammation of the prostate gland (Cancer Research UK, website). There is no specific PSA level that can distinguish CaP from BPH (Thompson et al., 2005). Therefore, men with high PSA levels need to undergo repeated biopsies to rule out CaP as these individuals are at a greater risk of developing CaP as they get older. A need for a more specific test to evaluate the requirement of biopsies in these patients has led to the recent development of the PCA3 (prostate cancer gene 3) molecular biomarker. The PCA3 gene encodes a prostate-specific mRNA that is highly over expressed in CaP cells compared with BPH (Marks et al., 2007). The PCA3 gene is detected in the nuclear material collected from cancer cells that have been shed into the urine after the prostate is massaged three times on each lobe during DRE (Groskopf et al., 2006). The PCA3 urine assay has very recently been introduced clinically in the UK.

Patients with localised CaP show considerable heterogeneity in biological aggressiveness and prognosis. Even though CaP is normally an indolent disease, 25-30% of the tumours behave aggressively (Crawford 2003). Presently there is no accurate way to predict the course of the disease in individual patients and no consensus on the most appropriate means of treating localized disease. However, radical prostatectomy is an effective treatment for patients whose cancer is confined to the prostate and has not spread to the seminal vesicles. Over 90% of men remained cancer free five years after the surgery (http://www.upmccancercenters.com).

Radiation therapy is used to treat patients whose cancers are confined to the prostate gland or has spread to nearby tissues. It is also used in advanced forms of cancers to reduce the size of the tumour. Androgen deprivation is the main treatment given to patients with locally advanced and early metastatic disease. Initially, between 70-80% of the patients respond to the therapy but the tumour eventually becomes hormone independent and more aggressive, leading to a poor prognosis (Crawford 2003; Cancer Research UK, website).

### 1.1.2 Epidemiology of prostate cancer

There are 32,000 cases of prostate cancer diagnosed in the UK every year, which is 12% of all cancers, causing 10,000 deaths. One in every 4 male cancers diagnosed in the UK is prostate cancer making it the most commonly diagnosed cancer in men. This may be explained by the fact that men are living longer and that there is enhanced usage of the PSA test (Cancer Research UK, website). Various exogenous (diet, geography and socio-economics situation) and endogenous (hormonal imbalance, epithelial and stroma interaction and family history) risk factors contributing to prostate cancer development have been suggested (Bostwick et al., 2004; Crawford, 2003).

#### 1.1.2.i Age, ethnicity and diet

Age is the major risk factor with incidence rate increasing sharply as men get older. The highest numbers of cases diagnosed in men are between the ages of 70 and 79 and CaP is rarely found in men under the age of 50 (Crawford, 2003; Cancer

Research UK, website). Prostate cancer is usually evident in men in their 60s or 70s even though pre-neoplastic lesions known as prostatic intraepithelial neoplasia (PIN) can be found in younger men and are quite common in men in their fifties. The incidence of PIN is much more extensive, affecting 1 in 3 men compared to the incidence of carcinoma which affects approximately 1 in 9 men. Morphological changes related to the initiation of the disease are commonly observed, occurring early in life whereas advancement of the disease to metastatic state affects a fewer number of older men (reviewed in Abate-Shen and Shen, 2000).

Geographical distribution of CaP cases also varies considerably, with the highest incidences of CaP in the western world and lowest in Asia. USA has twice the incidence rate of CaP than that of the UK but this may be due to higher usage of PSA testing in the USA (Collin et al., 2008; Shibata and Whittemore, 2001). Within the USA, African Americans have a higher incidence rate of CaP than white Americans, and the Chinese and Japanese have the lowest rate in the world, leading to the idea that dietary and environmental factors may play a significant role in prostate carcinogenesis (reviewed in Bostwick et al., 2004; Cancer Research UK, website). Effects of diet and environment have been shown by studies conducted on Japanese immigrants to the USA. The risk of prostate cancer correlated with the age of the individuals at the time of relocation to the USA and the period spent in the new environment (Cook et al., 1999). Adapting to a Western lifestyle has been proposed to account for higher risk of prostate cancer, especially high intake of food rich in saturated fat such as red meat and dairy products which are known to increase prostate cancer incidence and mortality (reviewed in Bostwick et al., 2004). High

intake of soybean, which is rich in isoflavones such as genistein and daidzin, is thought to possibly be related to low incidences of prostate cancer in Japan. It has been proposed that tyrosine-specific kinases, which are proteins involved in cell proliferation and transformation can be inhibited by genistein and thus constrain the progression of prostate tumours (Akiyama et al., 1987). It has been reported that daily intake of selenium, mainly found in grains and fish may have protective effects against prostate cancer in humans. Long term supplementation with α tocopherol (a form of vitamin E) has also been reported to lower the risk of CaP (Shirai et al., 2002; reviewed in Gronberg, 2003).

#### 1.1.2.ii Mortality

Significant variations exist between mortality rates due to CaP throughout the world. High rates of mortality are observed in the USA especially amongst the African Americans while mortality rates are much lower in China and Japan. African American men have more than double the rate of mortality compared to white Americans and approximately 10 times greater mortality than that for men in Hong Kong and Japan (Bostwick et al., 2004). This variation in the mortality rate may be explained by the differences in socioeconomic status of the patients which seems to be higher in lower status individuals. However, when healthcare is equally available to the African American men and white men, certain patient population may not avail the opportunities equally (reviewed by Hass and Sakr, 1997). In the UK 10,209 deaths were recorded in 2004 which was 13% of all male cancer deaths. Ninety three per cent of the prostate cancer deaths were in men aged 65 years or more (Cancer Research UK, website). Mortality has decreased in the USA and the UK, but the rate

of decline is much faster in the USA than in the UK. This could be due to intensive use of the PSA test for screening and monitoring the therapeutic efficacy or it may be due to the differences in the treatment of the disease or both (Collin et al., 2008; Shibata and Whittemore, 2001). The fact that most men with prostate cancer die from other causes rather than directly from it is well established. There is a 3% risk of an American man dying of prostate cancer and a 72% risk of dying with the disease but of unrelated causes (reviewed Bostwick et al., 2004). The main predictors of death due to prostate cancer is related to the disease being diagnosed at a young age, being of African American race, or disease at an advanced stage when diagnosed (Crawford, 2003).

### 1.1.2.iii Genetic susceptibility

The chance of developing prostate cancer doubles for individuals that have either their father or a brother affected by the disease. The risk is further increased if multiple relatives have prostate cancer and the onset of the disease is usually 6-7 years earlier in life compared with the sporadic form of the disease (Carter et al., 1992). Approximately 40% of patients of less than 55 years of age have hereditary CaP when diagnosed with the disease. Hereditary prostate cancers account for 5-10% of the disease incidence and there are no known clinical differences between hereditary and sporadic prostate cancer (Bratt, 2002). Familial prostate cancer is thought to occur from inheriting one or more susceptibility genes or being exposed to the same environmental factors (Crawford, 2003; Gronberg, 2003). Linkage analysis and refined mapping has identified 3 strong candidate genes for hereditary prostate cancer (HPC). *RNASEL* (1q25), believed to be a tumour suppressor gene involved in

regulating cell proliferation and apoptosis is found within the HPC1 (1q24-25) locus (Carpten et al., 2002). The other germline mutations are in the *MSR1* (8p22-23) and *HPC2/ELAC2* (17p12) genes. The involvement of these 3 putative susceptibility genes was very infrequent in sporadic CaP (Porkka and Visakorpi, 2004).

Linkage analysis and epidemiological studies of familial prostate cancer have identified other susceptibility loci on chromosomes X, 8, 17 and 20, but these loci have not yet been linked to any specific candidate genes (Simard et al., 2002). The search for CaP susceptibility genes has been difficult because the disease is usually diagnosed at an advanced age which often means it is impossible to obtain DNA samples from living relatives of more than one generation; also problematic is the complexity in identifying hereditary and sporadic forms of the cancers in individuals with high risk phenotypes and genetic heterogeneity (Simard et al., 2002).

Recently, Eeles et al (2008) employed genome-wide association study (GWAS) to investigate genetic susceptibility in CaP patients that were either diagnosed with the disease at ≤60 years or had family history of the disease. The control for this study consisted of individuals with very low PSA levels of <0.5ηg/ml. The authors analysed over half a million SNPs (single nucleotide polymorphisms) in the two populations and they identified seven new loci on chromosomes 3, 6, 7, 10, 11, 19 and X associated with CaP. In these new regions, the authors identified three candidate susceptibility genes for CaP. These are *MSMB* (microseminoprotein beta- which codes for an immunoglobulin binding factor family and is synthesized by the epithelial cells of the prostate) on chromosome 10, *LMTK2* (lemur tyrosine kinase 2)

on chromosome 7 and *KLK3* (kallikrein-related peptidase 3) on chromosome 19. It has been proposed that these candidate genes and their products may be used to screen CaP and potentially be used as therapeutic targets.

### 1.1.2.iv Effects of hormones on prostate cancer

There is strong evidence to suggest that steroid hormones play a significant role in the pathogenesis of prostate cancer but the precise mechanisms by which androgens affect this process is not clear. The growth and differentiation of the prostate gland which is composed of stromal and epithelial cells is regulated by androgens. The enzyme 5-alpha-reductase reduces testosterone to dihydrotestosterone (DHT), an active metabolite that controls the development of the prostate (reviewed in Haas and Sakr, 1997). Inhibition of the enzyme 5-alpha-reductase causes benign prostatic hyperplasia (BPH) whereas androgen ablation either surgically or with hormone agonists is used as therapy for advanced prostate cancer (reviewed by Crawford, 2003). Men who have congenital abnormalities in androgen metabolism do not develop prostate cancer or BPH nor do those men who were castrated before puberty (Haas and Sakr, 1997). Conflicting studies have been reported on the plasma levels of testosterone and DHT in prostate cancer patients and healthy controls of similar age (Ghanadian et al., 1979; Meikle and Stanish, 1982). In one study, African American men had 15% higher serum testosterone level compared with white Americans, reflecting increased risk of prostate cancer in that ethnic group (Ross et al., 1986). In another, Japanese men had lower levels of 5-alpha-reductase and therefore decreased levels of DHT compared with American men (Ross et al., 1992). Recently, Marks et al (2006) investigated serum and tissue testosterone and DHT

levels in normal African American men and white men and found no significant difference between the two groups.

### 1.1.3 Pathological and molecular mechanisms underlining prostate cancer

The molecular pathology of prostate cancer is extremely complex, involving multiple genes and environmental factors. Currently, the cellular and molecular events associated with the initiation, development and progression of the disease are unknown. However, identification of genetic alterations involved in these stages of CaP is gathering pace with the aid of modern molecular tools, raising expectations that soon it will be possible to distinguish the indolent cancers from the life threatening aggressive tumours by molecular analysis of suitably validated biomarkers (Hughes et al., 2005). Inherited prostate cancer is estimated to constitute less than 10% of CaP with the majority of cases being sporadic (Konishi et al., 2005). Therefore, only the most consistent cellular, biochemical and molecular changes that occur in sporadic prostate cancer will be reviewed here.

### 1.1.4 Cellular biology of benign prostate growth

Morphological characterization of prostate epithelium has revealed the existence of three distinct types of cells (Fig.1.1). The most prevalent cell type is the androgen dependent secretory luminal cell which secretes prostatic proteins such as PSA (prostate specific antigen). These cells express androgen receptor (AR), cytokeratins 8 and 18 and CD57 (a surface marker-cluster differentiation 57). The second epithelial cell type is the basal cell; these are situated between the luminal cells and

the basement membrane of the prostate gland. Basal cells mediate attachment to the stroma and they express p63 (a nuclear protein that has high homology to the tumour suppressor protein p53), cytokeratins 5 and 14, CD44 and low levels of AR; these do not secrete prostatic proteins. The third type of prostatic epithelial cell is the neuroendocrine cell; these support the growth of luminal cells. Neuroendocrine cells are androgen independent and express serotonin and several other peptide hormones (reviewed in Abate-Shen and Shen, 2000). There are now reports describing the existence of certain prostatic epithelial cells that co-express both luminal and basal associated markers, the prostate stem cell antigen marker first appears on these rare intermediate cells (Kelly and Yin, 2008). Stem cells express high levels of p63 and its expression by the basal cells has led to consensus that epithelial stem cells reside in the basal compartment of the prostate (Tokar et al., 2005).

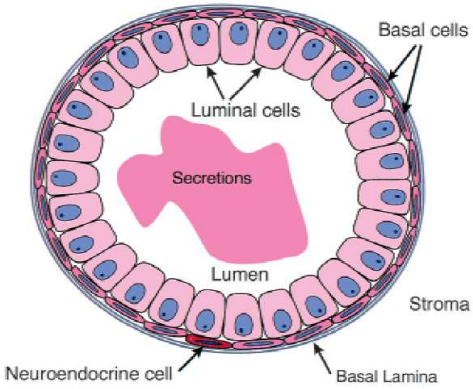



Figure 1.1: Different types of cells found in the human prostatic duct. The neuroendocrine cells are morphologically identical to the basal cells. Diagram reproduced from Abate-Shen and Shen, 2000.

Even though the three types of prostate epithelial cells differ in their marker expression and biological function they do originate from common pluripotent stem cells that are located in the basal cell layer (reviewed by Konishi et al., 2005; Abate-Shen and Shen, 2000; Foster et al, 2000). In normal prostate, there are only a small

number of neuroendocrine cells scattered along the basal cells. However, increased numbers of cells with neuroendocrine differentiation are present in more aggressive forms of prostate cancer (Cussenot et al., 1998).

### 1.1.5 Initiation and progression of prostate cancer

Prostatitis, Benign prostatic hyperplasia (BPH) and adenocarcinoma are the three major forms of prostatic disease. Although prostate cancer patients often have inflammation of the prostate, association between the two has not been clearly demonstrated (De Marzo et al., 2004; Karan et al., 2003). Seventy percent of CaP occurs in the peripheral zone of the prostate with 10-20% occurring in the transition zone and 5-10% arises in the central zone (Fig 1.2), whereas BPH (which is not a pre-malignant lesion or a precursor of prostate cancer) mainly evolves in the transition zone (Konishi et al., 2005).

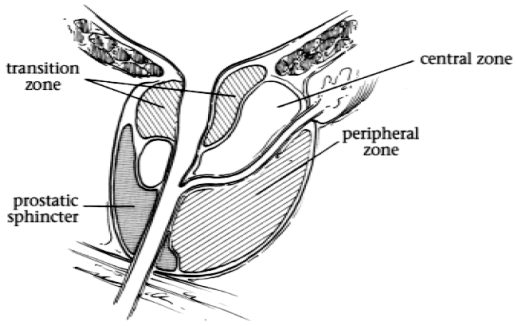



Figure 1.2: Anatomy of the human prostate. Diagram reproduced from Abate-Shen and Shen, 2000.

Prostate cancer in its initial stage is confined to the prostate gland and is androgen dependent. At this stage, the cancer is normally curable by surgical and/or radiation therapy. In most men, the disease is indolent but the aggressive form of CaP first invades the seminal vesicles and then metastasises to other organs, especially to the

regional lymph nodes and the bone system. Metastatic cancer is generally androgen independent and is the main cause of mortality, with the cancer spreading to bones in more than 70% of patients (Karan et al., 2003; Abate-Shen and Shen, 2000). Prostatic intraepithelial neoplasia (PIN) is the first morphologically recognizable stage of prostate cancer. Both PIN and advanced forms of CaP are multi foci lesions and have similar chromosomal abnormalities (Konishi et al., 2005). Genetic alterations in PIN have been linked to the development of CaP (Foster et al., 2000). PIN is normally divided into high and low grade using the Gleason grading system, with the high grade associated with the metastatic disease. However, high grade PIN is not an in situ carcinoma, in spite of being so closely associated with CaP, because PIN has intact basement membrane and does not invade into the stroma and produces small amounts of PSA (Konishi et al., 2005).

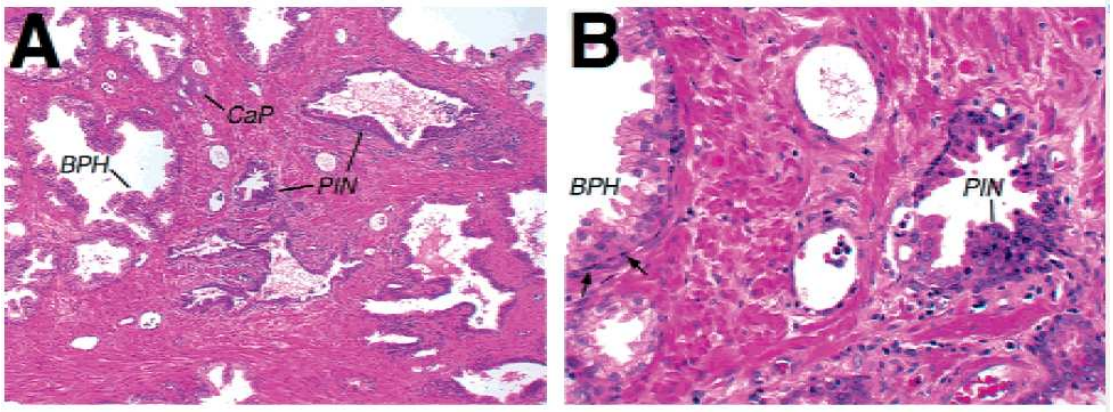



Figure 1.3: Histological images of prostate tissue stained with hematoxylin-eosin stains. A is a low magnification image showing heterogeneity of prostate tissue that contains BPH, PIN and CaP regions. B is a higher magnification view of the same image showing the BPH and PIN regions. Arrows are pointing towards the basal layer that surrounds the ducts at the BPH region, which is absent in the PIN region. Images reproduced from Abate-Shen and Shen, 2000.

Recently, proliferative inflammatory atrophy (PIA) has been identified as a possible precursor to PIN, making PIA an even earlier stage in the evolution of CaP. There

are several defined stages in the progression of normal prostate or BPH to metastatic CaP. These stages include PIA, followed by PIN, leading to localised CaP and subsequently to metastatic and/or hormone refractory prostate cancer (De Marzo et al., 1999).

Currently, the method used for predicting the grade for prostate cancer is the Gleason score where the most predominant cancer pattern and the next leading cancer pattern are given a grade of 1-5, with 1 being the most differentiated and 5 being the least differentiated. The two grades are added together. If there is only one principal histological pattern present in both cancers then the same grade is given so that the Gleason score ranges from 2 to 10 (Gleason et al, 1974).

### 1.1.6 Molecular mechanism of sporadic prostate cancer

The heterogeneity of CaP is a major factor that has made the identification of genetic alterations extremely difficult. Heterogeneity may be the result of multifocal tumours growing very close to each other and eventually terminating in fusion of separate lesions, creating difficulties in obtaining pure sample of cells from patients (Latil and Lidereau, 1998). Prostate cancer cells possess numerous somatic genomic alterations like all other epithelial cancers. Although genetic alterations in CaP have been widely studied little is known of the mechanisms that are involved in the progression of primary CaP to metastatic CaP. Therefore, distinguishing the indolent form of the disease from an aggressive form with current technology has not been possible so far (Saric et al., 1999).

### 1.1.6.i Chromosomal aberration associated with prostate cancer

Several methods, such as conventional cytogenetics (G banding), fluorescence in situ hybridization (FISH), comparative genomic hybridization (CGH) and loss of heterozygosity (LOH) have all been employed to study chromosomal aberrations in prostate cancer. The most successful technique has been comparative genomic hybridization (CGH) which allows loss and gain of DNA copy number to be detected. CGH studies have revealed that the loss of genetic material is higher than the gain in the early stages of prostate cancer, implying loss of tumour suppressor genes is involved in prostate tumorigenesis. Gains were observed in metastatic hormone-independent cancer indicating the activation of oncogenes at a later stage of the disease (Visakorpi et al, 1995). The most frequently recorded chromosomal losses are on 2q, 5q, 6q, 8p, 10q, 13q, 16q and 18q. More than 50% of the samples studied had losses in 8p and 13q chromosomal regions and these regions were also lost in high grade PIN, suggesting these chromosomal regions harbour one or more tumour suppressor genes. Gains have been recorded in metastatic CaP on chromosomal regions 2p, 11p, 1q, 3q, 4q, 7q, 8q, 11q, 12q and Xq (Porkka and Visakorpi, 2004; Karan et al, 2003).

### 1.1.6. ii The most commonly altered genes in prostate cancer

Heterogeneity in CaP found at the histological and clinical level is reflected at the molecular level too. There are no clearly defined genetic events that occur in its progression but different stages of CaP may possibly be associated with inactivation of one or more tumour suppressor genes (TSG) and functional activation of certain oncogenes (Ozen and Pathak, 2000). Not many CaP share all the same genetic

alterations but there are various genes that are most frequently altered in different stages of prostate cancer (Fig 1.4) (Jhavar et al., 2008; Tomlins et al., 2006; Hughes et al., 2005; Konishi et al., 2005; Porkka and Visakorpi, 2004).

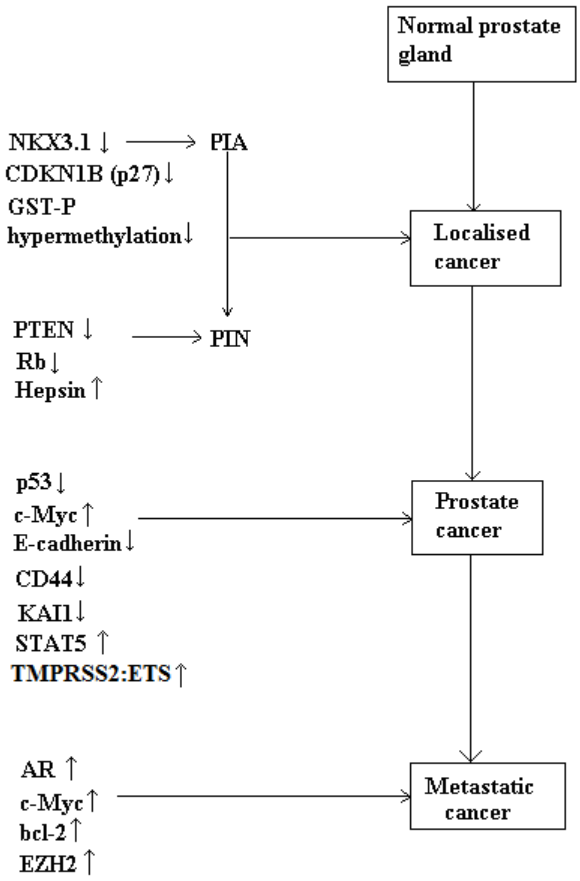



Figure 1.4: Proposed pathway for human prostate cancer progression. Different stages of CaP are associated with inactivation of candidate tumour suppressor genes and activation of oncogenes.

### 1.1.7 Chromosomal instability

The karyotype of prostate cancer has revealed both numerical and structural changes in a variety of chromosomes. Chromosomal instability contributes to molecular transformation of many epithelial cancers (Cahill et al, 1999), and one of the causes of chromosomal instability is dysfunctional telomeres (Counter et al, 1992).

Telomeres and telomerase, the ribonucleoprotein required to maintain telomere length, play an important role in prostate cancer. The biology of telomeres and telomerase and their involvement in cancer will therefore be reviewed next.

### 1.2 Cellular senescence: a tumour suppressor mechanism

Renewable tissues are essential for the viability of complex organisms such as mammals. However, these tissues are at risk of developing cancers because dividing cells acquire mutations much more easily than non-dividing cells. There is a greater possibility of mutations occurring during DNA replication leading to accumulation of genetic alterations that can result in tumorigenesis (reviewed in Deng and Chang, 2007). There is an association between human cancer development and advanced age. Adult cancers are predominantly carcinomas of epithelial origin arising in tissues that are continually being renewed throughout life. Tumour suppressor mechanisms have evolved to ensure the longevity of organisms and one such mechanism is replicative cellular senescence (reviewed in Campisi and di Fagagna, 2007; Kuliman and Peeper, 2009).

Cells from multicellular organisms were originally thought to possess an ability to proliferate indefinitely in culture. However, more than 40 years ago Hayflick and Moorehead (1961) discovered that human fibroblasts, when propagated in culture underwent robust cell division initially and thereafter cell proliferation gradually declined. All the cells in the culture eventually lost the ability to divide even though the cells were viable and there was ample space, nutrients and growth factors in the medium. This proliferative barrier was initially termed “the Hayflick limit” but is

now known as cellular or replicative senescence. The replicative capacity of cells depended on the origin of the tissue and the age of the organism from which the cells were isolated. A molecular clock was thought to exist in normal cells that registered the number of cellular replications and after a certain number of population doublings the cells underwent senescence (Harley et al., 1990). These observations generated two contradictory hypotheses at the time. The first theory stemmed from the fact that numerous cancerous cells proliferate indefinitely in culture, therefore cellular senescence was considered beneficial since it acted as a tumour suppressor mechanism in organisms to protect them from cancer. The second theory stemmed from the fact that tissue regeneration and repair deteriorates with age and cellular senescence was seen as a detrimental mechanism that led to the loss of regenerative capacity of cells *in vivo* (Reviewed in Campisi and di Fagagna, 2007; Stewart and Weinberg, 2006).

### 1.2.1 Senescence and immortalization prior to telomere discovery

Prior to the discovery of the involvement of telomeres with senescence and telomerase with immortalization, it was known that normal human fibroblasts proliferate between 50 to 80 population doublings. Subsequently, the cells senesce as they had reached a proliferative barrier, termed mortality stage 1 (M1). Replicative senescence is dependent on the presence of p53 and pRb tumour suppressor genes (Shay et al., 1991). M1 can be bypassed by transfecting the pre-senescent cells with viral genes such as the gene encoding the large T antigen of SV40 (simian virus) or co-transfection of genes encoding the HPV16 E6 and E7 transforming proteins (reviewed in Newbold, 2005). The HPV16 E6 and E7 viral proteins inactivate the

p53 and pRb tumour suppressor proteins respectively as does the large T antigen of SV40. Abrogation of both the p53 and pRb pathways is required to bypass senescence while inactivation of one of the two pathways did not suffice to bypass senescence. Evading the M1 barrier increases the proliferative capacity of the fibroblasts by 20 to 30 population doublings, rather than immortalizing the cells. The cells then enter another proliferative phase known as crisis or mortality stage 2 (M2), where their number remains approximately constant because successful cell division is balanced by cell death due to critically short telomeres (Wright et al,. 1989). Rarely, cells escape the crisis and form colonies of immortal cells but this a very infrequent event, occurring at $10^{-6}$ in epithelial cells and $10^{-7}$ in human fibroblasts (Shay and Wright, 2006) (Fig 1.5). The above findings indicate that SV40-LT antigen expression is essential but not sufficient for immortalization and that immortalization is a two-stage mechanism i.e. normal human somatic cells need to overcome the 2 proliferative barriers, senescence and crisis to become immortal (Wright et al., 1989; reviewed in Newbold, 2002; Newbold, 2005; Stewart and Weinberg, 2006).

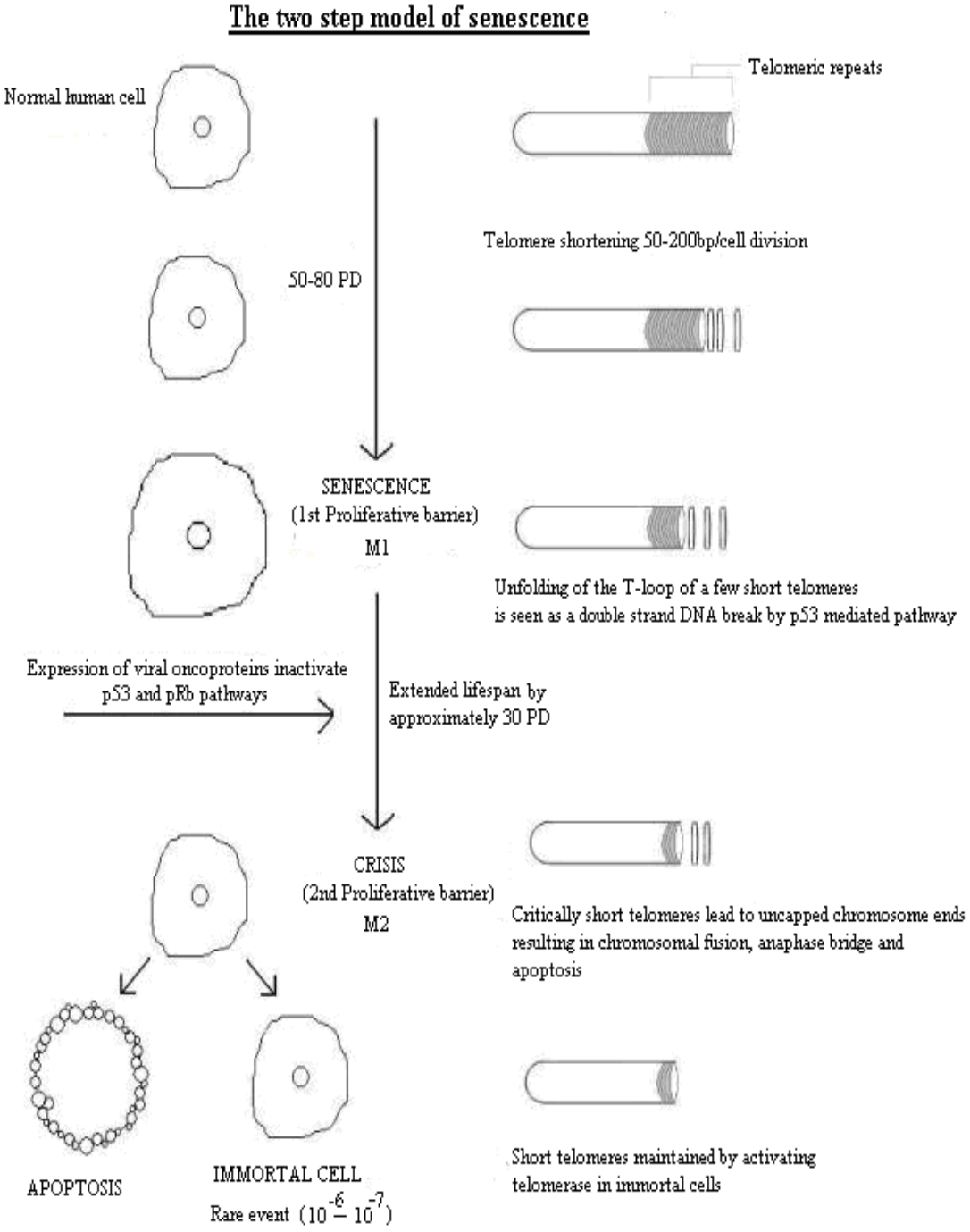



Figure 1.5: The two step model of cellular senescence: showing the two independent proliferative barriers, M1 and M2 that limit the indefinite division potential of normal cells.

### 1.2.2 Telomeres

Telomeres are composed of unique sequence of DNA and associated proteins at the ends of each chromosome. They allow cells to distinguish natural chromosome ends from DNA breaks by capping the 3' end, so prevent inappropriate DNA repair such as exonucleolytic degradation and ligation of one chromosome end to another (Smogorzewska and De Lange, 2004). In normal human cells, telomeres can consist of 5 to 15kbp of tandem repeats of TTAGGG (Moyzis et al., 1988). With every cell cycle an average of 50-150bp of nucleotides are lost due to the end replication problem at the 3' end (Fig 1.6) (Harley et al., 1990). This shortening of telomeres limits the cells from indefinite cell division hence acting as a potent tumour suppressor mechanism. In normal cells, once the length of a single or a few telomeres reaches a critical level the cells undergo replicative senescence and withdraw from the cell cycle (Reddel, 2003). However, germline cells and unicellular organisms have acquired telomerase activity to overcome the telomere shortening that leads to senescence (Shippen-Lentz and Blackburn, 1989). Telomere lengths in human germline cells are significantly longer than those of somatic cells and they are stable regardless of the age of the donor (Allsopp et al., 1992).

**The end replication problem**

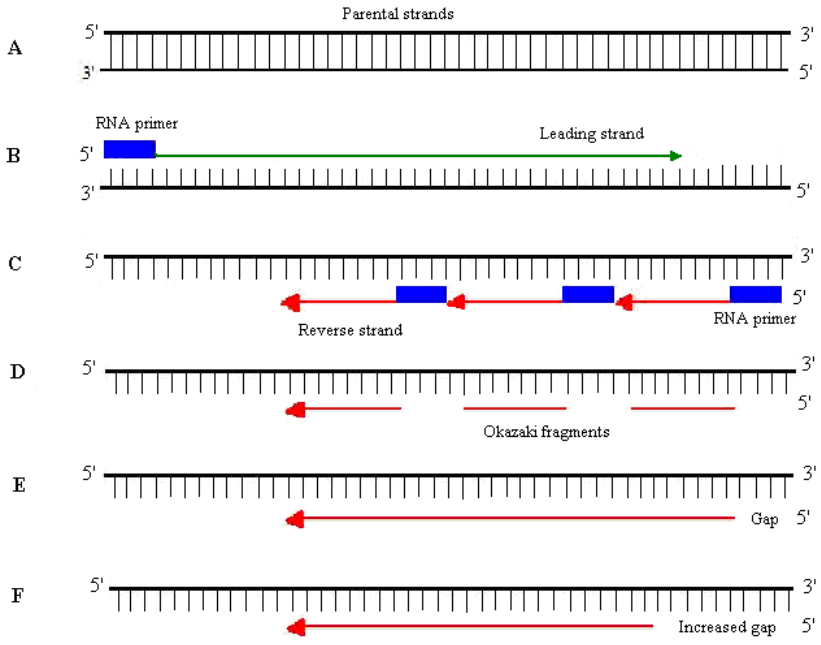



Figure 1.6: Diagram of the "end replication problem" which results in loss of DNA sequence with each cycle of DNA replication. (A) Double stranded parental DNA. (B) Parental strands open up to act as template to synthesize the daughter strands using DNA polymerase and the latter requires an RNA primer to initiate synthesis in the 5'-3' direction. The leading strand can be synthesized continuously until the end of the linear chromosome. (C) The lagging strand is synthesized as a sequence of fragments known as "Okazaki fragments" all of which require the RNA primer. (D) RNA primers are degraded. (E) The gaps left by the RNA primers between the newly replicated fragments are filled by DNA ligase, the exception being the gap at the very terminal 5'end which cannot be filled. (F) This gap is further increased by an apparent 5'-3' exonuclease which degrades 130-210 nucleotides that result in the further shortening of the 5' end of the telomere and a 3' G-rich overhang on the parental strand.

### 1.2.2.i Telomere dysfunction and senescence

Recent advances in molecular biology have revealed the involvement of telomeres with senescence and telomerase with immortalization. When cells are cultured *in vitro* they tend to senesce at a point when they still have an average of several kb of repeats remaining at each telomere (Counter et al., 1992). Unfolding of the T-loop

structure in these short telomeres is seen as a double strand break by the p53 mediated pathway resulting in replicative senescence. It is postulated that the cells stop dividing because the anti-proliferative mechanism, M1, has been activated (Shay et al., 1991). It has been hypothesised that in normal somatic cells telomere shortening in one or more of the 92 (23 pairs of chromosome in a normal cell with telomere at each end results in 92 telomeres) telomeric repeats may induce M1, even when an average of several kb of repeats remains on each of the other telomeres (Levy et al., 1992). Tumour suppressor proteins p53 and pRb, involved in cell cycle checkpoints for DNA damage are induced when a single chromosome of limited telomeric repeats produces a DNA damage signal (Wright and Shay, 1995). Cells that bypass the M1 mortality stage due to inactivation of p53 and pRb signalling pathways continue to divide with decreasing telomere lengths. When the telomeres become critically short they cannot protect the chromosome ends from being detected as double strand breaks and enter M2 or crisis (Newbold, 2005). M2 is characteristic of uncapped chromosome ends, end to end chromosomal fusions, anaphase bridges and a high number of apoptotic cells. End to end chromosome fusion resulting in dicentric chromosomes can initiate chromosomal instability through repeated breakage-fusion-bridge (BFB) cycles. During mitosis the two centromeres of a dicentric chromosome are pulled towards the opposite poles of the spindle causing the chromosome to break. These broken chromosomes can either translocate or form new dicentric chromosomes to continue the process of chromosomal instability. Such genomic instability is associated with extensive cell death making M2 distinguishable from M1 (reviewed in Stewart and Weinberg, 2006). Hence, crisis provides a potent barrier to tumour development but rarely a cell

in the M2 stage escape crisis and become immortal by reactivating or up-regulating telomerase resulting in infinite cell proliferation (Fig 1.5). Between 80 to 90% of tumour derived cells that have detectable telomerase activity also have short telomeres suggesting that telomerase activation leading to telomere stabilization, by arresting telomere shortening and stabilizing the frequency of dicentric chromosomes, occurs late during oncogenesis (Counter et al., 1992). The majority of immortalized cells express telomerase (Kim et al., 1994) and the rest use an alternative mechanism (ALT) to maintain the telomeric length with each cellular division (Reddel, 2003; Newbold, 2005; Shay and Wright, 2005; Stewart and Weinberg, 2006; Feldser et al., 2003).

### 1.2.2.ii Telomere function

Early cytogenetic studies revealed that chromosomes with broken ends fused together to form dicentric, ring or some other unstable form of chromosome i.e. inappropriate recombination (reviewed in Stewart and Weinberg, 2006). However, the natural linear ends of a normal chromosome were found to be stable, so this led to the theory that telomeres are specialised DNA structures at the ends of eukaryotic chromosomes which provide a protective cap and hence stability to the chromosomes (Shampay et al., 1984). The other very important function of telomeres is to prevent the loss of terminal bases at the 5'end of each newly synthesized strand of the DNA by allowing the linear ends to be replicated completely. This can only be achieved in the presence of telomerase but in its absence telomeres gradually get shorter with each successive cell division until they reach a critical threshold where cell replication is arrested. This phenomenon was termed replicative senescence by

Olovnikov (1973). Replication of the linear chromosomes presents a problem, known as the end replication problem first recognized by Watson in 1972 (Fig 1.6). The difficulty arises due to linear DNA being exclusively replicated in 5' to 3' direction and DNA polymerase requiring an RNA primer to initiate DNA replication. This results in a single strand overhang of the 3' end consisting of approximately 30-110 nucleotides in humans. Olovnikov (1973) first purposed replicative senescence to be attributed to the end replication problem due to telomere shortening which serves as an intrinsic clock like mechanism of aging that counts the number of cell division before growth arrest.

**1.2.2.iii Telomere structure**

Telomeres were originally thought to be linear structures but electron microscopy has shown that telomeres are in fact a large duplex structures consisting of 2 loops (Griffith et al., 1999). Telomere folds back onto itself to form a large telomere loop (T-loop) and the 3' end overhang binds to the double stranded telomeric sequence of the 5'end to form a displacement loop (D-loop) (Fig 1.7; Page 44). It is thought that T and D loops may cover the overhang structure so as to cap the telomeres and provide stability and capping may protect the telomeres from being recognized as DNA damage (Wright and Shay, 2005).

Alternative studies using the NMR (nuclear magnetic resonance) have shown that the single strand G rich 3'overhang can provide structural stability to the chromosome by folding back on itself to form a four stranded structure called G-quadruplex. G-quadruplexes can be stabilized by ligands such as $Na^+$ and $K^+$. Formation of the G-

quadruplex prevents the synthesis of further telomeric DNA repeats because the 3' end cannot be recognized by the RNA template of telomerase (Ambrus et al., 2006; Gilbert and Feigon. 1999). Both the T-loop and the G-quadruplex structures of telomeres have been observed *in vitro*. However, *in vivo* structures of telomeres are yet to be established, i.e. whether the telomeres form T-loops, G-quadruplexes or other structures is not yet clear.

### 1.2.3 Telomere, a multi-protein complex

The number of proteins being discovered in association with mammalian telomeres is constantly increasing. In humans, TRF1, TRF2, hRap1, TIN2, TPP1 and POT1 are the six proteins that form a multi protein complex at the telomeres known as "Shelterin" (de Lange, 2005). Shelterin is involved in telomere length control and it may inhibit telomerase from accessing the 3' overhang by forming a T-loop. The three main proteins that bind directly to the telomeres are TRF1, TRF2 (telomeric repeat-binding factors 1 and 2) and POT1 (protection of telomeres). TRF1 and TRF2 bind to the double stranded telomeric sequence of the T loop (van Steensel and de Lange, 1997; Smogorzewska et al., 2000), and POT1 binds to the 3' single stranded telomeric DNA overhang (Baumann and Cech, 2001). The structures of TRF1 and TRF2 are very similar but the TRF1 complex is involved in regulating telomere length whereas the TRF2 complex influences the length of the telomere as well as being involved in telomere capping. TIN2 binds to TRF1, TRF2 and TPP1 and these three proteins interact with each other through TIN2. In addition TPP1 binds to POT1 hence recruiting POT1 to the double stranded part of telomere and hRap1 interacts with TRF2 (reviewed in Smogorzewska and de Lange, 2004).

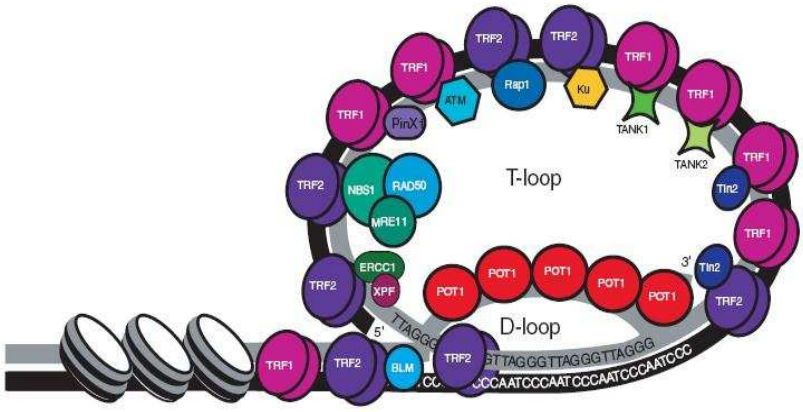



Figure 1.7: Telomeres form a protective t-loop structure in which the single stranded 3' G-rich overhang folds into the double stranded DNA forming a displacement loop. TRF1 and TRF2 bind specifically to the double stranded telomeric DNA and POT1 binds to the single stranded DNA. There are many additional proteins that bind to these core components forming a capped telomere. Diagram reproduced from Colgin and Reddel, 2004.

### 1.2.4 Telomerase

Telomerase is a ribonucleoprotein that functions as a reverse transcriptase to completely replicate the telomeric DNA and hence, by maintaining telomere length provides unlimited proliferative potential to the cells (Greider and Blackburn, 1985) (Fig 1.8). Telomerase activity is present in 90% of human cancers (Kim et al., 1994) making it the most common tumour marker (Shay and Bacchetti, 1997). Although most normal somatic cells do not express detectable levels of telomerase, mitotically active cells such as skin, hair follicle cells, lymphocytes, endometrial and cells of the colonic crypt express low levels of telomerase (Hiyama et al., 1995; Harle-Bachor et al., 1996; Yasumoto et al., 1996). Expression of telomerase is also associated with male germ cells, stem cells and embryonic cells whereas inhibition of telomerase is involved in apoptosis, cellular senescence and aging (Hahn et al., 1999). Telomerase is active during early embryonic development and it is progressively repressed

through differentiation in most somatic cells. Telomerase is switched off in the majority of cells starting at 20 weeks of gestation in the human embryo (Ulaner et al, 1998).

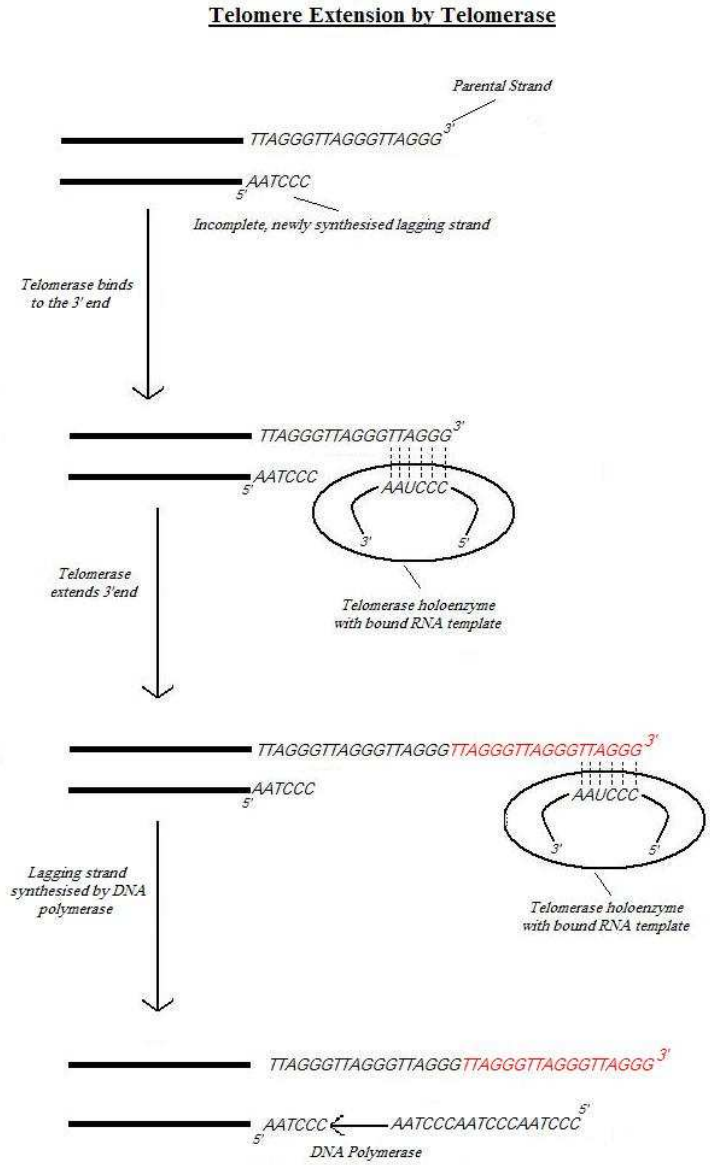



Figure 1.8: Simplified diagram of telomere extension by telomerase: the incomplete synthesis of the lagging strand in the replication fork uses telomerase to synthesize the G rich telomeric DNA sequence.

Telomerase is a large complex that is approximately 1000kDa. In humans, it has two main components; a functional RNA (hTR) that serves as a template for the addition of telomeric DNA to the 3' chromosomal ends and telomerase reverse transcriptase (hTERT) which is the catalytic subunit of telomerase with reverse transcriptase activity (Meyerson et al., 1997). There are additional proteins associated with telomerase including TEP1, hsp23 and hsp90, hStau and dyskerin. Hsp90 and hsp23 are required for the assembly of telomerase in the nucleolus and defects in dyskerin lead to *dyskeratosis congenita* (DC) (reviewed in Pendino et al, 2006). DC is a rare congenital disorder in which bone marrow failure is the major cause of premature mortality. Another feature of DC is predisposition to cancer. The principal cause of DC is the mutations in the *hTR* and *hTERT* genes resulting in defective telomere maintenance through telomerase (reviewed in Walne and Dokal, 2009).

### 1.2.4.i Human TR (hTR)

A single copy of the *hTR* gene is localized on chromosome 3q26.3 and it is transcribed by RNA polymerase II, which produces a mature transcript of 451 nucleotides (Soder et al., 1997). Human TR is found in both normal and cancerous cells and it contains 11bp sequence (5'-CUAACCCUAAC-3') template coding for the telomere repeats (TTAGGG)n (Feng et al., 1995). Even though the length and the sequence of the mature telomerase RNA has diversified between species, i.e. 153-192 nucleotides in ciliates and 1.3kb in *Saccharomyces cerevisiae*, the secondary structure has been largely conserved (Romero and Blackburn, 1991). As well as providing a template for the telomeres, telomerase RNA contains binding sites for the protein subunits (Gilley and Blackburn., 1999). Yi et al (1999) showed that the

level of *hTR* expression is five fold higher in tumour cells compared with normal cells and they suggested this increase in expression is due to upregulation of hTR transcription and a longer half-life of the transcribed RNA. The catalytic activity of telomerase is dependent on several factors involving hTR. These include its interaction with hTERT, its binding with the telomeric DNA and its role in the folding and assembly of telomerase (Holt et al, 1999).

### 1.2.4 ii Human TERT (hTERT)

Two related proteins, Est2p and p123, were initially identified as the catalytic subunit of telomerase in yeast *Saccharomyces cerevisiae* and in ciliate *Euplotes aediculatus*, respectively. Enzymatic activity of telomerase was abolished if these motifs were disrupted (Lingner et al., 1997). Substitution of a single amino acid in the reverse transcriptase motif of telomerase in yeast resulted in telomere shortening and senescence. This indicated the significance of these motifs in elongating telomeres via telomerase activity (Counter et al., 1997). Est2p and p123 homologues have since been identified in many organisms. Meyerson et al (1997) prepared cDNA encoding the human catalytic subunit of telomerase (hTERT) based on the conserved sequence information within the reverse transcriptase motifs of p123. Detectable levels of *hTERT* mRNA is found in telomerase positive tissues, cancer cell lines and tumours but not in cells and tissues that lack telomerase activity. Furthermore, the *hTERT* mRNA expression is induced when telomerase is activated during cellular immortalization (Kilian et al., 1997) and its expression is down regulated with telomerase activity when HL60, promyelocytic leukaemia cells were differentiated in vitro (Meyerson et al., 1997). These observations suggest *hTERT* mRNA is the rate-

limiting step in determining telomerase activity. Additional evidence exists that suggest hTERT is the most essential component required for functional telomerase. Transfection of the *hTERT* gene into telomerase negative cells resulted in cells expressing telomerase activity comparable to the levels seen in immortal telomerase positive cells (Counter et al., 1998). Weinrich et al (1997) demonstrated that mixing transcribed hTR and translated hTERT *in vitro* with rabbit reticulocyte lysate resulted in telomerase activity, indicating hTERT is the rate limiting factor for telomerase activity missing in normal somatic cells. Alternatively, a single amino acid change in any of the 8 RT specific motifs reduced or abolished telomerase activity providing direct evidence of hTERT being the catalytic subunit of telomerase (Cong et al., 2002, Wyatt et al., 2010).

#### 1.2.4.iii Multimerization of hTR and hTERT to form functional human telomerase

Separate *in vitro* syntheses of hTR and hTERT from human cell and rabbit reticulocyte lysate extracts have been co-assembled to produce functional telomerase. The level of telomerase activity produced by the hTR region spanning between +33 to +325 is comparable to the activity produced by the 451 nucleotide full length sequence (Tesmer et al., 1999). The authors also identified two fragments within this region which ranged between +33 to +147 and +164 to +325 nucleotides. Combining either of these fragments individually with hTERT failed to produce telomerase activity suggesting both are essential for the assembly of functional telomerase. Wenz et al (2001) later confirmed human telomerase contains two cooperating telomerase RNA molecules. The authors demonstrated that telomerase is

barely active when it consists of a heterodimer reconstituted from a mutant and wild-type telomerase RNA compared with a homodimer.

In another study, Beattie et al (2001) reported that two hTERT protein subunits are required to form functional hTERT. Functional hTERT multimerization occurs when the N terminus of one hTERT protein forms an association with a second full length hTERT protein that has an intact RT domain and a C terminus. These findings suggest that the catalytic activity of telomerase is achieved by two hTR and two hTERT subunits to form a tetramer complex (Weinrich et al., 1997).

### 1.2.5 Alternative telomere lengthening

Telomere length is maintained by upregulating telomerase activity in 90% of cancerous cells (Kim et al., 1994). The other 10% of cancers maintain their telomere lengths by one or more mechanisms referred to as an alternative lengthening of telomeres, ALT (Bryan et al., 1995). *In vitro* cells that have escaped crisis and become immortal maintain their telomere lengths by ALT mechanism. These cells have a mixture of variable telomere lengths, ranging from short to very long (Bryan et al., 1995). It is not known whether all telomerase negative immortal cell lines utilise the same ALT mechanism. Recombination as seen in the mutant yeast, in the absence of telomerase, has been proposed by McEachern and Blackburn (1996) as one of the alternative mechanisms. All the human cell lines examined either have telomerase activity or show evidence of ALT indicating the importance of telomere length maintenance in immortalization (Bryan and Reddel, 1997).

A recent study by Atkinson et al (2005) has suggested that the mechanism used for maintaining telomere length is determined by the chromatin environment of the telomerase promoters. Cells that utilize telomerase may reactivate the telomerase by chromatin remodelling of the *hTR* and *hTERT* promoters allowing a favourable state for transcription to occur. In contrast, the ALT mechanism might arise due to the tight repression of the *hTR* and *hTERT* promoters, caused by chromatin remodelling of the promoter sequence.

### 1.2.6 Telomerase and cancer

The development of cancer is a multi-step process that involves accumulation of numerous genetic and epigenetic alterations that have occurred to transform cells over a time period (Newbold, 1985). Multi-step carcinogenesis occurs through a process called clonal evolution where variant cells with increasing number of genetic abnormalities are repeatedly selected because they display a greater strength of survival and proliferate in a certain environment (reviewed by Newbold, 2005). This complexity of cancer development was not observed in a murine model where introduction of two cooperating oncogenes were sufficient for transformation (Land et al, 1983). However, epidemiology studies carried out by Armitage and Doll (2004) suggested 4 to 6 rate limiting events are required for cancer formation in humans. These findings have since been confirmed by cloning of the *hTERT* gene. Telomerase is the key enzyme required by cells to acquire immortality and this is reflected by its functional presence in over 90% of cancer cells (Kim et al., 1994). Significant levels of *hTERT* expression have also been reported in 90% of tumours whereas its absence from most normal somatic cells suggests transcriptional

activation of *hTERT* is required for cellular transformation (Kilian et al., 1997). Ectopic expression of *hTERT* and subsequent activation of telomerase activity, in telomerase negative cells, is sufficient to prevent telomere shortening that allows cells to overcome crisis. Therefore, activation of *hTERT* appears to be a critical step in tumour progression (Counter et al., 1998). It is essential, for this reason, to investigate the regulation of the *hTERT* gene so that molecular mechanisms of telomerase regulation, cellular senescence, immortalization and carcinogenesis in humans can be elucidated.

### 1.2.7 Human TERT regulation

The *hTERT* transcript was found to be absent in normal fibroblasts, pre-crisis cells and in telomerase negative immortalized (ALT) cells, but present in a large number of tumours that were surveyed. Matched pairs of pre- and post-crisis fibroblast and epithelial cells showed conversion from an *hTERT* negative to an *hTERT* positive status in parallel with telomerase activity (Kilian et al., 1997). These findings indicate that telomerase activity is tightly regulated by repressing the *hTERT* gene in normal cells and that its de-repression leads to activation of telomerase in tumour cells. The correlation between the *hTERT* mRNA expression and telomerase activity has strongly suggested that the *hTERT* gene is regulated at the transcriptional level (Meyerson et al, 1997). Multiple mechanisms seem to play roles in activation and repression of hTERT in cancer and normal cells respectively. Transcriptional, post-transcriptional and epigenetic modifications of the hTERT gene all seem to regulate its expression but transcription overwhelmingly predominates (reviewed by Kyo and Inoue, 2002).

### 1.2.8 Features of the *hTERT* gene and its promoter

The organization of the *hTERT* gene and its promoter region has been characterized by several groups (Kilian et al., 1997; Cong et al., 1999; Horikawa et al., 1999; Takakura et al., 1999). A single copy of the *hTERT* gene has been located on chromosome band 5p15.33 in human diploid cells and it is approximately 2Mb away from the telomeres (Leem et al., 2002). The *hTERT* gene is composed of 16 exons and 15 introns extending over 35kb and all the splice junctions at exon/intron boundaries conform to GT/AG sequence except for the last intron (Cong et al., 1999). The *hTERT* gene encodes a protein that consists of 1132 amino acids with a MW of 127kDa. Seven of the 8 hTERT protein motifs are the conserved form of the reverse transcriptases and the eighth is the non-RT but telomerase specific T motif (Meyerson et al., 1997) (Fig 1.9).

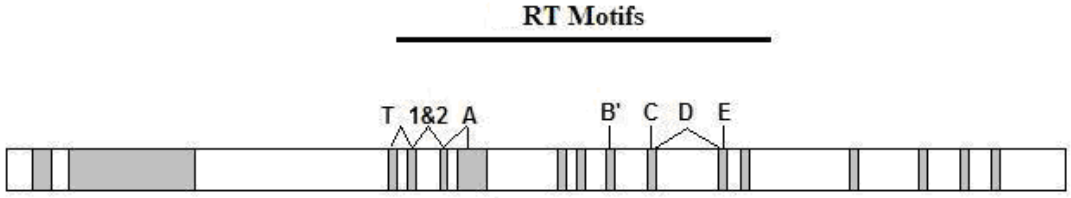



Figure 1.9: Organization of the *hTERT* gene showing the localization of 16 exons (shaded regions) and 15 introns. The *hTERT* gene encodes for a protein that has seven conserved motifs of the reverse transcriptases and a non-RT telomerase specific T motif

Sequence analysis immediate upstream of the *hTERT* promoter has shown the region to be GC rich without TATA or CAAT boxes but it does possess binding sites for several transcription factors (Fig 1.10). Two E-boxes (CACGTG) within the promoter region of *hTERT*, binding sites for the Myc/Max/Mad network, have been identified at 5' upstream of the initiating ATG at -165/-160 bp and at +44/+49 bp.

There are 5 GC-boxes that are binding sites for the general transcription factor Sp1 (stimulating protein 1) localized within 110 bp of the *hTERT* transcription initiating site, situated between the two E-boxes. The transcription initiation site maps to 60-120 bp upstream of the translation start site (Horikawa et al., 1999; Cong et al., 1999; reviewed in Janknecht R, 2004). Two binding sites for the estrogen receptor ER exist in the *hTERT* proximal promoter region. The first one is at -2677 to -2665 bp and the second at -873 to -859 bp upstream of the ATG. The latter consists of a half site for ER binding and has a Sp1 site adjacent to it. Complexes of ER and Sp1 often bind together to the DNA at this site (Cong et al., 1999; reviewed in Janknecht R, 2004).

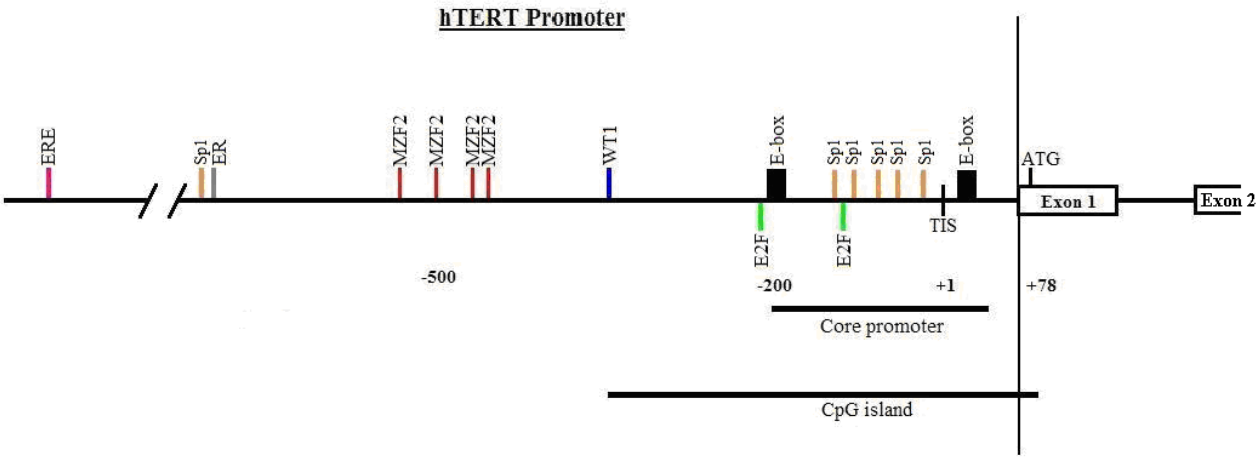



Figure 1.10: Representation of the *hTERT* promoter region showing the binding sites of selected transcription factors, +1 indicates the transcription initiation site (TIS) and the translation initiation codon ATG.

### 1.2.9 Transcription regulators of *hTERT*

Like telomerase, the *hTERT* promoter is inactive in normal and pre-immortal cells but active in immortal and cancer cells suggesting telomerase is regulated via *hTERT* at the transcriptional level (Cong et al., 1999; Takakura et al., 1999). In order to fully understand how telomerase is regulated by repressing the *hTERT* promoter, negative

transcription factors need to be identified. Several known transcription factors are involved in the positive and negative regulation of the *hTERT* gene and some of the major ones will be reviewed (Fig 1.11).

### 1.2.9.i c-Myc/Max/Mad network

*Myc* is a proto-oncogene that is a key regulator of cellular proliferation and differentiation. *Myc* encodes for a universally expressed nuclear phosphoprotein, Myc which consists of 439 amino acids and it is expressed in various human cancers. The c-Myc protein acts as a transcription factor by forming a heterodimer with the Max protein to activate *hTERT* expression by binding to the E-boxes, whereas Mad, an antagonist of c-Myc forms a heterodimer with Max to repress *hTERT* expression (Gunes et al., 2000; Grandori et al., 2000). Being antagonists, the levels of c-Myc and Mad are inversely regulated with *c-Myc* gene being active in proliferating and cancerous cells and *Mad* expressed in differentiating and resting cells (Grandori et al., 2000; Luscher B., 2001). A switch from Myc/Max to Mad/Max at the *hTERT* promoter down-regulates *hTERT* expression in promyelocytic leukemic HL60 cells during differentiation (Xu et al., 2001). Casillas et al (2003) reported a reverse switch when foetal lung fibroblasts were transformed causing the cells to acquire telomerase activity. These finding suggest antagonism between Myc and Mad may be an important factor in determining *hTERT* expression and telomerase activity.

### 1.2.9.ii Sp1

A large number of genes are activated by the general transcription factor Sp1 which binds to the GC-boxes located in the promoter and enhancer regions (Suske G., 1999). Sp1 sites in the core promoter of the *hTERT* are essential for its activity as it lacks a TATA box and mutation in these sites leads to the termination of promoter activity (Kyo et al., 2000). The exact regulatory mechanism of Sp1 is yet to be determined but Sp1 does seem to cooperate with cMyc to activate *hTERT* transcription. This cooperation is specific to the type of cells indicating that other transcription factors may be involved. Correlation between *c-Myc* and Sp1 expression levels and telomerase activity has been reported in SV40 LT antigen transformed fibroblasts at different stages of transformation during multi-step carcinogenesis (Kyo et al., 2000). *c-Myc* expression has been observed in a large number of tumours but some tumours lack this expression even in the presence of telomerase. Transfer of normal human chromosome 3 into a breast cancer cell line repressed *hTERT* expression without affecting *c-Myc* or *Mad* expression levels (Ducrest et al., 2001). Sp1 is also found in numerous normal cells where telomerase is absent suggesting *c-Myc* and Sp1 expression may be insufficient in inducing cancer specific telomerase activation.

### 1.2.9.iii Steroid hormones

Detectable telomerase activity in normal human endometrial cells correlates with its proliferative activity indicating steroid sex hormones may tightly regulate telomerase (Kyo et al., 1997). Estrogen acts as a positive transcriptional regulator of the *hTERT* gene by binding directly to the *hTERT* promoter in hormone sensitive tissues (Kyo et

al., 1999). Estrogen-induced proliferation is inhibited by its antagonist, progesterone which has been used to treat estrogen dependent cancers (Henderson et al., 1993). Estrogen-induced expression of the *hTERT* gene can be inhibited by exposure to progesterone (Wang et al., 2000). The negative effect of progesterone on *hTERT* regulation may be indirect through inducing the expression of p21, which is a cyclin dependent kinase inhibitor that negatively regulates the cell cycle (Holt et al., 1997). Depriving prostate cancer cells of androgens reduces telomerase activity in androgen sensitive cells. However, this effect is not observed in androgen insensitive cells (Soda et al., 2000). Conversion of androgens to estrogen is one possible mechanism that may be employed to activate telomerase; the precise mechanism is yet to be elucidated.

**1.2.9.iv Cell cycle regulators**

The p53 protein is a well documented transcriptional factor involved in many biological processes such as DNA damage, hypoxia and oncogene activation that results in cell cycle arrest and/or apoptosis. Absences of functional p53 allow cells to immortalize that may lead to neoplastic transformation (Asker et al., 1999). Inactivation of p53 and activation of telomerase is a common feature observed in many cancers indicating cell cycle regulators may be involved in regulating telomerase. Co-transfection of an *hTERT* promoter construct with wild type *p53* in HeLa cells resulted in inhibition of *hTERT* expression (Xu et al., 2000). Transcriptional repression of *hTERT* by p53 occurs through direct binding to TATA binding protein, CAAT binding factor and Sp1 (Bargonetti et al., 1997). The *hTERT* promoter lacks TATA and CAAT boxes but does possess 5 Sp1 binding sites, making it possible for p53 to form a complex with Sp1 and repress *hTERT* activity.

Mutation in Sp1 binding sites in the core promoter terminates *hTERT* repression by p53 (Xu et al., 2000). Telomerase inhibition is also observed by p21, p15 and p16 but this inhibition may be a secondary effect following the induction of cell cycle arrest (Kagawa et al., 1999).

#### 1.2.9.v E2F-1

It has been reported that over-expression of pRb and E2F-1 in a human squamous cell carcinoma cell line represses telomerase. The mechanism by which these proteins exert their effect is unknown but several mechanisms have been proposed. pRb and E2F may possibly repress *hTERT* directly or cooperatively (Crowe and Nguyen, 2001). Crowe et al (2001) later reported two putative E2F-1 binding sites proximal to the transcriptional start site of the *hTERT* promoter. Mutation in these sites resulted in increased promoter activity whereas its overexpression repressed *hTERT* promoter activity in reporter gene assay, suggesting E2F-1 to play an important role in *hTERT* repression.

#### 1.2.9.vi Wilms' tumour 1 tumour suppressor gene

Wilms tumour 1 (WT1) is a tumour suppressor protein that represses *hTERT* by directly binding to the core promoter region located at approximately -352 bp upstream of the ATG. Mutation in the DNA binding site of WT1 resulted in increased *hTERT* promoter activity in 293 renal cells but not in HeLa cells. Over-expression of the gene *WT1* in 293 cells led to the inhibition of mRNA expression and telomerase activity (Oh et al., 1999) indicating that WT1 may be a negative

transcription regulator. However, *WT1* is limited to the tissues in which it is expressed i.e. kidney, gonad and spleen suggesting its role as a repressor will also be limited (Kyo and Inoue, 2002).

### 1.2.9.vii Multiple Endocrine Neoplasia type 1 (*MEN-1*) tumour suppressor

*MEN-1* is a tumour suppressor gene, located at 11q13 that encodes for a protein called Menin. Mutations in the *MEN1* gene leads to multiple endocrine neoplasia type 1 disease. Development of tumours in patients with mutated *MEN1* may be a direct result of the loss of *hTERT* repression activity. Menin acts as a negative regulator of *hTERT* expression by binding to several positive transcription factors. It is not known through which protein menin exerts its effects but JunD and/or NF-κB are thought of as possible candidates for mediating this activity (Lin and Elledge, 2003).

### 1.2.9.viii Myeloid specific zinc finger protein 2 (MZF-2)

Transient transfection experiments using luciferase reporter assays on the *hTERT* promoter identified a silencer region between -776 to -378bp upstream from the ATG site. Cellular differentiation was found to increase the inhibitory effects of the silencer. Sequence analysis has shown MZF-2 to have multiple binding sites in the silencer region and its over-expression results in repression of *hTERT* promoter activity whereas mutation of these sites activates it (Fujimoto et al., 2000). However, MZF-2 is expressed in normal as well as cancerous cells therefore it is probably not a specific *hTERT* transcriptional repressor.

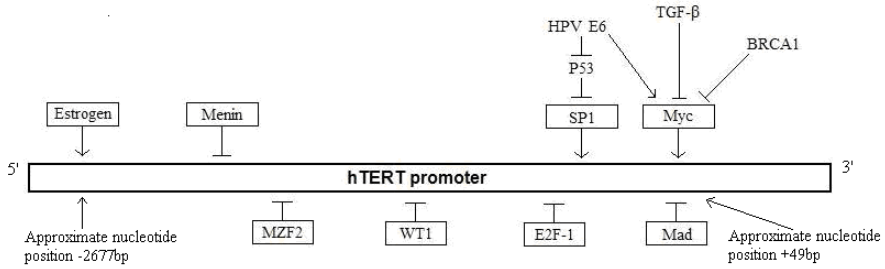



Figure 1.11: Binding sites of some of the main transcription regulators on the *hTERT* promoter.

### 1.2.9.ix E-Box mediated regulation of *hTERT*

Some of the *hTERT* transcription regulators mentioned above were identified by over expressing the proteins in question rather than the physiological protein levels that exist in the cells. Horikawa et al (2002) searched for an endogenous factor that caused differential *hTERT* transcription in a renal cell carcinoma cell line that was *hTERT* positive (RCC23) and the same cell line with transferred chromosome 3 which was *hTERT* negative (RCC23/chr 3). The authors claim that the proximal E-box downstream of the transcription initiation site is responsible for the *hTERT* differential transcription between the two cell lines. Even though over-expression of c-myc and Mad proteins did mediate their effect through this E-box, endogenous levels or binding activity of these proteins did not control *hTERT* transcription in the two similar renal cell lines. A different endogenous E-box mediated binding protein repressed *hTERT* transcription in RCC23/chr 3 cell line but not in RCC23 suggesting the inactivation of this repressor during carcinogenesis. This E-box mediated *hTERT* repression was also observed in normal fibroblasts and epithelial cells and inactive in some of the telomerase/hTERT positive cancer cells. However, when the *hTERT* promoter region was PCR amplified between -261 and +80 in 15 cell lines originating from breast, prostate and cervical carcinomas in our laboratory, no

mutations were observed, suggesting that the repression through the downstream E-box may possibly be specific to renal cell carcinoma.

Table 1.1: Summary of all the well documented *hTERT* transcription regulators

| ***hTERT* transcription factor** | **Proposed status of the *hTERT* regulator** |
|---|---|
| c-Myc/Max/Mad network | c-Myc/Max - activator<br>Mad/Max – repressor |
| Sp1 | Activator |
| Steroid hormones | Estrogen - activator<br>Progestrone – repressor<br>Androgen – activator in CaP cells |
| Cell cycle regulators | Repressor |
| E2F-1 | Repressor |
| Wilms' tumour suppressor gene (WT1) | Repressor |
| Multiple Endocrine Neoplasia type 1 (MEN-1) | Repressor |
| Myeloid specific zinc finger protein 2 (MZF-2) | Repressor |
| E-Box mediated regulation of *hTERT* | Repressor – probably specific for only renal cell carcinoma |
| *hTERT* repression by normal human chromosomes | Various chromosomes implicated in different cancers. See Table 1.2 |

### 1.2.9.x Repression of *hTERT* transcription by normal human chromosomes

Fusion of telomerase-positive immortal cells with normal somatic cells results in hybrids that have repressed telomerase activity. This indicates the loss, mutation or inactivation of telomerase repressor genes in the immortal cells which are required for the normal cells to senesce (Wright et al., 1996). In most somatic cells the absence of telomerase activity is due to the *hTERT* gene being repressed and it is proposed to be the loss of this *hTERT* gene repressor that allows the upregulation of *hTERT* expression which is normally observed in cancerous cells. Many groups have reported the repression of *hTERT* transcription and downregulation of telomerase activity in various cancer cell lines, following microcell mediated transfer of specific human chromosomes. These findings suggest the presence of transcriptionally active

*hTERT* repressor gene(s) that could be identified on normal human chromosomes using such an approach (Oshimura and Barrett, 1997).

Introduction of chromosome 3 into RCC23, a human renal carcinoma cell line, led to the repression of telomerase activity, gradual shortening of telomere length, and induction of cellular senescence. In contrast, microcell transfer of chromosome 7 and 11 into RCC23 resulted in hybrid cells that maintained their telomerase activity and telomere length as in the parental RCC23 cell line (Oshimura and Barrett., 1997). Horikawa et al (1998) reported that telomerase repression in RCC23 chromosome 3 hybrids is due to down-regulation of the *hTERT* gene. Our own group, (Cuthbert et al., 1999) described similar repressor activity after the transfer of chromosome 3 into 21NT, a breast cancer cell line. Powerful repression of telomerase activity and permanent growth arrest was observed with chromosome 3 after 10-18 population doublings. However, transfer of chromosome 8, 12 and 20 into 21NT cells did not repress telomerase activity. These findings suggest the presence of a telomerase repressor gene(s) on normal human chromosome 3 that induce cellular senescence in cancer cells. Tanaka et al (2005) used whole cell fusion to demonstrate that the defects in telomerase repression are corrected by different regions of chromosome 3 in breast and renal carcinoma. Hybrid cells generated from self-fusion of two renal cell lines, KC12 and RCC23 and the breast cancer cell line 21NT expressed telomerase activity as in the parental cells. Similarly, hybrids from whole cell fusion of KC12 and RCC23 also expressed telomerase activity indicating the same genetic defect is shared by the two renal cell carcinoma cell lines. However, fusion of 21NT

with KC12 and 21NT with RCC23 generated a significant number of hybrids that did not possess telomerase activity.

Another group reported that the transfer of normal human chromosome 7 into a telomerase positive human mesothelial cell line (MeT5A), resulted in telomerase repression which was associated with telomere shortening (Nakabayashi et al., 1999). In order to investigate which component was responsible for telomerase repression, the authors investigated mRNA levels of telomerase related genes. The expression of *hTERT* was markedly lower in telomerase repressed hybrids compared with non-repressed and revertants whereas there was no significant difference in the expression levels of *hTR* and *TEP1* between the hybrid clones, suggesting lower expression of the *hTERT* gene is responsible for reduced telomerase activity.

Nishimoto et al (2001) transferred chromosome 2, 4, 5, 10 and 16 into a telomerase positive hepatocellular carcinoma cell line, Li7HM. Only chromosome 10 repressed telomerase activity and the growth of the hybrid cells was arrested after 50 population doublings. Transfer of defined fragments of chromosome 10p narrowed down a region of 28.9cM on 10p15.1, which led to immediate telomerase repression and telomere shortening, strongly suggesting the presence of *hTERT* repressor gene(s) in this region. Another group reported the transfer of chromosome 6 and 11 into a HPV16-immortalized keratinocyte cell line, FK16A, and a cervical cancer cell line SiHa. Hybrid clones generated from the transfer of chromosome 6 for both cell lines underwent growth arrest which was associated with telomere shortening, suggesting the presence of a telomerase repressor (Steenbergen et al., 2001).

In contrast, Backsch et al (2001) transferred normal human chromosomes 3, 4, 6, and 11 into HeLa cells and found strong repression of telomerase activity in chromosome 3 and 4 hybrid cells but not 6 and 11, even though functional loss of chromosome 6 and 11 are implicated in cervical cancer. From these studies it can be concluded that telomerase activity and *hTERT* mRNA expression can be abolished in telomerase-positive cell lines by the transfer of a normal human chromosome, suggesting that normal human cells possess *hTERT* transcriptional repressors that may be tissue-specific and form part of a complementation series.

Table 1.2: Summary of the MMCT studies showing repression of either telomerase activity or *hTERT* transcription repression due to individual transfer of normal human chromosome into cancer cell lines originating from various different cancers.

| **Name of cell line** | **Tissue origin of the human cell line** | **Transferred chromosomes** | **Measured parameter** | **Reference** |
|---|---|---|---|---|
| RCC23 | Renal carcinoma cell line | 3, 7, 11 | Chromosome 3 repressed telomerase activity | Oshimura and Barrett. Eur J Cancer. 1997 Apr; 33(5):710-5 |
| RCC23 | Renal carcinoma cell line | 3 | Repression of *hTERT* transcription with chromosome 3 | Horikawa et al. Mol Carcinog. 1998 Jun; 22(2):65-72 |
| 21NT | Breast adenocarcinoma cell line | 3, 8, 12, 20 | Chromosome 3 repressed telomerase activity | Cuthbert et al. J Natl Cancer Inst. 1999 Jan 6; 91(1):37-45 |
| Li7HM | Hepatocellular carcinoma cell line | 2, 4, 5, 10, 16 | Chromosome 10 repressed telomerase activity | Nishimoto et al. Oncogene. 2001 Feb 15; 20(7):828-35 |
| FK16A<br><br>SiHa | Keratinocyte cell line<br><br>Cervical carcinoma cell line | 6, 11<br><br>6, 11 | Chromosome 6 repressed telomerase activity in both cell lines | Steenbergen et al. J Natl Cancer Inst. 2001 Jun 6; 93(11):865-72. |
| HeLa | Cervical carcinoma cell line | 3, 4, 6, 11 | Chromosomes 3 and 4 repressed telomerase activity | Backsch et al. Genes Chromosomes Cancer. 2001 Jun; 31(2):196-8. |
| MeT5A | Mesothelial cell line | 7 | Chromosome 7 repressed telomerase activity and *hTERT* expression | Nakabayashi et al. Expt Cell Research. 1999 252, 376-382. |

### 1.2.10 Splice variants of *hTERT* gene

Regulation of the *hTERT* gene is tissue-specific in eukaryotes and also involves alternative splicing of the mRNA (Adams et al., 1996). Several transcripts of the *hTERT* gene exist in human cells (Fig 1.12). They include the full length transcript and the well characterized α and β deleted variants. The α-spliced transcript has 36 nucleotides deleted from the 5' end of exon 6 including motif A, and the β-spliced transcript has 182 nucleotides deleted from exons 7 and 8 including motif β, and α- and β-spliced transcripts. Other splice variants include 159 nucleotides insertion of intron 14, a 38 nucleotide insertion of intron 4 and the replacement of exon 15 and part of exon 16 with the 600 nucleotides of intron 14. The proteins coded by these transcripts are not detected in tissue cultured cells but it is thought that these transcripts may be expressed during human development and are possibly tissue and age specific (reviewed in Pendino et al, 2006). It is only the full length transcript that is associated with telomerase activity (Ulaner et al., 2000). Alternative splicing of the *hTERT* gene removes critical reverse transcriptase motifs and hence provides an important mechanism of regulating telomerase activity. Suppression of telomerase activity during human foetal kidney development is thought to employ this mechanism (Ulaner et al., 1998). Lack of telomerase activity in some normal tissues containing both α-and β-spliced transcripts suggests the presence of a post-transcriptional mechanism and mRNA splicing has been lost during neoplastic transformation (Ulaner et al., 2000).

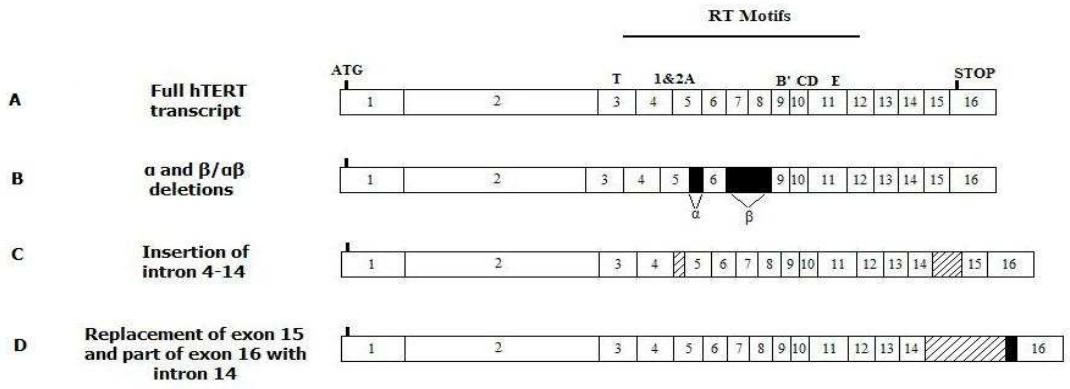



Figure 1.12: Splice variants of the *hTERT* gene. Only the full length transcript (A) is associated with telomerase activity. The location of translation initiation codon ATG and the stop codon are indicated as are telomerase specific T motif and the seven RT motifs. There are three types of deletion variants namely α, β and αβ (B) and various intronic insertions as mentioned in the text (C and D).

### 1.2.11 Epigenetic regulation of *hTERT* transcription

There are two types of epigenetic modifications involved in regulating gene expression. One is methylation of cytosine at the CpG dinucleotide by DNA methyltransferase (DNMT), which is the only known epigenetic modification of DNA in mammals and the other is the post-translational modification of histones (Robertson, 2005). Human cancers are often associated with irregular methylation leading to activation of proto-oncogenes, such as *c-Myc*, *Ha-ras*, *K-ras*, *c-Fos* and *Bcl-2* or repression of tumour suppressor genes such as *pRB*, *p16*, *BRCA1* and *hMLH1* (Laird and Jaenisch, 1994).

#### 1.2.11.i. DNA methylation status of the *hTERT* promoter

Large CpG islands in the GC rich *hTERT* promoter are usual sites for DNA methylation and alterations in chromatin structure involved in regulating the *hTERT*

expression. However, conflicting reports about this have been published in recent years. When Dessain et al (2000) analysed a number of cell lines and tissues for the methylation status of the *hTERT* promoter, no correlation between methylation and *hTERT* expression was established. In contrast, Guilleret et al (2002) found a positive correlation between hypermethylation of the *hTERT* promoter, mRNA expression and telomerase activity in various human tumour cell lines and in normal and tumour tissues derived from different organs. Furthermore, the same group conducted a demethylation study where 3 telomerase-positive cell lines, with hypermethylated promoters were treated with a demethylation agent 5aza-2'-deoxycytidine (5azadC). Strong repression of *hTERT* mRNA was observed with *hTERT* demethylation suggesting hypermethylation of the *hTERT* promoter is required for its expression (Guilleret and Benhattar, 2003). These finding are in complete contrast to general regulation of gene expression where promoter hypermethylation represses transcription (Robertson, 2005). Choi et al (2007) recently reported a complex pattern of *hTERT* promoter methylation, and they suggest methylation status of 3 specific CpG sites might be significant in activation of *hTERT* expression in colorectal carcinoma. They claim two CpG sites are more hypermethylated and a proximal exon region is hypomethylated in cancerous tissues compared with normal tissues. Zinn et al (2007) recently confirmed that the *hTERT* promoter is densely hypermethylated in most cancer cell lines. However, they found methylation of the *hTERT* promoter is very heterogeneous and the transcription start site lacks methylation in three different types of cell lines that were examined. The authors claim that the region around the transcription site of the *hTERT* promoter remains unmethylated for the gene to be expressed and that DNA methylation and *hTERT*

expression in cancerous cells conform to the well established concept of promoter methylation with gene silencing

### 1.2.11.ii Chromatin structure

Gene transcription is dependent on the structure of the chromatin, which in turn depends on the post-transcriptional modification of nucleosomal histones by the two enzymes histone acetyltransferase (HAT) and histone deacetylase (HDAC). Human *TERT* transcription can be induced by HDAC inhibitors in normal cells, indicating the importance of HDAC activity in repressing *hTERT* (Cong and Bacchetti, 2000).

Chromatin immunoprecipitation (ChIP) experiments have demonstrated that the switch from Myc/Max to Mad1/Max at the *hTERT* promoter during differentiation of HL60 cells is accompanied by a decrease in histone acetylation. In the promoter region of proliferating HL60 cells, the E-boxes are mainly occupied by c-Myc and a high level of acetylated histones H3 and H4. This suggests that c-Myc activates *hTERT* transcription by engaging HAT to the promoter region. Alternatively, in differentiating HL60 cells, Mad1 replaces c-Myc on the E-boxes and reduces acetylation of histones at the *hTERT* promoter considerably (Xu et al., 2001). These findings suggest that the Myc/Max/Mad network regulates *hTERT* expression by modifying the chromatin at the promoter for transcriptional activation or repression (Cong et al., 2002). However, mutation in the E-boxes of the *hTERT* promoter is able to respond to HDAC inhibitor in the same way as the wild type promoter (Cong and Bacchetti, 2000) indicating that HDAC may be directed to the *hTERT* promoter by more than one repressor.

In order to identify the *hTERT* regulatory mechanism in normal and tumour cells, Szutorisz et al (2003) scanned the *hTERT* gene for nuclease sensitive sites. Treating the chromatin of telomerase positive tumour cells and telomerase negative fibroblasts with DNase 1 and micrococcal nuclease (MNase) revealed a nuclease-sensitive site in the second intron of the *hTERT* gene in the telomerase positive tumour cells that was absent in the chromatin of normal fibroblasts. The authors examined the state of the chromatin at the second intron in 21NT, a breast cancer line and in *hTERT* repressed hybrids of 21NT that were generated by the transfer of a normal human chromosome 3. The 21NT cell line exhibited similar patterns of MNase sensitivity to telomerase positive cell lines whereas the 21NT chromosome 3 hybrids revealed similar patterns to the normal fibroblast. Hybrids that had lost the transferred normal copy of chromosome 3 showed MNase sensitivity patterns that were observed in telomerase positive cells and some of these hybrids reexpressed the *hTERT* gene. Therefore, the authors claim that the *hTERT* expression in tumour cells depends on an open state of chromatin at intron 2.

Wang and Zhu (2003) reported a similar study where they showed telomerase and *hTERT* mRNA could be induced in telomerase negative pre-crisis cells and ALT cells by treating the cells with HDAC inhibitor trichostatin (TSA). The *hTERT* expression was accompanied by an increase in the chromatin sensitivity to DNase 1 digestion with the appearance of a hypersensitivity site near the *hTERT* transcription initiation site. These results suggest that the *hTERT* promoter in telomerase-negative cells is repressed by a mechanism that involves chromatin organization and

immortalization of cells by activating telomerase may possibly involve chromatin remodelling resulting in loss of repression (Wang and Zhu, 2003).

### 1.2.12 Post-translational regulation of hTERT

It is indisputable that hTERT is regulated mainly at the transcriptional level but there is evidence to suggest posttranslational modification of the hTERT protein occurs as an additional step in controlling telomerase activity. Some normal ovarian and uterine leiomyoma cells had no detectable telomerase activity even when the cells expressed *hTR* and the full length transcript of *hTERT* (Ulaner et al., 2000). Similarly, Rohde et al (2000) found no correlation between *hTERT* mRNA expression and telomerase activity in normal and malignant renal tissue. These findings suggest *hTERT* expression is not always sufficient to induce telomerase activity in certain types of cells and posttranslational modification of the hTERT protein may determine the status of telomerase activity (reviewed by Cong et al., 2002).

#### 1.2.12.i Phosphorylation of the hTERT protein

Phosphorylation of the hTERT protein might be one way of activating telomerase. Protein kinase C and Atk kinases have been reported to phosphorylate the hTERT protein that is required for its translocation to the nucleus leading to telomerase activation (Kang et al., 1999). Activation of T-lymphocytes by hTERT phosphorylation leading to telomerase activity has been reported by Liu et al (2001). The authors discovered that the induced telomerase activity in CD4 T cells was

independent of the hTERT level but instead depended on the phosphorylation and translocation of hTERT from the cytoplasm to the nucleus. This indicates that telomerase activity may be regulated by translocation of phosporylated hTERT from the non-functional cytosolic location to a functional nuclear compartment, providing another mechanism of controlling telomerase activity that is independent of hTERT protein level.

In summary, telomerase regulation is highly complex with a range of regulators controlling its activity at various levels. The conclusion that can be drawn from this review is that the key transcriptional repressor(s) of hTERT have yet to be identified. The status of telomeres and telomerase and the involvement of known telomerase regulators implicated in prostate cancer will be briefly reviewed next.

### 1.3 Telomere shortening in prostate cancer

Recent data has confirmed that telomere dysfunction plays a significant role in genetic instability in prostate cancer (Meeker, 2006). Telomere lengths were measured in normal, BPH and CaP samples obtained from human radical prostatectomy specimens from the same patients. Cells from CaP tissue consistently possessed shorter telomere lengths compared with the telomere lengths of cells obtained from neighbouring normal tissue and BPH tissue (Sommerfeld et al., 1996).

Meeker at al., (2002) found telomere lengths were very short in 93% of high grade PIN (HGPIN) foci obtained from radical prostatectomy. Telomere shortening was also observed in HGPIN samples taken from needle biopsy from patients without

evidence of CaP. In another study, telomere lengths were reported to be short in 63% of HGPIN samples increasing to 80% in foci located 2mm away from the adenocarcinoma (Vukovic et al., 2003). The reduced telomere lengths observed in PIN is beyond the stage where the cell cycle checkpoint can respond suggesting a defective checkpoint in PIN. One of the checkpoints that may be damaged in PIN is 14-3-3 sigma, which is a protein induced by p53 in response to DNA damage (Hermeking, 2003). The expression of 14-3-3 sigma protein is decreased in 91% of prostate cancers compared with the non malignant areas (Lodygin et al., 2004).

### 1.3.1 Telomerase in prostate cancer

Detectable telomerase activity is reported in 84% of CaP samples (Sommerfeld et al., 1996). Telomerase activity is rarely found in normal or BPH tissues and the levels of telomerase activity reported are much lower than the CaP tissue. It is questionable if these normal and BPH tissues adjacent to CaP are truly positive or if it is contamination of these tissues by a few telomerase positive CaP cells. Normal and BPH tissue obtained from cancer-free prostate are always negative for telomerase. Inflammation of the prostate is a common phenomenon and the presence of activated lymphocytes may be another source of contaminating telomerase activity in non-cancerous cells (Sommerfeld et al., 1996). Around 10-20% of prostate cancers are negative for telomerase. These tumours may have a better prognosis as the tumour cells may have a limited proliferative capacity. Currently, there is not enough data to determine if there is a correlation between telomerase activity and tumour grade (Meeker, 2006).

### 1.3.2 Telomerase and hTERT regulation in prostate cancer

There are several known transcription regulators of *hTERT*, of which the positive regulators are the c-myc and CBX7 and the negative regulators include Mad1, SIP1, and menin (Lin and Elledge, 2003). One of the most frequently reported positive regulator in prostate cancer is the *c-myc* oncogene. High levels of *c-myc* expression have been reported in prostate cancer cell line, LNCaP and in prostatic adenocarcinomas (Nag and Smith, 1989). Latil et al (2000) investigated the effects of *myc* expression on *hTERT* regulated mRNA expression levels in 33 sporadic prostate cancer samples by real time quantitative PCR. *myc* was detected in 58% and *hTERT* in 67% of tumour samples. A significant correlation was observed between *myc* over-expression and increased levels of *hTERT* expression, suggesting the upregulation of telomerase activity is in part due to transactivation of *hTERT* by myc in prostate cancer. However, the authors observed no correlation between over-expression of *myc* or *hTERT* expression and the pathological stage of the disease.

Immortalization of normal human epithelial cells requires the inactivation of the $p16^{INK4a}$/Rb pathway and maintenance of telomere length (Kiyono et al., 1998). Gil et al (2005) investigated the role of hTERT, c-myc and other viral oncoproteins in prostate cancer progression by introducing a combination of genes into primary human prostate epithelial cells (HPrEC) to simulate different stages of cancer. The authors discovered that overexpression of *c-myc* alone resulted in complete immortalization of HPrEC. This immortalization was due to c-myc having the ability to upregulate *hTERT* expression and hence activating telomerase to maintain telomere lengths and also to suppress the proteins that regulate the cell cycle, such as

$p16^{INK4a}$/Rb. Vitamin D3 and retinoic acid have both been reported to reduce *hTERT* mRNA expression and telomerase activity in two prostate cancer cell lines PC-3 and LNCaP (Ikeda et al., 2003).

Recently, several regulators of *hTERT* expression have been reported in CaP. The demethylation reagent, 5-azacytidine (5-aza-CR) was shown to act as an antineoplastic drug in prostate cancer. Telomerase activity was significantly reduced by 5-aza-CR in TSU-PR1 but not in DU145 prostate cancer cell line. RT-PCR revealed telomerase inhibition was related to down-regulation of *hTERT* mRNA expression in TSU-PR1 cells. Transient expression assays have shown 5-aza-CR repressed transcriptional activity of the *hTERT* promoter through the E-box. The authors demonstrated by western blot analyses that 5-aza-CR reactivates p16 expression and represses c-myc expression in TSU-PR1 cells but not in DU145 cells (Kitagawa et al., 2000). Jagadeesh et al (2006) showed genistein, an abundant isoflavone found in soybean, has antiproliferative effects on prostate cancer. Genistein decreased *hTERT* and *c-myc* expression and transcriptional activity in a dose dependent manner indicating genistein represses *hTERT* by down regulating *c-myc* expression.

### 1.4 Telomerase: a target for cancer therapeutics

Maintenance of telomeres through the expression of telomerase is a hallmark of cancer cells. Absence of telomerase expression in most normal cells has allowed telomerase to become a universal marker for human cancers for a number of years. This fact has made inhibition of telomerase an attractive target for cancer therapy.

The approaches that have been used to target telomeres and telomerase include oligonucleotide based therapeutics, immunotherapy, gene therapy and combination therapy (Shay and Wright, 2006).

Human TERT and hTR, the two main components of telomerase, have been independently targeted using dominant mutants of hTERT and hTR. Small molecule nucleoside AZT and the most extensively studied non-nucleoside BIBR1532 both inhibited telomerase. BIBR1532 has advantage over AZT because the former inhibits telomerase specifically whereas AZT lacks selectivity for telomerase compared with other polymerases (reviewed in Zimmermann and Martens, 2007). RNA template of telomerase has directly been targeted with GRN163 which is a potent antagonist of hTR. GRN163L is a derivative of GRN163 that contains a palmitoyl lipid carrier to facilitate its transport into the cells. GRN163L is currently being used in clinical phase I/II trials for chronic lymphocytic leukaemia but current data suggests it has the potential to be a universal anticancer agent (reviewed in Zimmermann and Martens, 2007; Shay and Wright, 2006).

Telomeres have also been targeted for cancer therapeutics. Formation of G-quadruplex structures at the ends of telomeres has made it a legitimate target for cancer therapy. Acridine derivatives are very potent inhibitors of telomerase and a trisubstituted acridine compound BRACO-19 has been developed as a ligand to stabilize the G-quadruplex structures. BRACO-19 binds to the single stranded 3' overhang and displaces POT1 thereby uncapping the 3' telomere ends. The growth inhibition and senescence response to BRACO19 was observed in human cell lines

within days rather than weeks (Gunaratnam et al., 2007). Another potent G-quadruplex ligand that is used to stabilize telomeres is telomestatin. TRF2 and POT1, the two telomeric proteins that are involved in the capping the telomere by forming a t-loop are modulated by telomestatin. *In vitro* exposure of tumour cells to telomestatin causes rapid degradation of the telomeres and delocalization of POT1 and TRF2 from the telomeres (Gomez et al., 2006).

The argument against targeting telomerase is that it can also affect the proliferative cells such as the germ cells, stem cells and lymphocytes, but these cells have longer telomeres than tumour cells therefore the damage would be limited (Zimmermann and Martens, 2007). Therapies targeting telomerase is a novel approach that has seen rapid progress in the past 2 years and it could lead to an effective cancer treatment without side effects.

# Chapter 2

# General materials and methods

## 2.1 General equipment used for routine cell culture

All cell culture was performed in a LaminAir HB2448 (Heraeus Instruments) safety cabinet that had been cleaned and swabbed with 70% industrial methylated sprit (IMS) prior to any cell manipulation. Cells were cultured in fully humidified incubators. Hera Cell (Heraeus) incubators were set at either 6.5% or 10% $CO_2$ and at 37°C. An inverted phase contrast microscope, Olympus CK40, was used for visualizing the cells. Digital images of cells were captured using an Olympus IX71 microscope attached to a coolSNAP cf camera (Photometrics). Cells were routinely spun down at 1500rpm for 5min in a Sorvall Legend T bench centrifuge. Foetal Bovine Serum (FBS-European) was purchased from Sigma and it was batch tested and reserved if suitable.

## 2.2 Details of cell lines used

### 2.2.1 PC-3

PC-3, a prostate epithelial cell line established from a human prostatic grade IV adenocarcinoma from a 62 year old male Caucasian, was used as a model system for locating telomerase repressor gene(s) on normal human chromosomes that may be

involved in prostate cancer. PC-3 was cultured in Ham's F-12 Nutrient mix with 7% FBS and 2mM L-glutamine. Cells were cultured in 6.5% $CO_2$ at 37°C in a fully humidified incubator. Confluent cultures were split 1:2 to 1:6 and routinely fed every 3 days if sub-culturing was not required. PC-3 cells were kept in culture for a maximum of 3 weeks and thereafter a new batch was thawed out.

### 2.2.2 Human: rodent monochromosomal cell hybrids

Human: rodent monochromosomal hybrid panel was constructed by Professor R. Newbold's group for use in human gene mapping and gene function. These hybrids were used to individually transfer normal human chromosomes into PC-3 cells. The normal human chromosome tagged with the HyTK fusion gene allowed positive selection of cells that had incorporated the tagged chromosome by culturing the cells in hygromycin B. The nomenclature used for the human: rodent hybrid panel is A9HyTK1, A9HyTK2 etc. The number and HyTK denote the chromosome number tagged with the hygromycin resistant gene in a mouse fibroblast, A9, background. The human: rodent hybrid panel was routinely cultured in DMEM containing 10% FBS, 2mM L-glutamine and 400U/ml of hygromycin B and cultured in 10% $CO_2$ at 37°C in a fully humidified incubator. The cells were routinely sub-cultured at 1:6 to 1:8 when 80% confluent and fed every 3 days if sub-culturing was not required.

## 2.3 Cryopreservation of cells

Healthy cells growing in log phase were fed with fresh culture medium 24 hr prior to freezing them down. The culture medium was aspirated off and the adherent cells

washed once with appropriate volume of Versene (0.04% EDTA in PBS). The cells were detached with warm 0.05% trypsin made up in EDTA. The detached cells in trypsin were neutralised with complete cultured medium and the cells spun down at 1500rpm for 5min. The supernatant was aspirated off and the cell pellet flicked and resuspended in freezing medium consisting of 90% FBS and 10% DMSO. Aliquots of 0.5 or 1.0ml of cells were frozen down in 2ml ampoules by placing them into gaseous phase of liquid nitrogen before transferring them into liquid phase. Cells were routinely frozen down at 3-5x$10^6$ from P-100 dishes and at 1-2 x$10^6$ from P-60.

### 2.4 Recovery of cells from liquid nitrogen

Ampoules of cells were taken out of liquid nitrogen and swabbed with 70% IMS. Caps were loosened to release the pressure before rapidly thawing the cells at 37°C. Cells were transferred into 10ml of warm complete culture medium and centrifuged at 1500rpm for 5min. The supernatant was aspirated off and the pellet gently flicked to disperse the cells before resuspending them into appropriate volume of complete medium.

### 2.5 Testing cells for mycoplasma contamination

#### 2.5.1 The Hoechst 33258 stain method

Antibiotics can suppress the growth of mycoplasma in cells therefore cells were cultured for at least 2 weeks in the absence of all antibiotics including hygromycin, geneticin, neomycin etc. After 2 weeks, cells were seeded onto sterile glass cover-slips placed in Petri dishes or in slide chambers. Cells were fixed directly with

freshly made Carnoy's fixative (methanol and acetic acid 3:1 ratio) when they were approximately 70% confluent. Three millilitres of fixative was added to 5ml cultures for 2min at room temperature. The medium and the fixative was aspirated off and 5ml of fixative was added to the cells and left to incubate at room temperature for 5min. The cells were fixed once more with fresh fixative and left to air-dry. The cells were stained with 0.05µg/ml of Hoechst 33258 made up in PBS pH 7.0 for 5min in the dark at room temperature in a coplin jar. The slides were rinsed twice with PBS pH 7.0 and visualized under a fluorescence microscope.

### 2.5.2 The nested PCR method

The parental cell lines and hybrids that were routinely used in the laboratory were subjected to mycoplasma contamination tests at regular intervals. Genomic DNA was isolated from the cells using a DNA isolation kit (Wizard, Promega).

For the first round amplification of the mycoplasmal DNA, we used the GPO1 primer (5'ACTCCTACGGGAGGCAGCAGTA 3') and MGSO primer (5'TGCACCATCTGTCACTCTGTTAACCTC 3'). For a 20µl reaction volume, 1.0µl (100ηg) of DNA was added to 1.0µl (0.4µM) each of the GPO1 and MGSO primers and 17µl of Reddymix Mastermix. The amplification was carried out for 35 cycles with denaturing at 95$^{o}$C, annealing at 55$^{o}$C and extension at 72$^{o}$C. A final extension step at 72$^{o}$C for 10min was added.

For the second round amplification of the mycoplasmal DNA, we used the GPO2 primer (5'CTTAAAGCAATTGACGGGAACCCG 3') and MGSO primer. For a

20µl reaction volume, 1.0µl of the product from the first round of amplification was added to 1.0µl (0.4µM) each of the GPO2 and MGSO primers and 17µl of Reddymix Mastermix. PCR amplification conditions and cycles were the same as above. The PCR products from both rounds were run on a 1.5% agarose gel containing ethidium bromide.

A strong positive sample produces a band at 720bp from the first round of amplification and a band at 145bp from the second amplification. A weak positive sample produces a single band at 145bp form the second round of amplification. Only the cells negative for both rounds of amplifications were used in my study.

**2.6 Harvesting colonies**

Plates were examined under an inverted microscope in order to locate colonies. Once the colonies were identified they were circled with a marker at the bottom of the plate. Culture medium was aspirated off and an appropriate sized cloning cylinder was pressed into a plate of autoclaved Vaseline using a pair of sterile forceps. The cylinder was carefully lowered onto a colony and pressed down firmly. Trypsin, 100-200µl depending on the size of the cylinder being used was pipetted and cells were left to incubate at room temperature until they detached. The cells were retropipetted using a Pasteur pipette and diluted into 5ml of complete culture medium, containing appropriate drug if required, into a P-60 plate.

## 2.7 RNA extraction using Trizol reagent

Monolayer of cells, at approximately 80% confluence, was washed twice with 5ml of PBS. One millilitre of Trizol reagent (Sigma) was added to the cells and left for at least 1min at room temperature. The cell lysate was pipetted well and the content stored in 1.5ml microcentrifuge at -80°C until required. Once all the samples had been collected for extraction they were thawed at room temperature and incubated for 5min at 15-30$^{o}$C. Two hundred microlitres of chloroform was added to the samples and the tubes were shaken vigorously by hand for at least 15sec. The samples were then incubated for 2-3min at 15-30$^{o}$C prior to spinning them at 13000rpm for 20min at 4°C in a bench centrifuge (Eppendorf centrifuge 5415R). The clear upper aqueous phase that contains RNA exclusively was carefully pipetted into a fresh microcentrifuge tube. Isopropyl alcohol, 750µl, was added to each sample and mixed gently prior to incubation for 10min at 15-30$^{o}$C. Samples were centrifuged at 13000rpm for 10min at 4°C. RNA precipitate forms a gel-like pellet normally on the side of the tube. The supernatant was poured off and the RNA pellets washed once with 75% ethanol. The samples were mixed well by vortexing prior to centrifugation at 8000rpm for 5min at 4°C. The 70% ethanol was poured off and the RNA pellets air-dried at room temperature for 10-15min. RNA was dissolved in 20µl of DEPC treated water for a minimum of 1hr on ice and absorbance was read at 260ηm.

# Chapter 3

# Generation of human monochromosome hybrids by microcell-mediated chromosome transfer (MMCT)

## 3.1 Introduction

MMCT is a well established technique in our laboratory that allows individual normal human chromosomes tagged with a selectable marker to be transferred into any mammalian cell. The transferred chromosome is maintained in the recipient cells as an intact functional and structural entity. Individual transfer of the complete complement of normal human chromosomes into any mammalian cells permits the entire genome to be scanned for the existence of active genes that could be identified by a particular phenotype on the basis of genetic complementation (Cuthbert et al., 1995; Newbold and Cuthbert, 1998; Saxon et al., 1985). The structural characteristics of the introduced chromosome can easily be defined by STS mapping and chromosome painting in a heterospecific human: rodent hybrid (Newbold and Cuthbert, 1998). This fact was taken into account when the monochromosomal hybrid panel was constructed by Professor Newbold's group.

We have used genetic complementation studies to locate telomerase/hTERT repressor genes in breast cancer cells and, in the present study, in a prostate cancer

cell line. Identifying the telomerase repressor gene required the whole genome to be screened for repressive function. This was made possible by the availability of the complete panel of human: rodent monochromosomal hybrids in our own laboratory (Cuthbert et al., 1995). Each hybrid contained a single intact human chromosome tagged with the selectable HyTK fusion gene. The HyTK bifunctional gene was constructed by fusing the bacterial hygromycin phosphotransferase (hph) gene with the herpes simplex virus type 1 (HSV-1) thymidine kinase (TK) gene. The reason for choosing the HyTK fusion gene as a mammalian selectable marker was to promote the facility for the transferred chromosome to be selected "in" or "out" of the recipient cells. Positive selection (to maintain the transferred chromosome in the recipient cells) of the integrated HyTK marker is achieved by adding hygromycin B in the culture medium and negative selection (loss of the transferred chromosome) by culturing cells in a synthetic prodrug called ganciclovir (Newbold and Cuthbert, 1998). The reverse selection makes use of the fact that the HSV thymidine kinase (TK) on the HyTK selectable marker phosphorylates the non-toxic prodrug ganciclovir (GCV) to GCV-phosphate. The latter is further phosphorylated to GCV-triphosphate by endogenous kinases which causes DNA chain termination and single strand breaks once it is integrated into the DNA inducing apoptosis in the target cells (Beltinger et al., 1999). The dual properties of the HyTK marker allows a specific phenotype associated with the transferred chromosome (in my case telomerase activity and *hTERT* transcription determined by TRAP assay and quantitative RT-PCR respectively) to be confirmed by the loss of the phenotype by reverse selection of the transferred chromosome in the hybrid clones (Cuthbert et al., 1995).

### 3.1.1 Construction of human: rodent monochromosomal hybrid panel

The HyTK fusion gene was inserted into a small (approximately 4.2 kb) Moloney-based retrovirus (tgLS(+)HyTK) which was placed under the transcriptional control of the proviral 5' long terminal repeat (LTR) (Lupton et al, 1991). The initial step in tagging normal human chromosomes with the selectable fusion gene involved the transvection of the producer cell line PA317 with the retroviral vector tgLS(+)HyTK. Amphotropic pseudotype viral supernatants collected from PA317 cells were used to infect the normal diploid adult human male fibroblast strain IBR.2; the cells were subsequently selected with hygromyin B and cryopreserved before utilizing them as human chromosome donors. Our monochromosomal hybrid panel was constructed by direct microcell transfer of the virus-infected human IBR.2 cells into A9, a mouse fibroblast cell line. A9 clones resistant to hygromycin B were cryopreserved prior to cytogenetic and molecular analysis. Monochromosomal hybrids were further analysed by STS-PCR using primers that amplified the telomeric proximal regions on the p and q arms. Clones that were positive for both markers were selected for more thorough characterization, essentially to remove the likelihood of the clones containing a chimeric chromosome. The identity and integrity of the transferred chromosome was additionally confirmed by G-banding and FISH analysis using whole chromosome specific paints after 40 population doublings, to confirm the presence of a stable single human chromosome (Cuthbert et al., 1995).

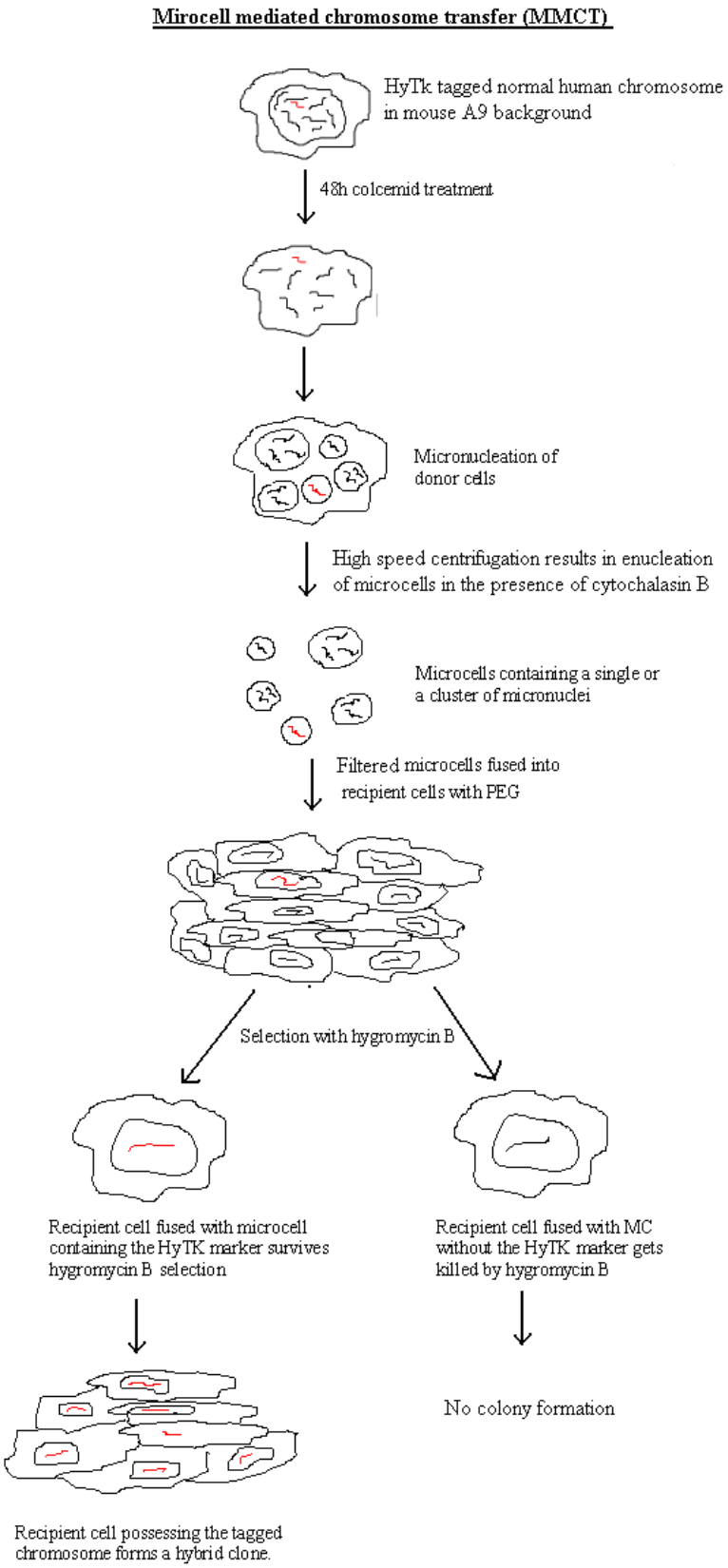



Figure 3.1: Diagram showing the main steps involved in the MMCT technique.

In the past, the MMCT has proved to be an invaluable tool in successfully identifying several genes in various disorders by using the donor panel constructed by Professor Newbold's team. These include the MMADHC gene which is responsible for defects in cobalamin metabolism (Coelho et al., 2008); SUMF1 gene which is involved in multiple sulphatase deficiency (Cosma et al., 2003) and SURF1 gene which plays a role in biogenesis of cytochrome c oxidase in a human neurological disorder (Zhu et al., 1998). Our donor panel has also been successfully used in cancer studies. A number of chromosomal regions have been identified where tumour suppressor (Dafou et al., 2009) and telomerase repressor gene(s) (Steenbergen et al., 2001; Cuthbert et al., 1999) may possibly be located.

The initial aim of this study was to identify a chromosome that may have a repressive effect on telomerase activity when transferred into a prostate cancer cell line. This was achieved by individually transferring the 22 autosomal chromosomes and the X chromosome into PC-3 cells to screen the whole genome for the telomerase repressor gene(s).

## 3.2 Materials and Methods

### 3.2.1 Microcell Mediated Chromosome Transfer (MMCT)

Human: rodent monochromosomal cells, referred to as a "donor" cell line, were seeded at the cell number shown in Table 3.1. The cells were set up in 12x24cm$^2$ flasks (Nunclon$^{TM}$ Surface, Nunc) in DMEM containing 20% FBS without hygromycin B, in a final volume of 5ml. The cells were incubated at 37$^o$C for 24hr in

a fully humidified incubator with 10% $CO_2$. PC-3; the recipient cell line was seeded at 3, 3.5 and $4x10^6$/10cm dish in duplicate in 15ml of complete culture medium on the same day as the donor cells. The PC-3 cells were incubated at 37$^oC$ for 72hr in a fully humidified incubator with 6.5 % $CO_2$. After approximately 24hr of incubation, colcemid (for concentrations refer to Table 3.1) was added to the donor cells and the cells were left to micronucleate for 48hr. The cell seeding number and the colcemid concentrations had previously been optimized for each donor cell line. I had optimized the cell number to produce 70-80% confluent monolayer cells after 24hr of incubation and the colcemid concentration was optimized to produce a maximum number of micronuclei with the least number of non-viable cells after 48hr of incubation. The culture medium was aspirated off from each of the 24 flasks and 30ml of 10μg/ml of cytochalasin B (Sigma) prepared in serum free DMEM was pipetted into each flask. The flasks were weighed accurately to two decimal places and paired to 0.03g differences. 75ml of warm water was poured into each bucket of the pre-warmed rotor (Sigma, 6X250ml) and the paired flasks placed opposite each other into the rotor at 45 degrees with the cell monolayer facing inwards. The flasks were centrifuged at 9500g for 1hr 10min at 37°C in a Sigma laboratory centrifuge 6K15 (Philip Harris). The flasks were weighed again and any loss or gain in weight of more than 0.02g was discarded. DMEM containing the cytochalasin B was poured out of the flasks leaving behind 1-2ml. The pellets at the two corners of the flasks were flicked and microcells from 6 flasks were pooled into one 15ml conical tube. The microcells were spun down at 4500rpm for 5min. The supernatant was aspirated off and the pellet flicked before adding 2ml of warm serum free DMEM. The microcells were pipetted thoroughly in order to obtain single cell suspension. The

volume was made up to 12ml in each tube with serum free DMEM and the microcells mixed well prior to sequentially filtering through an 8μm and two 5μm polycarbonate filters (Whatman: Nucleopore Track-Etch Membrane, Swinlock Holders 25mm diameter) to maximize the yield of microcells carrying only a single chromosome. The microcells were spun at 4500rpm for 5min. The supernatant was aspirated off and the pellet flicked and resuspended in 3ml of serum free Ham's F-12 medium, containing 100μg/ml Phytohaemagglutanin (PHA-P, Sigma). Meanwhile the plate containing the most evenly distributed PC-3 cells was selected and washed three times in warm SF Ham's F-12 medium and the microcells pipetted on to the recipient cells. The plate was incubated at 37$^{o}$C and 6.5%$CO_2$ for 25min to allow the microcells to attach to the recipient cells. Microcells that had not attached were carefully aspirated off and 3ml of PEG solution containing 42.5% polyethylene Glycol 1000 (Sigma) and 8.5% DMSO in SF Ham's F-12 medium was added slowly to the edge of the plate. After exactly one minute at room temperature the PEG solution was carefully aspirated off and the plate was washed three times with warm SF Ham's F-12 medium with each wash lasting 1min. Fifteen ml of complete medium was added to the plate and the cells were incubated at 37$^{o}$C and 6.5% $CO_2$ overnight. The next day, the cells were subcultured into ten P-100 dishes and incubated for 3 days before gradual selection with 100U/ml of hyg B for 3 days and thereafter with 200U/ml of hyg B until colonies appeared. Clones were picked and cultured with half the dose of hyg B for maintaining the transferred chromosome in the recipient cells. Two ampoules of each clone were cryopreserved in liquid nitrogen. A mock fusion was also performed as a control for the MMCT.

Table 3.1: Monochromosomal hybrids were seeded at the densities shown for MMCT experiments, and the colcemid dose used for each hybrid is the optimum that produced maximum micronucleation with the least number of non-viable cells.

| **Human:rodent monochromosomal hybrids** | **Cell seeding density Number of cells/flask (x$10^6$)** | **Colcemid dose (μg/ml)** |
|---|---|---|
| A9HyTK1 | 2.0 | 0.12 |
| A9HyTK2 | 1.5 | 0.05 |
| A9HyTK3 | 1.75 | 0.075 |
| A9HyTK4 | 1.5 | 0.15 |
| A9HyTK5 | 1.5 | 0.10 |
| A9HyTK6 | 1.5 | 0.075 |
| A9HyTK7 | 1.5 | 0.06 |
| A9HyTK8 | 2.0 | 0.10 |
| CHOHyTK9 | 1.0 | 0.05 |
| CHOHyTK9 (del p) | 1.0 | 0.05 |
| A9HyTK10 | 1.75 | 0.10 |
| A9HyTK11 | 1.25 | 0.05 |
| A9HyTK12 | 1.5 | 0.05 |
| A9HyTK13 | 1.5 | 0.065 |
| A9HyTK14 | 1.5 | 0.06 |
| A9HyTK15 | 1.25 | 0.04 |
| A9HyTK16 | 2.25 | 0.075 |
| A9HyTK17 | 1.5 | 0.075 |
| A9HyTK18 | 2.0 | 0.10 |
| A9HyTK19 | 2.0 | 0.06 |
| A9HyTK20 | 2.0 | 0.075 |
| A9HyTK21 | 1.0 | 0.05 |
| A9HyTK22 | 1.0 | 0.05 |
| A9HyTKX | 2.0 | 0.10 |

## 3.3 Results

### 3.3.1 Microcell mediated chromosome transfer of all 22 autosomal normal human chromosomes and the X chromosome into PC-3, a prostate cancer cell line

Heterospecific human: rodent hybrids containing a single copy of a normal human chromosome in mouse A9 cells were used as microcell donors. A complete, well characterized panel, consisting of 22 autosomal chromosomes plus the X chromosome, has been constructed by Professor Newbold's group (Cuthbert et al., 1995). The donor cell lines available for transfer in our laboratory were all at under passage 15. Microcells isolated individually from each of these donor cells were fused into PC-3, a prostate epithelial cell line (see Materials and Methods) and hybrids were selected with 200U/ml of hygromycin B; colonies appeared 3 to 4 weeks after fusion. Hybrid colonies were marked whilst being examined under the microscope after approximately two weeks. On average 2-3 colonies per plate were formed within this time period. Any colonies appearing thereafter were considered to be satellites. The possibility of satellite colonies forming within the two weeks was deemed to be low since the colonies are very small. Often a dozen or more colonies were observed on a plate after 4 to 5 weeks. The previously marked colonies were picked using disposable plastic cloning cylinders once they were large enough to fill the x5 magnification viewing field. The hybrid colonies were expanded as separate cell lines to be assayed for telomerase activity.

### 3.3.2 Microcell Mediated Chromosome Transfer (MMCT)

The initial step in MMCT involved micronucleation of the donor cells (Fig 3.2). This was achieved by incubating the donor hybrids with the mitotic inhibitor colcemid which blocks the cells in metaphase by preventing the formation of a functional spindle. The donor cells were treated with colcemid for 48hr at concentrations that had produced the highest number of micronuclei with the lowest number of non-viable cells in the pilot study (refer to Table 3.1). The non-viable cells were determined by the number of cells that had detached from the plate. Incubating the donor cells with the correct concentration of colcemid is vital to the success of the MMCT procedure as high dose of this mitotic inhibitor resulted in increased cell toxicity and decreased number of micronucleated cells.

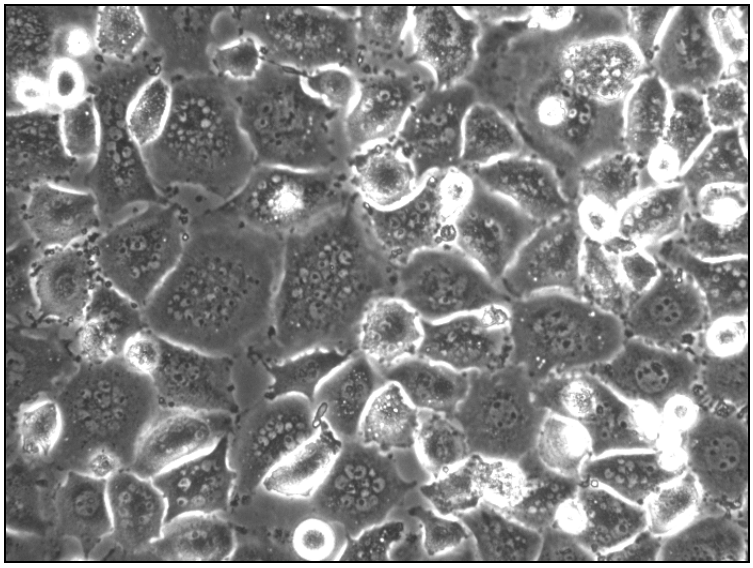


Figure 3.2: Incubating the donor cells with colcemid for 48hr results in micronucleation of the majority of cells. Example of micronucleated A9HyTK 11 donor cells is shown above. (Magnification of the image is x20).

Incubating the micronucleated cells with cytochalasin B resulted in nuclear extrusion (Fig 3.3). Centrifuging these micronucleated cells attached to the plastic culture surface at high speed in the presence of cytochalasin B caused extruded nuclei to break away from the cells, grasping a small amount of cytoplasm to produce microcells.

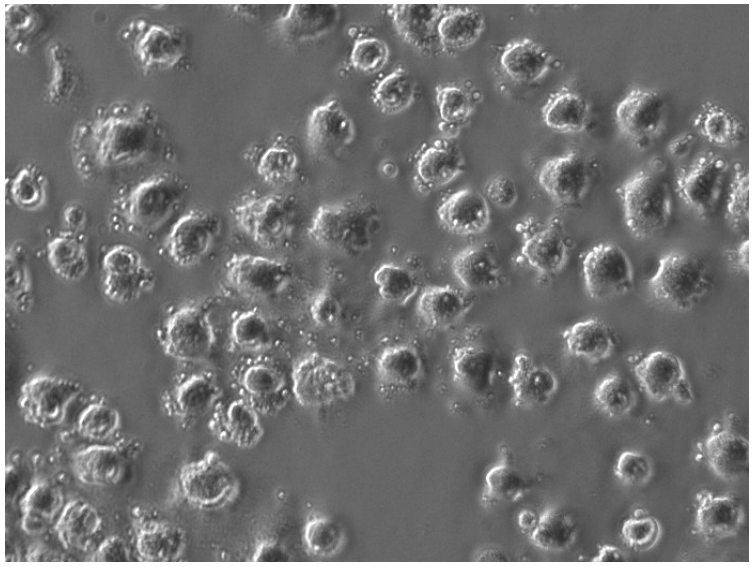


Figure 3.3: Incubating the micronucleated cells in cytochalasin B for approximately 20min caused nuclear extrusion which later helps the micronuclei to break away from the cell when they are being centrifuged. (Magnification of the image is x20).

Microcells produced by enucleation of the micronucleated cells were size-selected by serially filtering through a single 8µm followed by twice through 5µm polycarbonate filters to enrich the population of microcells containing a single normal human chromosome and to remove the contaminating whole A9 cells and cell debris (Fig 3.4).

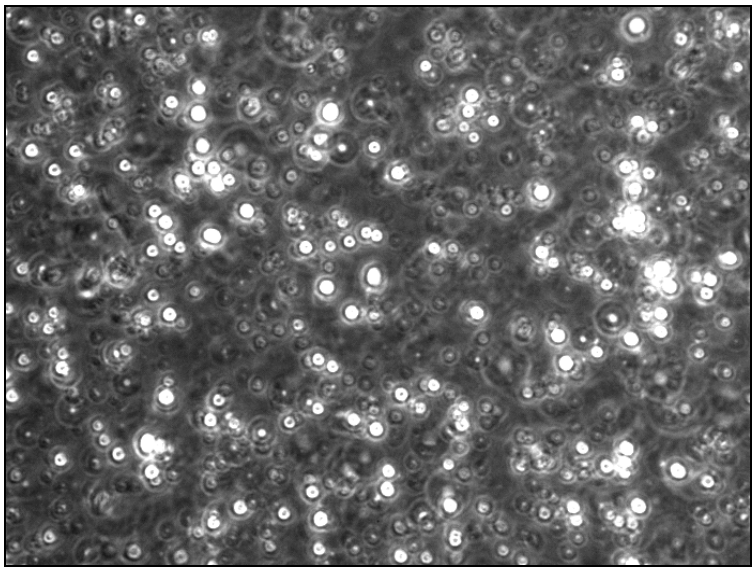


Figure 3.4: Microcells are filtered through 1x 8µm and 2 x 5µm polycarbonate filters prior to fusion with the recipient cells. Image of filtered microcells prepared from A9HyTK 11 cells. (Magnification of the image is x40).

An evenly distributed recipient (PC-3) monolayer was used for fusion when the cells were approximately 90% confluent (Fig 3.5).

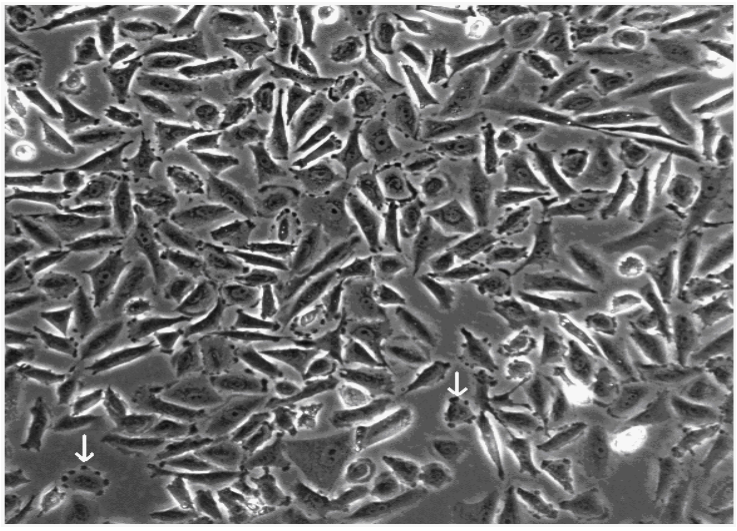


Figure 3.5: A monolayer of PC-3, a prostate cancer cell line used as recipient cells. The arrows are pointing at the "ruffles" in the cytoplasm and should not be confused with microcells (an image of microcells is shown in Fig 3.6). (Magnification of the image is x10).

Microcells were incubated with the recipient cells in the presence of phytohaemagglutinin (PHA-P) prior to fusing them using polyethylene glycol (PEG). Treating cells with PHA before exposure to 44% PEG results in reduced cytotoxcity caused by PEG. McNeil and Brown (1980) demonstrated the combined effects of PHA with PEG increases the fusion capacity of microcells with whole cells. PHA also helps microcells to adhere to the recipient cells so that the fusion with PEG is more efficient (Fig 3.6).

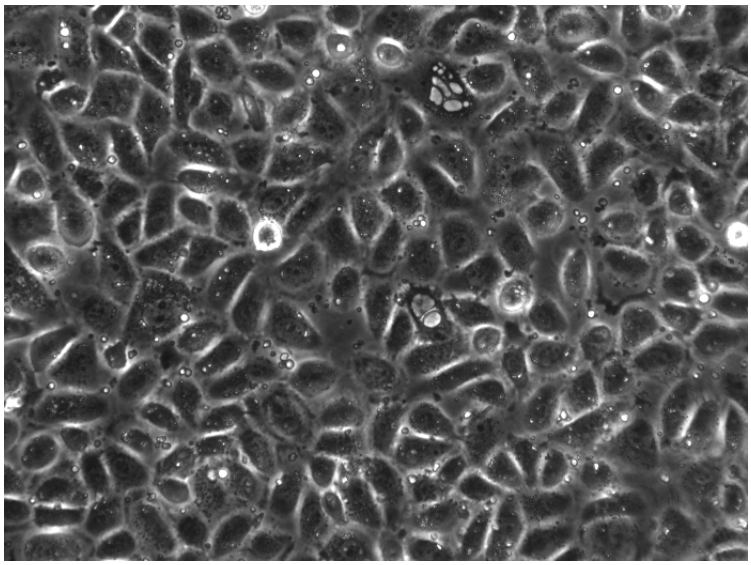


Figure 3.6: Image of microcells co-incubating with the recipient PC-3 cells in the presence of PHA, before fusing them with PEG. (Magnification of the image is x20).

Transfer of a tagged normal human chromosome into the recipient cells confers resistance to hygromycin B in the hybrids, which then proliferate to form a clone (Fig 3.7). Cells that do not possess the chromosome with the selectable HyTK marker are not resistant to hygromycin B and hence fail to proliferate into colonies. Hybrid clones were picked and each cultured as a separate cell line.

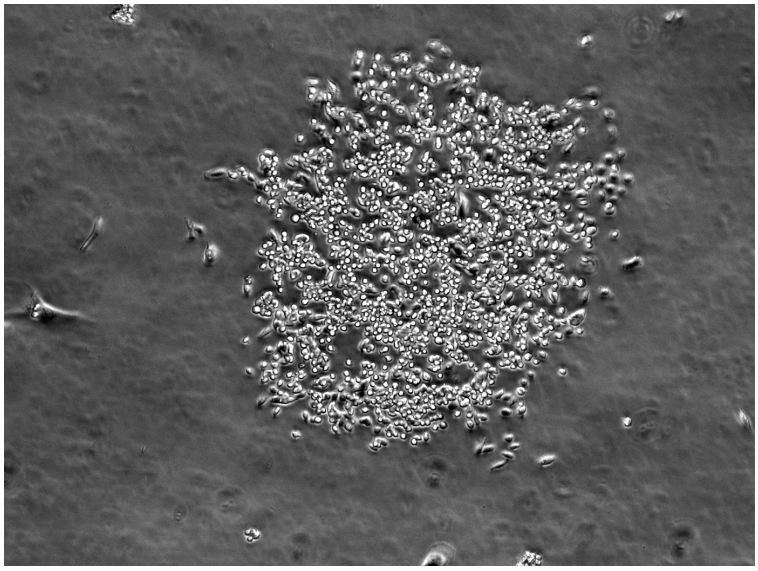


Figure 3.7: Image of a typical highly proliferative clone of PC-3 microcell hybrid cells resistant to hygromycin B. (Magnification of the image is x5).

With certain normal human chromosomes (Table 3.2) occasional hybrids were found to undergo senescence after they were picked and cultured in a P-60 plate to proliferate as an independent cell line (Fig 3.8). A possible reason for recipient cells undergoing senescence in this way is the presence of a telomerase repressor sequence in the transferred chromosome. Alternatively, there is also a possibility that the transferred chromosome possesses other senescence-inducing gene(s). However, in my study, the infrequent occurrence of these senescent colonies suggests that this is probably a phenomenon observed in cell culture where cells rarely undergo senescence.

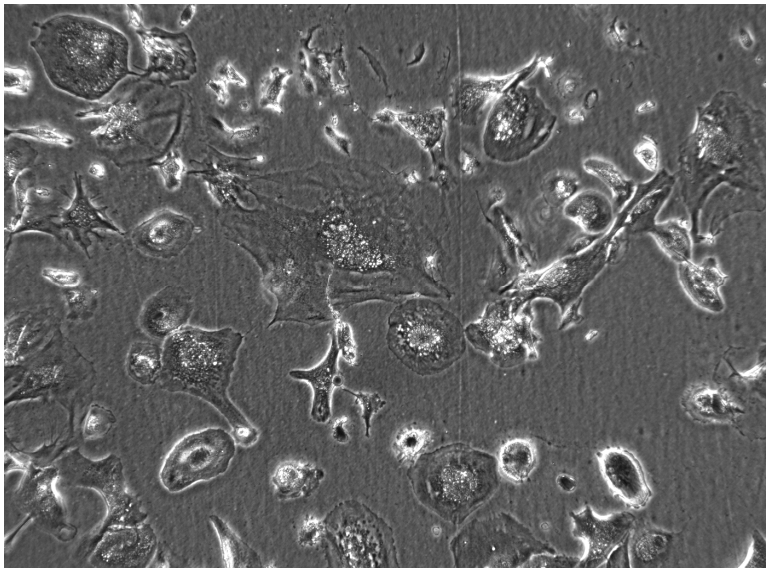


Figure 3.8: Image of a PC-3/chr 11 hybrid clone that underwent senescence. The difference in the morphology of senescent and normal proliferating cells is very evident. Senescent cells are much larger, flatter, and granular; they are metabolically active cells that have lost the ability to divide. (Magnification of the image is x10).

A complete set of hybrid clones was generated from the transfer of each normal human chromosome (Table 3.2) and cultured as independent cell lines. However, chromosome Y could not be transferred into PC-3 cells because a donor cell line containing an individual normal human chromosome Y was unavailable in our laboratory. During the construction of the "donor" panel Cuthbert et al (1995) were unable to isolate a Y chromosome, possibly due to lack of integration of the retrovirus carrying the HyTK marker.

The panel of hybrids generated by individual transfer of normal human chromosomes is a very valuable resource to screen the entire normal human genome for any gene of interest for which there is a clear cellular phenotype, i.e. tumour suppressor genes can be identified by either *in vitro* or *in vivo* growth suppression assays, metastasis suppressor genes by migration assay and telomerase repressor genes, as in this study,

can be identified by the TRAP assay or by the repressed expression levels of *hTERT*, the catalytic subunit of telomerase.

Table 3.2: Summary of the number of hybrid clones generated by MMCT (a single experiment was performed with each transferred chromosome) to study genetic complementation in PC-3, a prostate cancer cell line. The number of clones that underwent senescence from each MMCT is shown in the last column.

| **Hybrids** | **Number of colonies picked and cultured as separate cell lines** | **Number of clones that senesced on the P-60 plate** |
|---|---|---|
| PC-3/chr1 | 19 | |
| PC-3/chr2 | 25 | |
| PC-3/chr3 | 17 | 1 |
| PC-3/chr4 | 20 | |
| PC-3/chr5 | 15 | 1 |
| PC-3/chr6 | 18 | 1 |
| PC-3/chr7 | 16 | |
| PC-3/chr8 | 26 | |
| PC-3/chr9 | 1 | |
| PC-3/chr9 (del p-arm) | 23 | |
| PC-3/chr10 | 20 | 2 |
| PC-3/chr11 | 17 | 1 |
| PC-3/chr12 | 18 | 2 |
| PC-3/chr13 | 38 | |
| PC-3/chr14 | 28 | 1 |
| PC-3/chr15 | 21 | |
| PC-3/chr16 | 24 | |
| PC-3/chr17 | 23 | |
| PC-3/chr18 | 26 | |
| PC-3/chr19 | 5 | |
| PC-3/chr20 | 28 | |
| PC-3/chr21 | 30 | |
| PC-3/chr22 | 18 | |
| PC-3/chrX | 21 | |

## 3.4 Discussion

On average, transfer of most normal human chromosomes into PC-3 cells produced 15-30 hybrid clones from microcells prepared from 12x24cm$^2$ flasks. The two chromosomes that gave a markedly reduced number of clones were chromosomes 9 and 19 (Table 3.2). It is a phenomenon well known in this kind of work that some chromosomes are easier to transfer than others. Generally, I found many experimental factors that can influence the efficiency of a transfer and produce a higher number of clones; these included: (i) the use of donor cells of the lowest passage number possible (because, in my hands, with increasing passage number, donor cells generally produced fewer micronuclei) and (ii) employing an optimal colcemid concentration that produced the highest number of micronuclei with the lowest number of nonviable cells. Later in the project when fragmented chromosome 11 was transferred into the telomerized PC-3 cell line (see section 7.2.1) fewer hybrid colonies were obtained. However, I found that increasing the number of microcells available for fusion with the recipient cells resulted in a larger number of colonies. This was achieved by preparing the microcells from donor cells seeded in 24 flasks compared with 12 flasks for whole chromosome transfers.

The presence of a powerful anti-proliferative gene on human chromosome 9 became evident during the construction of the monochromosomal hybrid panel in our laboratory. Cuthbert et al (1995) were unable to recover intact human chromosome 9 in mouse A9 background when it was directly transferred from diploid human fibroblast into the A9 cells. However, Chinese hamster ovarian (CHO) cells could tolerate intact human chromosome 9 and it was available in our laboratory as part of

the human monochromosome hybrid panel. Later, England et al (1996) provided strong evidence by STS-PCR deletion mapping of segregants generated from monochromosome transfer experiments that it was the $p16^{INK4a}$ (CDKN2A) gene that was responsible for growth suppressive effect of chromosome 9 in A9 cells. The anti-proliferative effect of chromosome 9 was not confined to A9 cells alone. Transfer of chromosome 9 into 5XH11, an X-ray-induced Syrian hamster dermal (SHD) cell line produced one clone. The authors provided further evidence of $p16^{INK4a}$ involvement in rapid growth suppression by transfecting CDKN2A cDNA into 3 human malignant melanoma cell lines.

In my study, MMCT of chromosome 9 generated only a single clone and this is most likely to be due to the presence of the cyclin dependent kinase (cdk) inhibitor gene $p16^{INK4a}$ located on chromosome 9p21 that blocks the cell cycle at G1 resulting in senescence. This pathway is often inactivated in the development of epithelial cancers including prostate cancer. Perinchery et al (1999) reported LOH on 9p21 in 72.5% of prostate cancer samples and they found the expression of the p16 protein to be either absent or very low suggesting the loss of p16 may be involved in prostate carcinogenesis. However, when Tamimi et al (1996) investigated the frequency of $p16^{INK4a}$ mutations/deletions in 20 primary prostate tumours and four established prostate cancer cell lines, they only found two mutations; one in DU145 cell line and the other in a primary tumour, suggesting that mutation of $p16^{INK4a}$ gene is not a frequent event in prostate cancer. Nevertheless, when I transferred chromosome 9 with a deleted p-arm into PC-3 cells, 23 clones were produced, providing further evidence that the $p16^{INK4a}$ tumour suppressor gene or some other gene located on

chromosome 9p may have contributed to the low colony number when whole chromosome 9 was transferred.

Transfer of chromosome 19 produced the second lowest number of clones (5). Gao et al (1999) had earlier reported a region on chromosome 19p13.1-13.2 that is responsible for suppressing *in vitro* and *in vivo* growth of both human (TSU-pr1) and rat (Dunning-R3327 AT6.1) prostatic cancer cells. Therefore, there is a possibility that the low colony number may be due to the presence of a growth suppressor gene(s) in the 19p13.1-13.2 region.

Even though the human chromosomes are intact and stable in our donor cell lines, as characterized by comprehensive cytogenetic and molecular analyses such as G-banding, FISH analysis and STS mapping (Cuthbert et al., 1995), the MMCT technique can result in either large or small deletions of the transferred chromosome once it has been fused into the recipient cells. This fact can be turned into a useful advantage in any gene mapping exercise, since heterogeneity in the genetic content of clones that are generated (with respect to the transferred chromosome) can permit sub-chromosomal localization of a gene of interest. With respect to a telomerase repressor sequence, one might therefore expect to see a mixture of hybrid clones that were repressed and others that were not. This of course assumes that the gene of interest is not positioned in close proximity to the selectable marker.

# Chapter 4

# Identification of the chromosome that may carry a gene (or genes) involved in telomerase repression in prostate cancer

## 4.1 Introduction

Normal human somatic cells undergo only a limited number of cell divisions before entering replicative senescence (RS). RS is an irreversible growth arrest that has been suggested to act as a protective barrier against cancerous transformation (Collado et al., 2007). In contrast, tumour cells overcome replicative senescence and grow indefinitely i.e. become immortal (reviewed in Campisi and di Fagagna., 2007). Telomeres are specialized DNA structures at the ends of linear chromosomes and their maintenance is a prerequisite for immortalization and cancer formation that commonly occurs through activation of telomerase (reviewed in Shay and Wright, 2005, Newbold 2005). Telomerase is tightly regulated in normal human somatic cells but the majority of tumour and immortal cells have elevated levels of telomerase activity in order to maintain telomere lengths (Kim et al., 1994). Inactivation or loss of both alleles of a critical gene that functions to repress telomerase could be an important event in the transition of normal cells into cancerous cells. Several studies have shown that introduction of a normal copy of a specific human chromosome by MMCT can be used to induce repression of telomerase activity in various cancers

(Horikawa et al., 1998; Cuthbert et al., 1999; Steenbergen et al., 2001; Nishimoto et al., 2001). The purpose of this part of my study was to measure telomerase activity in hybrids that I previously generated (Chapter 3) by the individual transfer of normal human chromosomes into PC-3 cells and hence identify a chromosome(s) that can repress telomerase activity in prostate cancer cells.

## 4.2 Materials and Methods

### 4.2.1 Determination of telomerase activity by conventional TRAP assay

#### 4.2.1.i Sample preparation

The surface of the hood and the pipettes were cleaned with RNase-Away solution before protein isolation. Cell hybrids from MMCT were cultured in P-60 dishes and trypsinized as normal when 50-80% confluent. The cell pellet was flicked and resuspended at approximately 3-5x$10^5$ in complete culture medium containing 0.1mM PMSF. Cells were counted and 2 times $10^5$ cells for each hybrid were spun down in 0.5ml microcentrifuge tubes. The supernatant was carefully removed with a Gilson pipette and the pellets frozen down immediately on dry ice. The samples were stored at -80$^o$C until required.

#### 4.2.1.ii Protein isolation

The samples to be assayed were thawed out on wet ice and 200µl of cold x1 CHAPS lysis buffer was added to each $10^5$cell pellet. Pellets were retropipetted vigorously and left to incubate on ice for 30min. Samples were centrifuged at 13000rpm for

20min at 4°C. Approximately 160µl of the supernatant was pippeted into a microcentrifuge tube and rapidly frozen down on dry ice for telomerase activity measurement. Twenty microlitre of the supernatant was used for protein determination.

**4.2.1.iii Protein assay**

Coomassie protein assay reagent (Pierce Solution) was used to determine the protein concentration in the samples. A standard curve was set up with BSA (Bovine serum albumin), dissolved in x1 CHAPS lysis buffer between 0 to 20µg protein. One millilitre of the Pierce solution was added to each tube set up for the standard curve and the unknown samples (20µl). The standards and the samples were incubated for exactly 5min at room temperature. The absorbance was read at 595ηm. The protein concentration of the unknown samples was determined from the standard curve and the samples were diluted to 50ηg/µl in CHAPS lysis buffer.

**4.2.1.iv Telomeric repeat amplification protocol (TRAP) assay**

TRAPeze, a telomerase detection kit (Intergen Company) was used to determine telomerase in actively dividing cells. All the samples to be assayed were thawed out on ice. TS primer was End labelled with $\gamma$-$^{32}$P-ATP (Amersham Biosciences) using the following reagents provided in the kit:

| | | |
|---|---|---|
| For 10 assays | TS primer | 10µl |
| | 10x KinaseA buffer | 2µl |
| | $T_4$ Poly Kinase | 0.5µl (10U/µl) |

| | | |
|---|---|---|
| | $dH_2O$ | 5µl |
| | $\gamma^{32}$P-ATP | 2.5µl |

The tube was placed in a PCR block for 20min at 37$^{o}$C and for 5min at 85$^{o}$C.

The PCR mastermix was prepared for the number of samples to be assayed in 1.5ml eppendorf tube using the following reagents:

| | | |
|---|---|---|
| For each assay | 10x TRAP buffer | 5µl |
| | 50x dNTP | 1µl |
| | TRAP primer mix | 1µl |
| | Taq (5units/µl) | 0.4µl (2 Units) |
| | $ddH_2O$ | 38.6µl |
| | $\gamma^{32}$P TS primer (end label) | 2µl |

Radioactive end label was added to the master mix at the very end and mixed well.

To 46µl of master mix 4µl of the sample to be assayed, 4µl of x1CHAPS lysis buffer (negative control), 2µl of $H_2O$ and 2µl of TSR8 (positive control) was added to the PCR tubes. The tubes were placed into a PCR machine (Tetrad, Peltier Thermal Cycler PTC-225) and the samples incubated at 30$^{o}$C for 30min and amplified using a two-step PCR at 94°C for 30sec, 59°C for 30sec for 30 cycle.

**4.2.1.v Polyacrylamide gel electrophoresis**

Non-denaturing 12.5% polyacrylamide gels were prepared in x0.5 TBE buffer and poured into 16 x 16cm plates. Ten microlitre of loading buffer was added to the PCR products and 30µl samples were loaded onto the vertical gel. The gel was allowed to run at constant 200V for approximately 3 to 3.5hr or until the bromophenol blue ran

out of the gel and xylene cyanol blue ran 70-75% of the gel length. Cold water was circulated through the system to prevent overheating of the gel; this prevents distortion of bands from excessive heat. The gel was dried and exposed overnight onto a phosphorimager screen in a cassette at room temperature before scanning the screen in the phosphorimager (Storm 820, Amersham Pharmacia Biotech).

## 4.3 Results

### 4.3.1 Effects of normal human chromosomes on telomerase activity in a prostate cancer cell line, PC-3, following microcell fusion

There was no published data available concerning the involvement of normal human chromosomes in telomerase repression in prostate cancer cells. Therefore, the entire chromosome panel was transferred into PC-3 as a complete screen of the human genome. There was no indication as to which chromosome may harbour a telomerase repressor from the number of hybrid clones recovered after MMCT or indeed the growth rate of hybrid cells collected from different transfers.

The hybrids generated from transferring normal human chromosome 3, 4, 10, 11, 17 and 21 into PC-3 cell line were selected to determine the level of telomerase activity in their respective clones. The reason for selecting these hybrids in the first phase of the study was because: (i) a region on the small (p) arm of chromosome 3 has already been found to repress telomerase activity in human breast cancer cell line in our laboratory (Cuthbert et al., 1999) and a different region on chromosome 3 had been found to repress telomerase in a human renal carcinoma cell line (Oshimura and

Barrett., 1997); (ii) chromosome 4 had been shown previously to repress telomerase in HeLa, a cervical cancer cell line (Backsch et al., 2001) and Bertram et al (1999) had identified a senescence gene on chromosome 4q; (iii) chromosome 10 had been shown to repress telomerase activity in hepatocellular carcinoma cell line (Nishimoto et al. 2001); (iv) chromosome 17 hybrids were selected because loss of the *TP53* gene is common in a variety of human cancers possibly leading to increased proliferative potential and ultimately to clonal evolution and malignant progression. Several groups have reported mutations in the *p53* gene in prostate cancer cells (Isaacs et al., 1991; Chi et al., 1994). (As described in section 1.2.9.iv, there is also evidence that p53 is directly involved in telomerase repression in normal cells); (v) chromosome 11 hybrids were selected because the loss of heterozygosity on both arms of chromosome 11 has been reported in prostate cancer (Dahiya et al., 1997); (vi) chromosome 21 hybrids were selected randomly. Telomerase activity was assessed in each of the hybrids at the same early growth stage, i.e. Passage 1, after transfer to mass culture using the commercially available TRAPeze kit.

The initial plan was to screen all of the hybrids. However, the observed repression by chromosome 11 led us to focus on this chromosome and map the critical gene.

### 4.3.2 Phosphorimager scans of TRAP gels showing telomerase activity in clones of various hybrids obtained after transfer of normal human chromosomes.

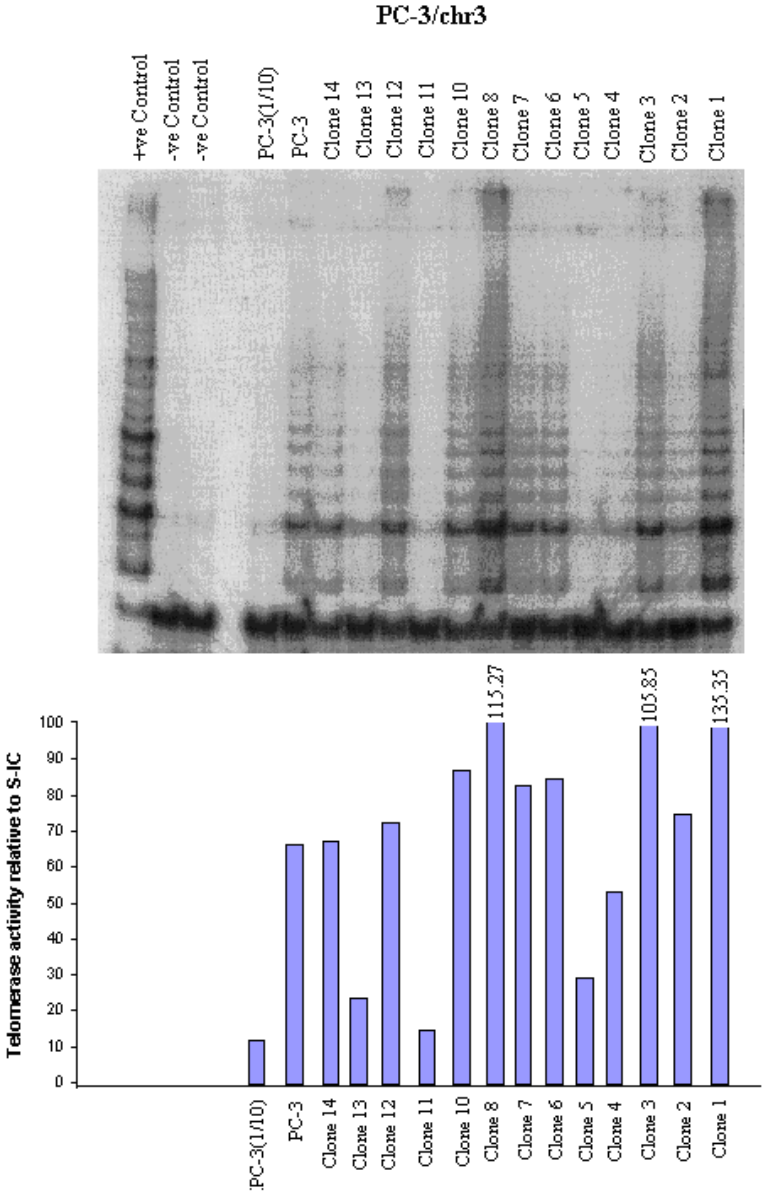



Figure 4.1: Levels of telomerase activity in hybrid clones generated by the transfer of normal human chromosome 3 into the PC-3 cell line. The corresponding histogram shows the level of telomerase activity relative to the internal control, S-IC. Control PC-3 values were obtained from mass cultures of PC-3 cells not subjected to MMCT.

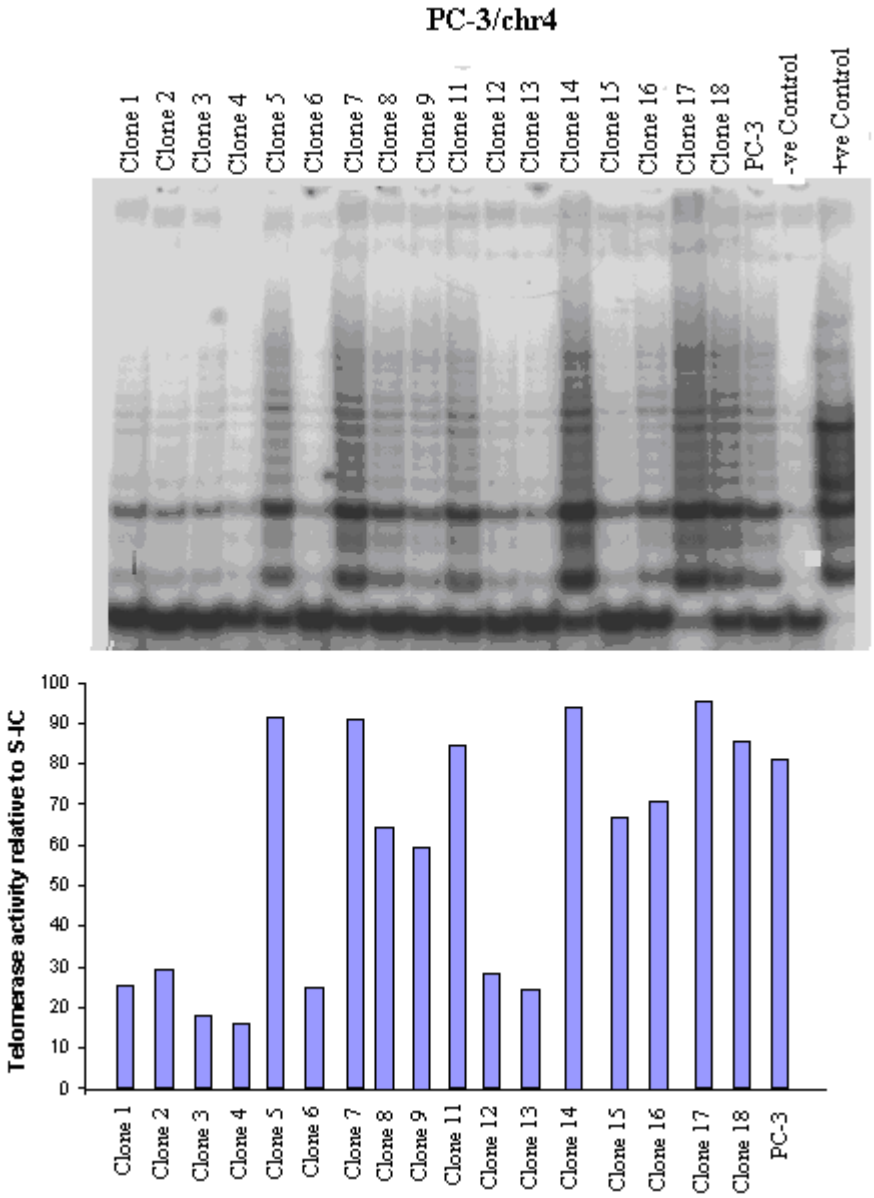



Figure 4.2: Levels of telomerase activity in hybrid clones generated by the transfer of normal human chromosome 4 into the PC-3 cell line. The corresponding histogram shows the level of telomerase activity relative to the internal control, S-IC. Control PC-3 values were obtained from mass cultures of PC-3 cells not subjected to MMCT.

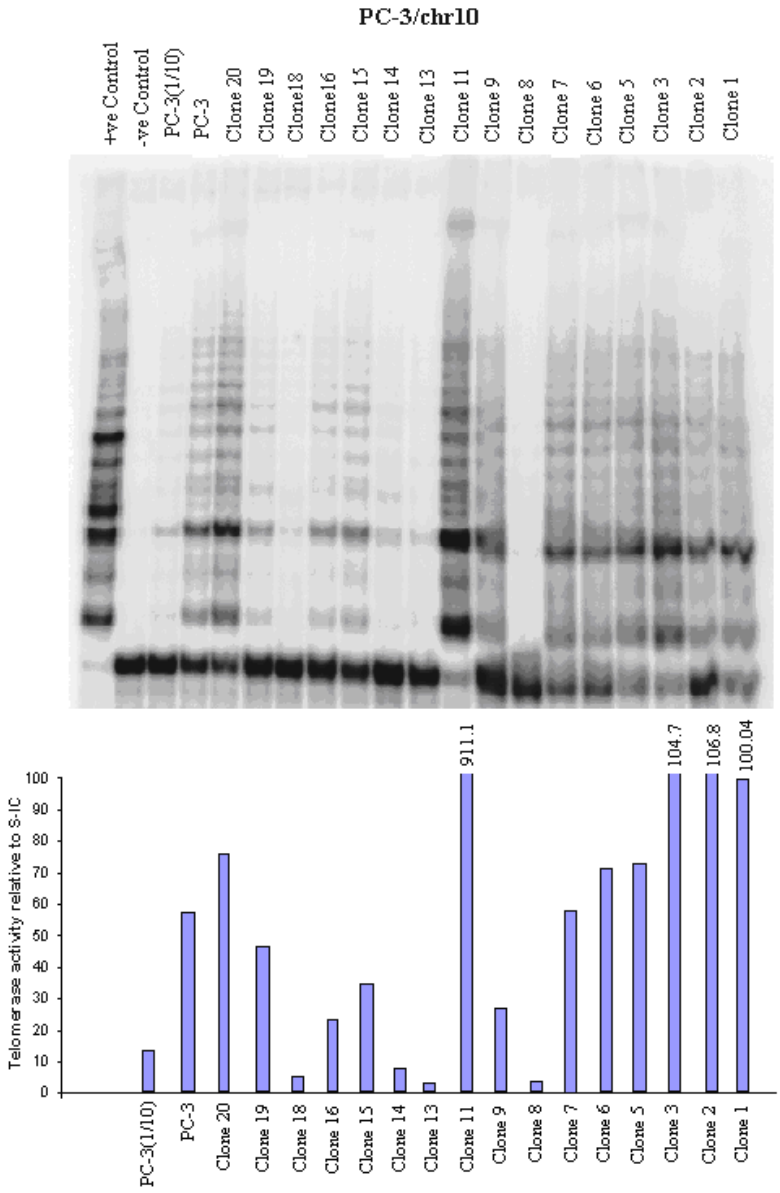



Figure 4.3: Levels of telomerase activity in hybrid clones generated by the transfer of normal human chromosome 10 into the PC-3 cell line. The corresponding histogram shows the level of telomerase activity relative to the internal control, S-IC. Control PC-3 values were obtained from mass cultures of PC-3 cells not subjected to MMCT.

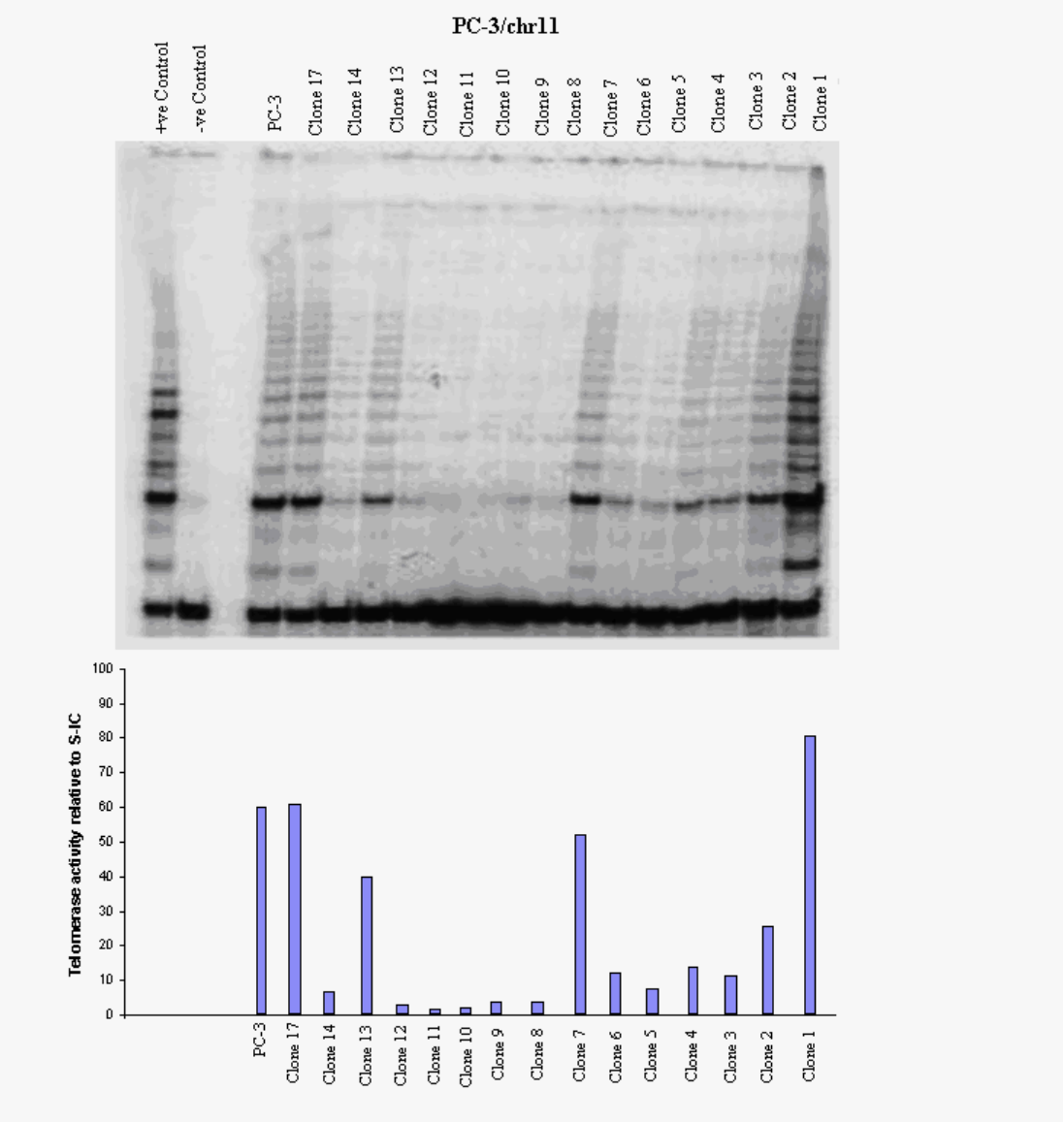



Figure 4.4: Levels of telomerase activity in hybrid clones generated by the transfer of normal human chromosome 11 into the PC-3 cell line. The corresponding histogram shows the level of telomerase activity relative to the internal control, S-IC. Control PC-3 values were obtained from mass cultures of PC-3 cells not subjected to MMCT.

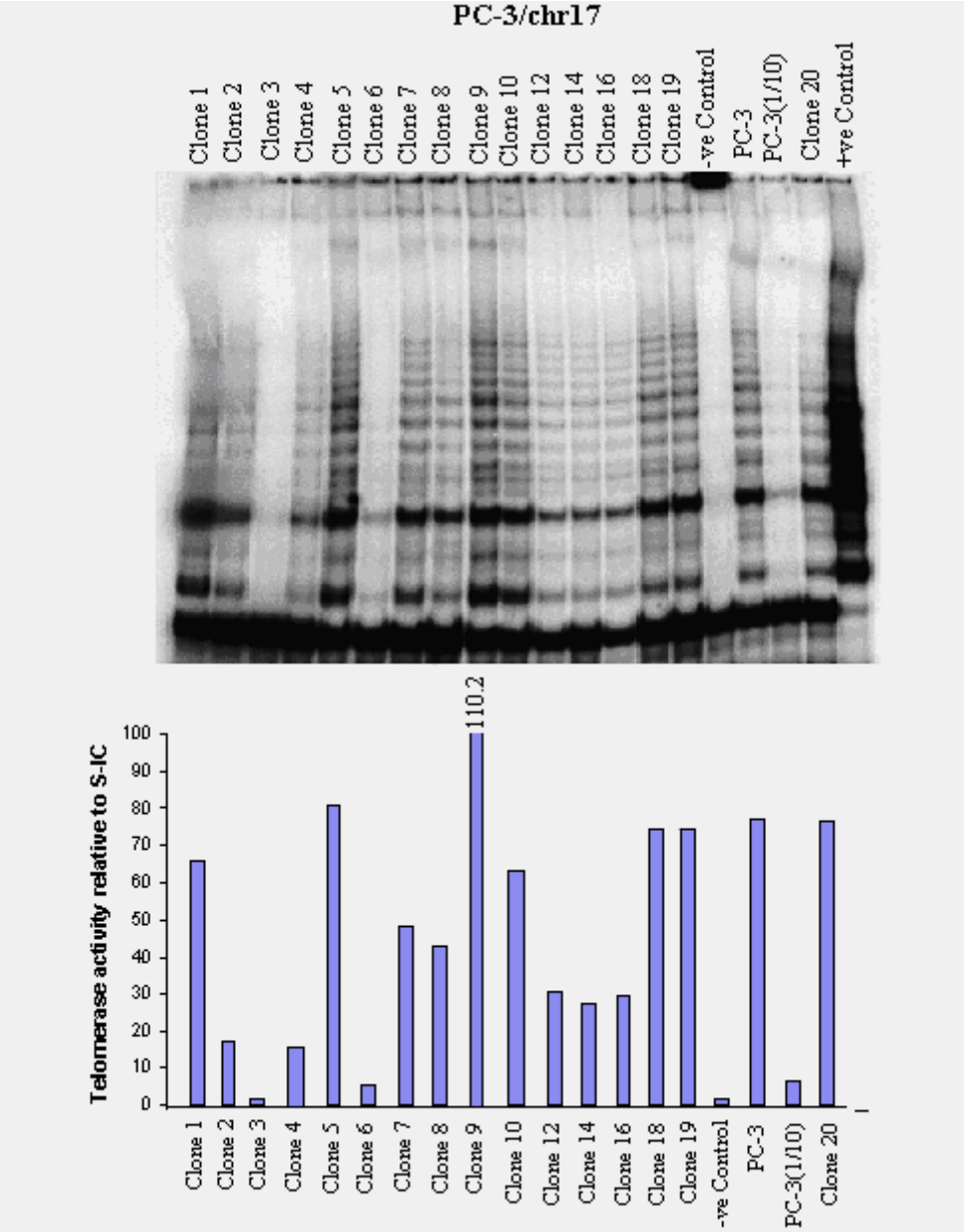



Figure 4.5: Levels of telomerase activity in hybrid clones generated by the transfer of normal human chromosome 17 into the PC-3 cell line. The corresponding histogram shows the level of telomerase activity relative to the internal control, S-IC. Control PC-3 values were obtained from mass cultures of PC-3 cells not subjected to MMCT.

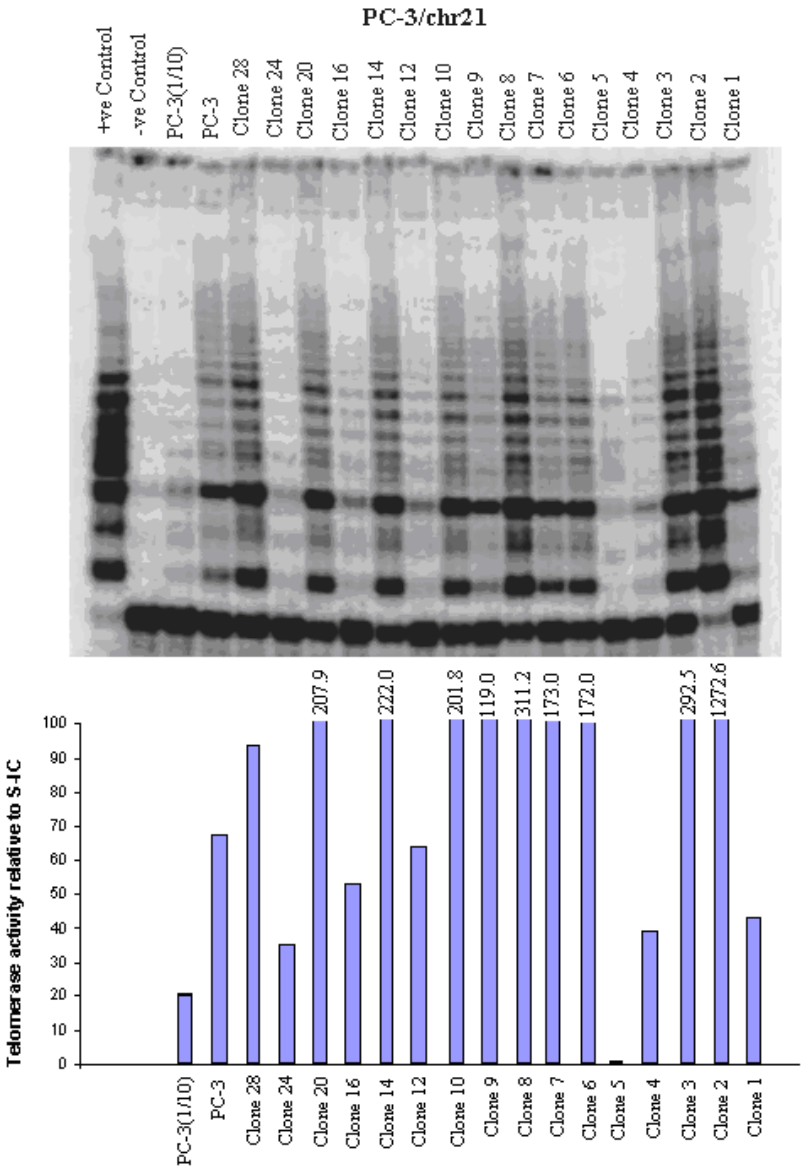



Figure 4.6: Levels of telomerase activity in hybrid clones generated by the transfer of normal human chromosome 21 into the PC-3 cell line. The corresponding histogram shows the level of telomerase activity relative to the internal control, S-IC. Control PC-3 values were obtained from mass cultures of PC-3 cells not subjected to MMCT.

ImageQuant analysis of phosphorimager scans of TRAP gels are represented as a histogram (Figs 4.1-4.6), showing telomerase activity in various hybrids obtained following MMCT of chromosomes 3, 4, 10, 11, 17 and 21 into PC-3 cells. A

summary comparing telomerase activity between the various hybrid clones and PC-3 cells is shown in Table 4.1.

In each set of hybrids, I found a proportion of clones that expressed low levels (less than 50% of the parent PC-3 cells) of telomerase activity, e.g. clones 11 and 13 (2/13) of PC-3: chromosome 3 hybrids (Fig 4.1), and clones 1-4, clones 6, 13 and 14 (7/17) of PC-3: chromosome 4 hybrids (Fig 4.2). The difference in telomerase activity between these hybrids could possibly be due to clonal variation that is normally observed when the parent line is seeded at clonal density and telomerase activity is measured randomly in selected clones. Savre-Train et al (2000) studied the level of telomerase activity and telomere length in telomerase-positive tumour-derived human cell lines and clones of these cell lines and found clonal heterogeneity existed in both telomerase activity and telomere length in all the cell lines that were examined.

Chromosome 10: PC-3 hybrid clones 8, 13, 14, and 18 (Fig 4.3) possessed very low levels of telomerase activity (less than 10% of the parent PC-3 cell line), as did chromosome 17: PC-3 hybrid clones 3 and 6 (Fig 4.5). Certainly there is a possibility that more than one normal human chromosome carries a sequence that appears to repress telomerase activity in prostate cancer as has been reported for HeLa, a cervical cancer cell line, by MMCT of normal human chromosomes 3 and 4 (Backsch et al., 2001). However, the apparent level of strong telomerase repression in chromosome 11 hybrids was remarkably higher (40%) compared with chromosome 10 (18.7%) and 17 (12.5%) hybrids (Fig 4.4).

A very high level of telomerase activity was observed in 56% (9/16) of chromosome 21: PC-3 hybrid clones (Fig 4.6). The telomerase activity was at least 2 fold higher than the parent PC-3 cells in all the nine clones. There is a possibility that an unknown positive regulator of telomerase activity exists on chromosome 21.

Table 4.1: A summary comparing the levels of telomerase activity in hybrids generated by MMCT of normal human chromosomes into PC-3 cells; the first column shows the percentage of hybrid clones that had less than 10% of telomerase activity of the parental PC-3 cell line. The second column shows the percentage of hybrid clones that had 20% lower telomerase activity than that of PC-3 mass cultures.

| **Hybrids** | **Percentage of hybrid clones with less than 10% telomerase activity of PC-3 mass cultures** | **Percentage of hybrid clones with 20% lower telomerase activity than that of PC-3 mass cultures** |
|---|---|---|
| PC-3/chr3 | 0 (0/13) | 31 (4/13) |
| PC-3/chr4 | 0 (0/17) | 47 (8/17) |
| PC-3/chr10 | 19 (3/16) | 44 (7/16) |
| PC-3/chr11 | 40 (6/15) | 80 (12/15) |
| PC-3/chr17 | 13 (2/16) | 56 (9/16) |
| PC-3/chr21 | 6 (1/16) | 25 (4/16) |

The data shows that chromosome 11 was the most persuasive candidate for harbouring a telomerase repressor sequence. Due to the conventional TRAP being a semi-quantitative method, the decision was made next to measure *hTERT* mRNA quantitatively in the hybrids, since it has been shown that there is a strong correlation between *hTERT* mRNA expression levels and telomerase activity (Meyerson et al., 1997). As will be seen in the following Chapters, hTERT analysis provided more

convincing evidence for a telomerase repressive sequence located on normal human chromosome 11.

## 4.4 Discussion

Screening the hybrid clones, generated by the individual transfer of normal human chromosomes into PC-3, for repressive effect on telomerase activity suggests that a telomerase repressor sequence is located on chromosome 11. Several groups have studied the effects of MMCT on telomerase repression by individual transfer of normal human chromosomes into telomerase-positive cancerous cell lines originating from various tissues. Different regions of normal human chromosome 3 have been reported to repress telomerase activity in breast, renal and cervical cancer cell lines (Cuthbert et al., 1999; Horikawa et al., 1998; Backsch et al., 2001 respectively). However, transfer of chromosome 3 into the prostate cancer cell line, PC-3, showed no convincing effect on telomerase activity. Collectively, studies carried out on other tumour cell lines suggest that genes regulating telomerase could be tissue-specific as proposed by Tanka et al (2005). These authors provided evidence from genetic complementation that different putative telomerase repressors were inactive in different types of cancers by whole cell fusion experiments involving two renal cell carcinoma cell lines (KC12 and RCC23) and a breast cancer cell line (21NT). In these studies fusion of KC12 and RCC23 generated hybrids with comparable telomerase activity to that of the parent cell lines, indicating that both renal carcinoma cell lines possessed the same defect. Fusion of 21NT cells with either KC12 or RCC23 cells produced a significant number of telomerase-negative hybrids, suggesting that the defect gene responsible for telomerase repression in the two renal cell lines differed from that in the breast cancer cell line.

In my study, further evidence that tissue-specific telomerase repressors are inactivated in different types of cancers emerged from the individual transfers of chromosome 10 and chromosome 4 into PC-3 cells. Normal chromosome 10 and chromosome 4 were relatively ineffective in reducing telomerase activity compared with chromosome 11, although chromosome 10 has been reported to repress telomerase activity in hepatocellular carcinoma (Nishimoto et al., 2001) and chromosome 4 in a cervical carcinoma cell line (Backsch et al., 2001). The fact that gene(s) on different normal human chromosomes appear to be able to repress telomerase activity in different tissues could indicate that not all the tumours have the same defective gene that regulates telomerase activity (Oshimura and Barret, 1997) and that some telomerase repressors may be subjected to inactivation in a tissue-specific manner (Tanaka et al., 2005). Alternatively, there may be more than one target gene in a pathway regulating telomerase. Several cell lines from each cancer type would need to be analysed to establish this.

In my study, telomerase activity was measured in hybrids generated by the transfer of normal human chromosomes into PC-3 cells. A significant level of telomerase repression in PC-3 chromosome 11 hybrids was observed at passage 1 in the absence of replicative senescence. Rapid senescence was recorded only in a few clones of different hybrids (see Table 3.1) and these clones were not analysed for telomerase activity due to the limited cell number. The most likely reason for not observing replicative senescence in a larger number of PC-3 chromosome 11 hybrids is that the hybrid cells were not cultured beyond passage one and that there may have been a delay in telomere shortening (approximately 150 to 200bp are lost with each cell

division) and the activation of replicative senescence, since our Q-FISH results (see section 5.3.2.) have shown that PC-3 cells have moderately long average telomere (5.6Kb). Hence, there is a significant probability that replicative senescence may have been observed if I had cultured the cells further than Passage 1. In this connection, Oshimura et al (1995) reported a higher growth potential of RCC23 chromosome 3 telomerase repressed hybrids (at 23-43 population doublings) compared with that reported by Cuthbert et al (1999) in 21NT chromosome 3 repressed hybrids (at 10-18 population doublings). The difference in growth potential observed in these renal and breast carcinoma cell lines could well have been due to the difference in original telomere lengths of the parent cell lines, which were 6kb and 3kb respectively.

# Chapter 5

# Construction of a PC-3 recipient cell line ectopically expressing *hTERT* cDNA

## 5.1 Introduction

Unlike normal human cells, human tumour cells can sometimes be established as permanent immortal cell lines. The presence of active telomerase is thought to be responsible for tumour cell immortality (Kim et al., 1994). Several groups have cloned the genes encoding the two main components of telomerase, the hTR (Feng et al., 1995) and hTERT (Meyerson et al., 1997). Subsequent work has shown that only the expression of the *hTERT* gene encoding the catalytic component of telomerase correlated with telomerase activity (Takakura et al., 1999; Meyerson et al., 1997). *hTERT* mRNA was detected in telomerase positive cell lines and tumours but not in telomerase negative cells. Furthermore, the expression of *hTERT* mRNA was induced upon telomerase activation during cellular immortalization (Kilian et al., 1997) and down-regulated when human promyelocytic leukaemia HL60 cells were induced to differentiate in vitro (Meyerson et al., 1997). These findings strongly suggested that *hTERT* mRNA expression is the rate limiting component of telomerase activity.

Subsequent studies have shown that the life–span of normal human cells can be extended by ectopically forcing the expression of telomerase by stable transfection of telomerase-negative cells with an *hTERT* cDNA construct (Counter et al., 1998; Bodnar et al., 1998). In my study, I wanted to ensure that I did not encounter replicative senescence when a normal human chromosome carrying a telomerase repressor sequence was transferred into the PC-3 cell line. Therefore, in the next phase of the project, PC-3 cells were stably transfected with an *hTERT* cDNA plasmid construct to ectopically express the *hTERT* gene. With the design of PCR primers that amplify cDNA sequence cloned from only immature (pre-spliced) endogenous *hTERT* mRNA, quantification of endogenous *hTERT* in the absence of replicative senescence would be possible.

## 5.2 Materials and Methods

### 5.2.1 Human telomerase reverse transcriptase (hTERT) plasmid construction

Empty viral vector pBABE puro (5.05kb) and the viral construct pBABE puro *hTERT* were gifts from Professor Wynford-Thomas, Cardiff. A 4kb *hTERT* cDNA insert from pBABE puro *hTERT* was cloned into the EcoR1 cloning site of a mammalian expression vector, pCI-neo (Stratagene) to create PCI-neo *hTERT* (by Dr. Scott Ellis, Brunel Institute of Cancer Genetics) (Fig 5.1). The orientation of the *hTERT* cDNA insert was confirmed by sequencing the construct (which has to be 5'-3' with respect to the CMV promoter).

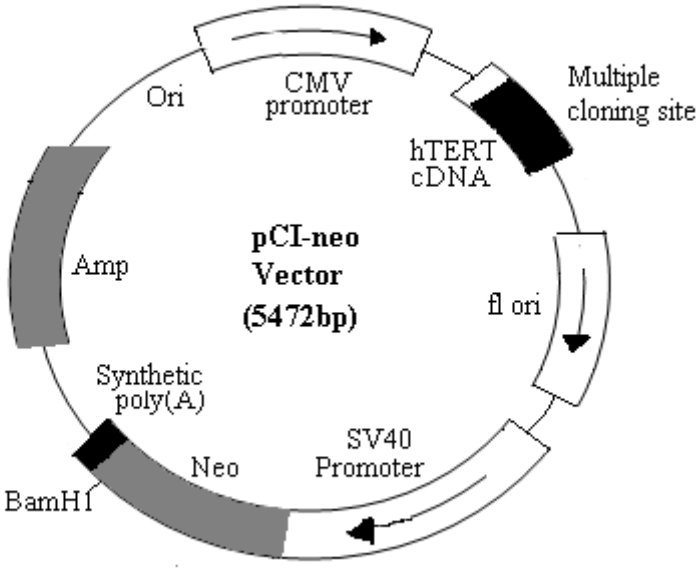



Figure 5.1: Diagram of pCI-neo vector.

The pCI-neo carries the human cytomegalovirus (CMV) promoter region that promotes the expression of the cloned DNA insert. The neomycin phosphotransferase gene in the vector allows the transfected cells to be selected with the antibiotic G418.

### 5.2.2 Stable transfection of PC-3 with *hTERT* plasmid construct using TfX$^{TM}$-20 liposomes

PC-3 cells were plated at 1.0x $10^6$ / 100mm dish, in triplicate in Ham's F-12 medium containing 7% FBS and left to proliferate overnight. Six millilitres of SFM pre-warmed to 37°C was placed into 3 tubes and 22.5µl of TfX20 reagent (Promega) was added to all the tubes; the first tube was the negative control, to the second tube 0.5-1.0µg of the empty plasmid pCI-neo was added (positive control) and to the third tube 0.5-1.0µg of pCI-neo-*hTERT* was added (test). The content of the tubes were

vortexed immediately and left to incubate at room temperature for 10-15min. The culture medium was aspirated from the 3 dishes of PC-3 cells and the content of each tube was added. The plates were returned to the incubator for 1hour. Twelve millilitres of complete medium was then added to each dish and the dishes left to incubate overnight. The cells were split 1:6 and cultured for 3 days prior to selection with 400μg/ml of geneticin (G418). Cells were fed once a week until colonies appeared. Once picked, the clones were maintained in 200μg/ml of G418. Subsequently total telomerase activity and immature (pre-spliced) *endogenous hTERT* mRNA expression levels (see section 5.2.4.iii) were determined in all the clones by quantitative TRAP analysis and real time qRT-PCR respectively.

### 5.2.3 Quantitative TRAP assay

A quantitative method developed by Henning, et al (2003) was used for assaying total telomerase activity (endogenously and exogenously expressed). Cell samples were lysed in CHAPS lysis buffer and the protein concentration measured as for the conventional TRAP assay. The total volume of the reaction mixture was 25μl/sample. Each reaction mixture consisted of 1μl of protein sample and 24μl of SYBR Green Mastermix, which contained 1x SYBR Green buffer, 0.1μg of TS primer (5′-AATCCGTCGAGCAGAGTT-3′) and 0.05μg of ACX primer (5′GCGCGG$(CTTACC)_3$CTAACC-3′). 24μl of SYBR Green mastermix and 1μl of 250ηg/μl protein sample was dispensed into each well of a microlitre plate. Each sample was assayed in triplicate. The PCR was performed on an ABI PRISM 7900 HT Sequence Detection System. The reaction mixture was first incubated at 25°C for

20min to allow the telomerase in the protein extracts to elongate the TS primer by adding TTAGGG repeat sequence. PCR was then started at 95°C for 10min to activate the Taq polymerase followed by a two-step PCR amplification of 35 cycles at 95°C for 30s and 60°C for 90s. A standard curve was generated from serially diluted telomerase positive prostate cell line; PC-3 extracts ($10^4$ to $10^1$ cells). The threshold cycle values ($C_t$) of the unknown samples were read off against the standard curve, and the level of telomerase activity compared to the number of PC-3 cells.

### 5.2.4 Determination of immature *endogenous hTERT* and *GAPDH* mRNA expression levels using real-time quantitative RT-PCR

#### 5.2.4.i Complementary DNA (cDNA) preparation

**DNase 1 treatment of RNA samples**

Total RNA was treated with DNase 1 (deoxyribonuclease 1, Amplification grade: Invitrogen, life technologies) prior to RT-PCR using the volumes of the reagents per sample as below:

4µg of total RNA

2µl X10 Buffer

0.5µl of RNase inhibitor (RNase Out)

2µl of DNase 1

The reaction volume was made up to 20µl with DEPC treated water. The samples were incubated for 1hr at room temperature to digest single and double stranded DNA. The DNase 1 was inactivated by adding 2µl of 25mM EDTA solution to the

reaction mixture followed by incubation at 65$^{o}$C for 15min. The samples were chilled on ice.

**First strand cDNA synthesis using SuperScript$^{TM}$11 for RT-PCR**

The following were placed in a PCR tube to reverse transcribe the *GAPDH* and the *hTERT* genes using gene specific primers:

5µl of DNase treated RNA.

1µl of reverse primer for GAPDH 3407 (100µM freshly diluted to 1:200)

1µl of reverse primer for hTERT 13174 (100µM freshly diluted to 1:200)

1µl of 10mM dNTP mix (10mM each of dATP, dGTP, dCTP, dTTP).

4µl of water

The above was incubated at 70$^{o}$C for 10min in a PCR machine. The tubes were chilled on ice and the following was added to the tubes.

4µl of X5 First Strand Buffer

2µl of 0.1M DTT

0.5µl of RNase Out

1µl (40U) of SUPERSCRIPT II

The final volume was made up to 20µl with DEPC treated water and the reaction mixture incubated at 42$^{o}$C for 50min. The reaction was inactivated by heating at 70$^{o}$C for 15min. The resulting cDNA was stored at -20$^{o}$C.

### 5.2.4.ii PCR amplification of GAPDH to check the quality of the cDNA

This step was used to check the quality of the cDNA prepared. Forward and reverse primers for the *GAPDH* gene were used for this purpose. One microlitre of cDNA template was added to 1µl of primer mixture (8.3µM of forward primer 1457 (5'GAAGGTGAAGGTCGGAGT 3') and 8.3µM of reverse primer 3407(5'GAAGATGGTGATGGGATTTC 3')) and the final volume was made up to 20µl with Reddymix Mastermix (*AB*Gene). The samples were initially incubated at 95$^{o}$C for 5min followed by 30cycles of 95$^{o}$C for 40sec denaturation, 55$^{o}$C for 45sec annealing and 72$^{o}$C for 45sec extension. A final extension step of 72$^{o}$C for 10min was added. Ten microlitre product of the PCR reaction was loaded onto 2% agarose gel, made up in TBE buffer containing 5µl of 10mg/ml Ethidium Bromide, 10µl of 1kb ladder was also loaded in order to estimate the size of the amplicon. The mini-gel was allowed to run at constant voltage at 40V for approximately 90min and the gel visualized on Alpha Imager (Alpha Innotech Corporation).

### 5.2.4.iii Quantification of immature *endogenous hTERT* and *GAPDH* mRNA levels using Real-Time PCR

I used a quantitative RT-PCR assay developed by our collaborators (Ducrest et al., 2001) to measure only the immature (pre-spliced) *endogenous hTERT* mRNA expression levels in the cDNA samples prepared from the hybrids. The primer pair that was used to amplify cDNA from immature nuclear endogenous *hTERT* spanned exon 2 and 256 nucleotides of intron 2. This immature nuclear *hTERT* correlated

with telomerase activity, indicating that *hTERT* RNA levels are regulated in the nucleus (Ducrest et al., 2001).

To x1 TaqMan Mastermix (Applied Biosystems) 0.4μM of *hTERT* forward primer 12896 (5'GAGCTGACGTGGAAGATGAGC 3' sequence localized at exon 2), 0.4μM of *hTERT* reverse primer 13156 (5'GGTGAACCTCGTAAGTTTATGCAA 3' localized at intron 2) and 0.1μM of TaqMan probe was added. To 22.5μl of the prepared mastermix 2.5μl of the cDNA was added and placed into a well of a microtiter plate to give a final volume of 25μl. Each sample was assayed in triplicate. Thermal cycling conditions consisted of a step at 50$^{o}$C for 2min, a denaturing step at 95°C for 10min followed by 50 cycles at 95°C for 15s and 60°C for 1min (ABI PRISM 7900 HT Sequence Detection System). The number of *hTERT* copies in the unknown samples was determined by constructing a standard curve using dilutions of a plasmid of known concentration that had been converted into copy number for the *hTERT* gene. The *hTERT* plasmid used for this purpose was generated by cloning the PCR product, amplified by the primers 12896(fw) and 13174(rv) into PCR 2.1 TOPO (by Dr Terry Roberts, BICGP). The PCR machine plots the cycle number at which the product is above the background (Ct) against the log of the number of template molecules. The efficiency of the assay is indicated by the slope of the line and the accuracy by linear regression coefficient of the standard curve. To determine the copy number of the *GAPDH* molecules in the samples, to 1X Sybr Green Mastermix (Applied Biosystems) 0.4μM of forward primer probe 1457 (5'GAAGGTGAAGGTCGGAGT 3' sequence localised at exon 1) and 0.4μM of reverse primer probe 3407 (5'GAAGATGGTGATGGGATTTC 3' sequence

localised at exon 3) was added. To 22.5µl of the prepared mastermix 2.5µl of the cDNA was added. The thermal cycle conditions were the same as for the *hTERT* assay except the samples ran for 40 cycles. As for *hTERT*, the numbers of *GAPDH* copies in the unknown samples were determined by constructing a standard curve using dilutions of a plasmid of known concentration that had been converted into copy number of the *GAPDH* gene. The *GAPDH* plasmid was generated by cloning the PCR product, amplified by the primers 1457(fw) and 3407(rv) into PCR 2.1 TOPO (by Dr Terry Roberts, BICGP).

### 5.2.5 Quantitative Fluorescence In Situ Hybridization (Q-FISH)

Q-FISH is a rapid technique used readily to measure the telomere length of individual chromosomes in a metaphase spread. It can also reveal any telomeric fusions that may have occurred. It has advantages over the traditional Southern blot method that only gives the average value of the terminal restriction fragments (TRF, which includes the telomeric and the sub-telomeric regions).

#### 5.2.5.i Slide preparation

Freshly fed cells growing in logarithmic phase were incubated with 0.1µg/ml of colcemid for 2hr. The cells were trypsinized as normal and the pellet resuspended in approximately 200µl of the culture medium. Ten millilitres of hypotonic solution (75mM KCl) was added to the cells very slowly and the suspension allowed to incubate at room temperature for 30min. The cells were centrifuged at 1500rpm for 5min. The supernatant was aspirated off and the pellet resuspended drop-wise in

15ml of freshly prepared fixative, consisting of Methanol: Acetic acid (3:1). The cells were incubated at room temperature for 30min and then centrifuged at 1500rpm for 5min. The last step of fixation was repeated 3 more times and finally the pellet resuspended in 1ml of fixative. Slides were rinsed in tap water and then dried with clean tissue prior to dropping 15µl of the cell suspension. Slides were aged overnight at 50$^{o}$C on a heating block.

**5.2.5.ii Fixation of metaphase slides**

The slides were washed, whilst shaking in PBS at room temperature for 15min in a Coplin jar followed by fixation in 4% formaldehyde for 2min on the bench. The slides were then washed three times in PBS for 5min each on the shaker prior to incubating them in 1mg/ml of pepsin solution (50ml of water acidified with 0.5ml of 1M HCl, pH 2.0, containing 10% pepsin solution) at 37$^{o}$C for 10min. The slides were subsequently washed twice in PBS for 2min each on the shaker followed by fixation in 4% formaldehyde for 2min on the bench. The slides were washed three times in PBS for 5min each on the shaker before dehydrating them serially in 70%, 90% and 100% ethanol for 5min each on the bench. Slides were left to air dry.

**5.2.5.iii Hybridization with the telomeric and centromeric probes**

Stock (1ml) hybridization mixture for the telomeric probe was made up of 700µl deionized formamide, 5µl blocking reagent (Boehringer Mannheim), 50µl $MgCl_2$ buffer ( 2.5M $MgCl_2$, 9mM $Na_2HPO_3$, pH 7.0), 10µl Tris (1M, pH7.2), 152µl dd$H_2O$ and 83µl of PNA solution (6µl/ml FITC labelled $C_3TA_2$ peptide nucleic acid, PE

Biosystem). The centromeric probe (ready to use) was purchased from DAKO. Twenty microlitres of the telomeric probe and 1µl of the centromeric probe were pipetted on each slide and a 22x50mm coverslip gently lowered on the mixture. The slides were denatured at 80$^{o}$C for 3min on a heating block before placing them in a dark saturated chamber to incubate at room temperature for 2hr.

### 5.2.5.iv Post-hybridization wash

The slides were initially washed twice in 70% formamide (70ml formamide, 10ml 2xSSC and 20ml dd$H_2O$) in the dark for 15min to remove the coverslip and then three times in PBS for 5min each in the dark on the shaker. Slides were dehydrated by placing them sequentially into 70%, 90% and 100% ethanol for 5min each. Twenty microlitres of Vectashield with fluorescence DAPI mounting medium (Vector, Vectashield) was placed onto the slide and covered with a 22x50mm coverslip before sealing the edges with rubber cement.

### 5.2.5.v Image capture

Images of the metaphase spreads were examined on a digital fluorescence microscope (Zeiss Axioskop 2) with filters that detected DAPI (blue), FITC (green) and Cy3 (red). CCD camera (Jai) was used to capture the images and FishimagerI software (in situ imaging system (ISIS) Metasystems) was used to analyse the metaphases. The telomeric intensity is expressed relative to the centromeric signal. Ten metaphases were captured for each sample.

## 5.3 Results

### 5.3.1 Stable transfection of PC-3 cell line with the *hTERT* plasmid construct

When normal human chromosome 3 was transferred into 21NT, a breast cancer cell line, in our laboratory, 90% of the clones underwent replicative senescence after telomerase (*hTERT*) repression induced by the transferred chromosome. Initially the cells within the clones were found to proliferate normally with a slightly increased doubling time compared with that of the parental cell line. Subsequently, reduced growth rate was observed in these cells with morphological changes that are typical of senescence i.e. enlargement, multinucleation, vacuolation and positive senescence-associated (SA)-β-galactosidase staining (Cuthbert et al., 1999). Subsequent mapping experiments following chromosome 3 fragment transfer were conducted in a telomerized 21NT clone, 21NT/hTERT. Similarly, I wanted to ensure that I did not encounter senescence in my sets of PC-3 hybrids after MMCT of normal human chromosomes carrying telomerase repressor sequence. Therefore, as in our 21NT study, the PC-3 cell line was stably transfected with an *hTERT* plasmid construct to express the *hTERT* gene ectopically. We reasoned that if the transferred chromosome represses telomerase, then (as in the 21NT study) the clone may senesce. Providing telomerase ectopically would allow me to study *hTERT* transcriptional repression in the absence of senescence.

Quantitative RT-PCR has become the most widely used technique for detecting and quantifying gene expression (mRNA) levels. It is a highly sensitive method for quantification of low abundance transcripts. For this reason the qRT-PCR technique

was used to determine the level of immature (pre-spliced) *endogenous hTERT* mRNA expression level in the transfected hybrid clones.

Several critical factors that govern gene expression analysis by qRT-PCR were considered. DNA-free undegraded RNA and accurate assessment of the starting RNA is vital for accurate quantification. The reverse transcription step is known to cause most of the variability in RT-PCR, as the yield and quality of cDNA can be highly variable. Therefore, *GAPDH,* a housekeeping gene present in high abundance, was used as a reliable endogenous control of cDNA synthesis. The quantitative assay for *hTERT* mRNA expression in the hybrid clones was conducted using TaqMan RT-PCR. TaqMan technology uses a gene-specific fluorescent probe that generates their fluorescence via hydrolysis of the probe by Taq polymerase's 5'-3' exonuclease activity. The hydrolysis separates fluorescein from a quenching dye and results in an increased fluorescein signal. *GAPDH* mRNA levels were determined by Sybr Green which is a non-sequence-specific fluorescent dsDNA binding dye.

The efficiency of the *GAPDH* standard curve varied between 95-100% and the *hTERT* curve varied between 90-94%. The lower efficiency for the *hTERT* standard curve may possibly be due to primers not being fully optimized. It is generally recommended (by companies producing the primers) that the size of the amplicon should be between 100-150bp for qPCR reaction to be as close to 100% efficiency as possible and our *hTERT* primers produced an amplicon length of 260bp. However, it is also recommended that the efficiency of the qPCR reaction is at least 90% and our levels at 90% or higher was acceptable according to the guideline.

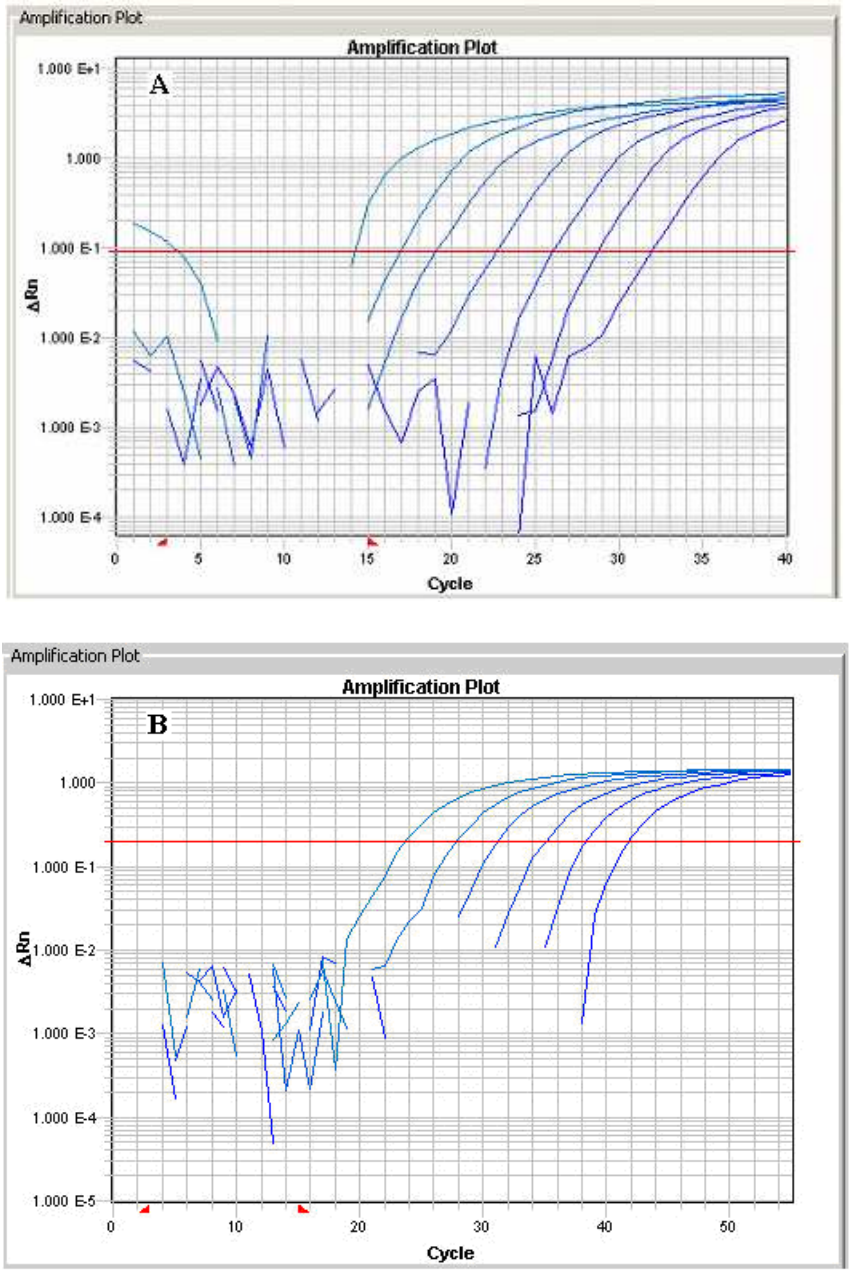
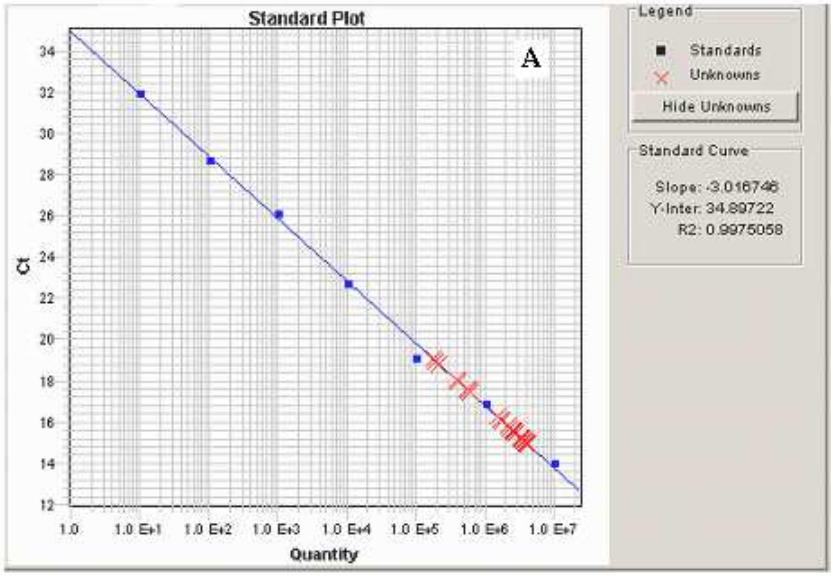



Figure 5.2: Typical images of real-time qRT-PCR amplification plots of (A) *GAPDH* mRNA; (B) *hTERT* mRNA. The dilutions of the *GAPDH* plasmid DNA used were $10^1$-$10^7$ and the *hTERT* plasmid dilutions used were $10^1$-$10^6$. The red line denotes the threshold level, a point at which the fluorescence signal from the sample exceeds the background fluorescence i.e. a point at which all samples have the same concentration of DNA.



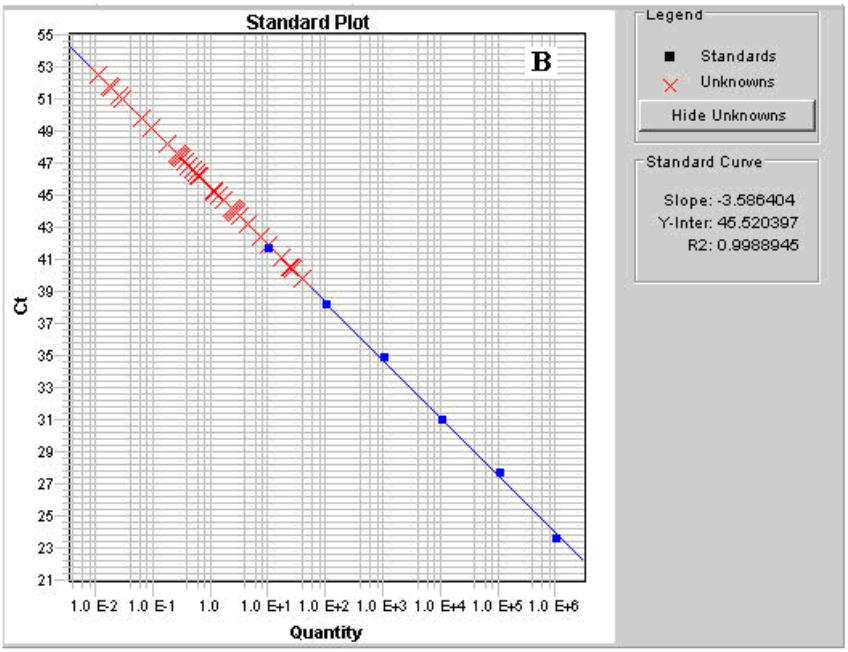




Figure 5.3: Standard curves for (A) *GAPDH*; (B) *hTERT* constructed from the amplification plots in Fig 5.2. The slope of the line can be used to determine the exponential amplification which is $10^{(-1/slope)}$ and efficiency of the PCR reaction is $(10^{(-1/slope)})$-1. The red diagonal lines are the unknown samples.

The 19 hybrids that were generated, following the transfection of the PC-3 cells with the *hTERT* plasmid construct, were subjected to *hTERT* mRNA expression analysis by qRT-PCR (Fig 5.4). Normalization of the samples was achieved by measuring the number of *hTERT* transcripts and expressing it relative to the number of *GAPDH* transcripts (Fig 5.4).

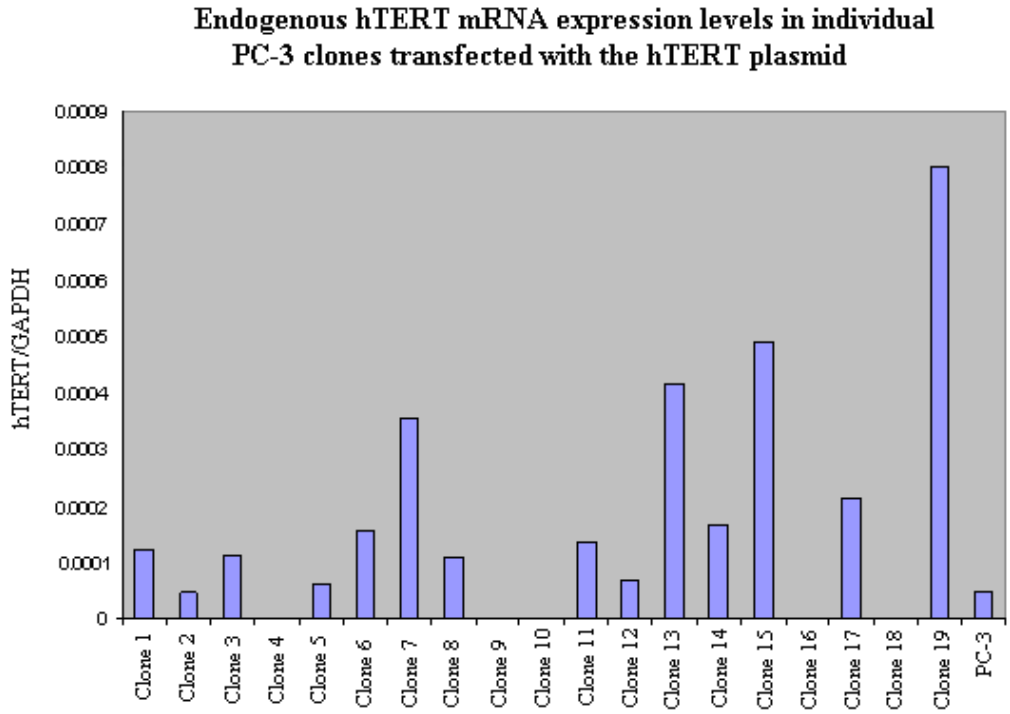



Figure 5.4: The PC-3 cell line was transfected with the *hTERT* plasmid construct. G418 resistant clones were selected and cultured as separate cell lines. The immature endogenous *hTERT* mRNA expression levels were measured by real time qRT-PCR in the PC-3 clones in triplicates.

I observed considerable variation in the normalized endogenous *hTERT* levels in the telomerized clones of PC-3 cells. This level of variation had been previously observed in 21NT, a breast cancer cell line that was also transfected with the *hTERT* plasmid construct in our laboratory (data not shown). Ducrest et al (2001) reported 0.2 to 6 molecules of spliced *hTERT* RNA per cell (by using 2 sets of primer pairs

that amplified cDNA only from spliced *hTERT* RNA) in telomerase-positive cell lines whereas the number of *hTERT* RNA molecules was below the detection limit in telomerase-negative cells. In my study, expression of endogenous *hTERT* was completely undetectable in clones 4, 9, 10, 16 and 18 (Fig 5.4), whereas substantially higher levels of *hTERT* was observed in clones 7, 13, 15, 17 and 19 compared with the PC-3 cells. All the other clones expressed *hTERT* to at least the level seen in the parent PC-3 cells.

Next, I selected the five G418 resistant clones (namely clones 7, 13, 15, 17 and 19) that expressed high levels of *endogenous hTERT* and subjected them to quantitative TRAP analysis in order to evaluate in each the expression of the transfected *hTERT* gene at the level of telomerase enzyme activity (Fig 5.5). Telomerase activity was 7 fold higher in clone 15 compared with the parent PC-3 cells. Clones 7 and 17 expressed a comparable level of telomerase activity to PC-3 cells whereas clone 13 expressed reduced levels. Telomerase activity was absent in clone 19 as in GM847, a telomerase negative cell line used as a control. Clone 19 should have expressed some telomerase activity since the endogenous *hTERT* was 8 fold higher than the parental PC-3 cells (Fig 5.4). The variation in telomerase activity observed in the other clones may possibly reflect the differences in plasmid integration mechanisms. In stable transfections the plasmid becomes linear prior to inserting itself into the host genome. The number of copies of the transfected gene being inserted and the position where the plasmid opens up is random; hence these factors can govern gene expression leading to large variations in telomerase activity levels in different clones.

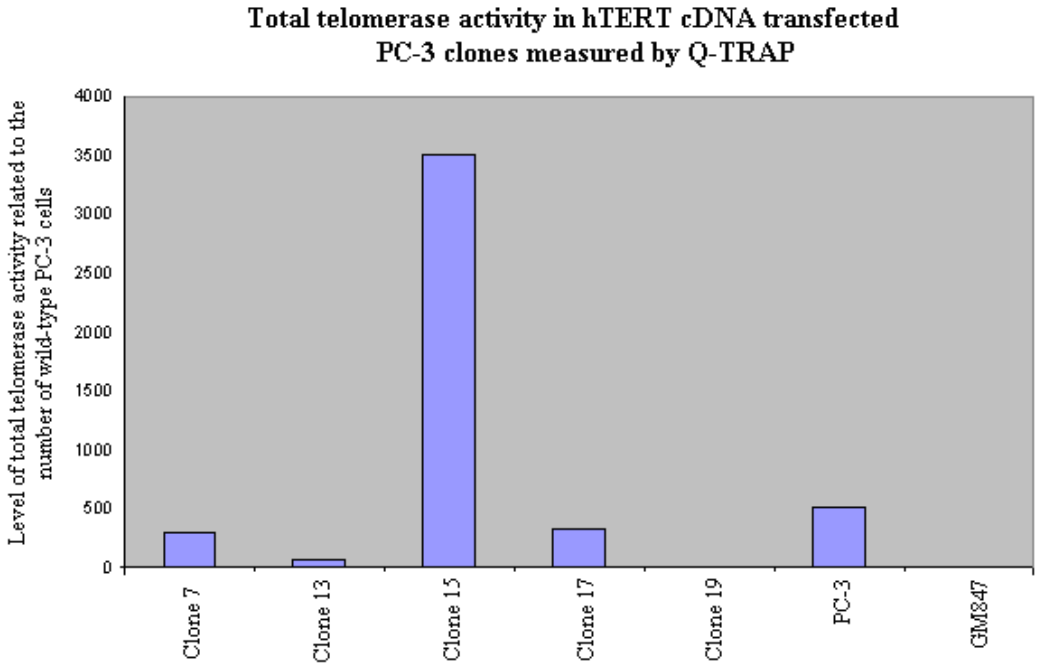



Figure 5.5: Expression of the transfected *hTERT* was assessed by measuring telomerase levels in the G418 resistant clones by quantitative TRAP.

I selected clone 15 into which I could individually transfer normal human chromosomes and measure the effect of the transferred chromosomes on *endogenous hTERT* transcription, in an immortal background. The resulting hybrids would be prevented from undergoing replicative senescence since clone 15 ectopically expressed the highest level of telomerase activity after transfection with the *hTERT* plasmid construct. It also showed the second highest endogenous *hTERT* transcriptional levels. This particular clone was named PC-3/hTERT and it was grown in mass culture and a large stock of ampoules cryopreserved at a low passage number.

### 5.3.2 Quantitative Fluorescence In Situ Hybridization (Q-FISH)

In order to investigate if there was a significant difference in the telomere lengths between the two types of cells, Q-FISH was performed on the wild type PC-3 cell line and the selected PC-3/hTERT clone (PC-3/hTERT 15). The average length of the telomeres in metaphase spreads of both samples were captured and measured by ISIS (in situ imaging system) software.

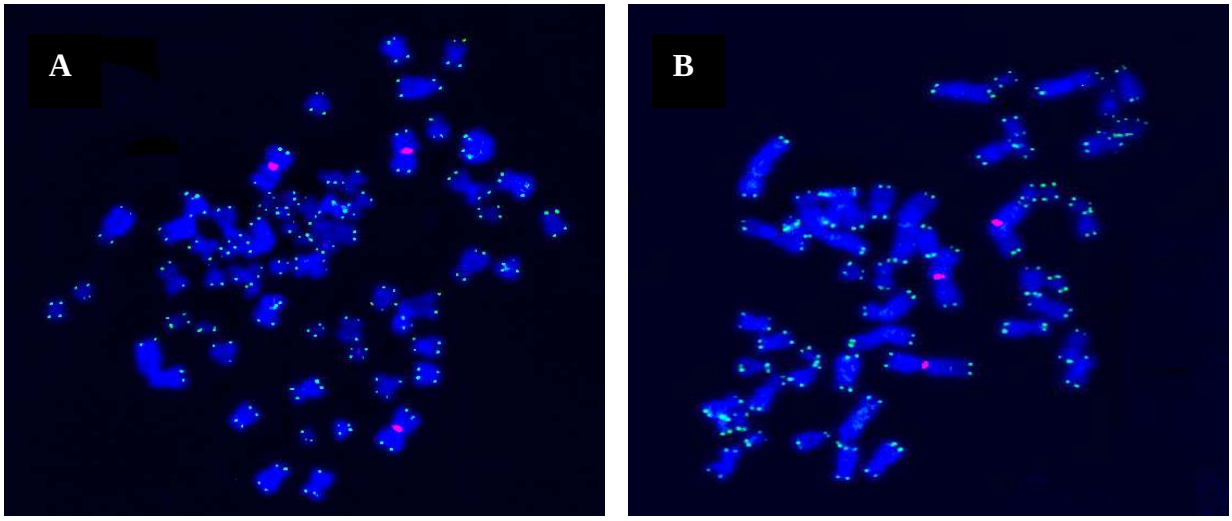



Figure 5.6: Metaphase spreads prepared from PC-3 parental cell line (A) and the telomerized PC-3/hTERT cell line (B). Telomeres were labelled with FITC probe (green) and centromere of chromosome 2 was labelled with Cy3 probe (red).

Ten metaphase spreads were captured and the average telomere length from each spread was used to determine the mean difference between PC-3 and PC-3/hTERT cell lines.

Table 5.1: The length of telomeres was measured in twenty-three pairs of chromosomes in each metaphase prepared from the wild-type PC-3 and the selected telomerized clone 15 (PC-3/hTERT)

| **Number of metaphase** | **Average telomere length in one metaphase of PC-3 (Wild-type) cells (kb)** | **Average telomere length in one metaphase of PC-3/hTERT 15 cells (kb)** |
|---|---|---|
| 1 | 4.503 | 7.68 |
| 2 | 3.244 | 6.577 |
| 3 | 2.915 | 5.414 |
| 4 | 5.023 | 6.589 |
| 5 | 5.062 | 8.190 |
| 6 | 6.873 | 6.32 |
| 7 | 4.389 | 5.99 |
| 8 | 8.658 | 5.48 |
| 9 | 8.016 | 9.46 |
| 10 | 7.240 | 8.39 |
| Average telomere length calculated from 10 metaphases | **5.592** ±1.99 | **7.009** ± 1.35 |

The average length of the ten metaphase spreads measured were 5.6 kb for PC-3 and 7.01kb for PC-3/hTERT (Table 5.1). The unpaired t test showed the difference between the two cell lines is significant ($p<0.05$), and reflects the increased telomerase activity of the telomerized cells.

## 5.4 Discussion

In this part of the study, I wanted to construct a cell line that expressed high levels of *endogenous hTERT* mRNA and high total telomerase activity. So that transfer of normal human chromosome into such a cell line would allow me to measure, if any, reduction in the *endogenous hTERT* levels in the hybrid clones that would be generated, in the absence of senescence.

Maintenance of telomeres in most immortal cells derived from human cancers is achieved by endogenous expression of telomerase. In this study, the immortal PC-3 cell line that, like most cancer cells, already expressed endogenous telomerase to maintain telomere length was forced to overexpress exogenous telomerase by transfecting the PC-3 cells with an *hTERT* cDNA construct and the changes in the telomere length was analysed by Q-FISH. The level of total telomerase measured by quantitative TRAP was 7 fold higher in the selected telomerized clone (PC-/hTERT) compared with the telomerase level observed in the parental PC-3 cell line (Fig 5.5). Similarly, the level of *endogenous hTERT* mRNA expression was approximately 10 fold higher in the selected PC-3/hTERT clone compared with the parent PC-3 cells (Fig 5.4). The Q-FISH results showed that providing the catalytic subunit of telomerase exogenously produced significant lengthening of telomeres in the telomerized PC-3 cell line. I believe that this should permit the measurement of repressed *endogenous hTERT* mRNA levels in hybrids that would be generated by the transfer of normal human chromosomes, carrying a putative telomerase repressor sequence, into PC-3/hTERT in the absence of replicative senescence.

# Chapter 6

# Repression of endogenous *hTERT* transcription by normal human chromosome 11

## 6.1 Introduction

Several studies have reported significant correlation between telomerase activity and immature *hTERT* mRNA expression levels in various cancerous cells, whereas other subunits of the telomerase were ubiquitously expressed in both normal and cancerous cells (Meyerson et al., 1997; Kirkpatrick et al., 2003; Snijders et al., 1998). Regulation of the *hTERT* gene at transcriptional, post-transcriptional, and translational levels is well documented but transcriptional deregulation of the *hTERT* gene is arguably the major mechanism activating telomerase in human cancers (Meyerson et al., 1997; Kilan et al., 1997). To date, the mechanism by which *hTERT* transcription is regulated in normal cells is largely unknown even though several general activators and repressors of *hTERT* transcription that act directly or indirectly on the 5' promoter region have been identified by various groups (see section 1.2.9).

Ducrest et al. (2001), in collaboration with our group, found a substantial number of *hTERT* RNA transcripts exclusively in the nucleus of telomerase positive cells that

had mainly retained intron 2. The level of this immature endogenous nuclear *hTERT* mRNA correlated with telomerase activity, indicating *hTERT* is regulated at the transcriptional level in the nucleus. The authors also demonstrated that our transfer of normal human chromosome 3 into 21NT (Cuthbert et al., 1999) completely abolished *endogenous hTERT* (immature nuclear *hTERT* RNA) even when the 21NT cells transfected with an *hTERT* cDNA plasmid construct were ectopically expressing the gene. In this study, I also wanted to identify a normal human chromosome that could down-regulate *endogenous hTERT* in telomerized PC-3 cells, as chromosome 3 could in 21NT cells by using the same methodology.

## 6.2 Materials and Methods

### 6.2.1 Transfer of normal human chromosomes into PC-3/hTERT cell line

The MMCT method was performed as described in section 3.2.1. The only difference was the recipient PC-3/hTERT cell line.

### 6.2.2 Determination of endogenous *hTERT* and *GAPDH* levels by qRT-PCR

The synthesis of cDNA and the quantification of *hTERT* and *GAPDH* mRNA expression levels were measured by the methods described in sections 5.2.4.i and 5.2.4.iii respectively.

## 6.3 Results

The powerful method of quantitative RT-PCR allowed me to determine which normal human chromosome (if any) may carry a DNA sequence that regulates *hTERT* mRNA transcription. The qRT-PCR method permits reliable quantification of small differences in the transcript levels. Therefore, any change in *endogenous hTERT* in PC-3/hTERT hybrids containing the individually transferred normal human chromosome should be detectable.

Chromosomes 3, 8, 10, 11, 13 and 17 were selected to be individually transferred into the telomerized PC-3 cell line so that the *endogenous hTERT* mRNA expression levels could be determined by qRT-PCR in the hybrids that would be generated. Chromosomes 8 and 13 were chosen instead of chromosomes 4 and 21, because in PC-3 cells I had shown earlier that chromosome 4 and chromosome 21 did not significantly repress telomerase activity when measured by the semi-quantitative TRAP assay (Figs 4.1 and 4.6). The reason for selecting chromosomes 8 and 13 was based on the fact that CGH studies have shown these chromosomes to be frequently altered in prostate cancer (Dong, 2001; Nupponen and Visakorpi, 2000).

Clonal variation in the level of *endogenous hTERT* mRNA expression in the PC-3/hTERT cells had to be determined first prior to measuring its level in hybrids that would be generated by the individual transfer of selected normal human chromosomes into the telomerized PC3/hTERT cell line by MMCT. I assessed the clonal variation by seeding the PC-3/hTERT cells at $10^4$ per dish; the resulting clones were picked and assayed for *endogenous hTERT* mRNA level (Fig 6.1).

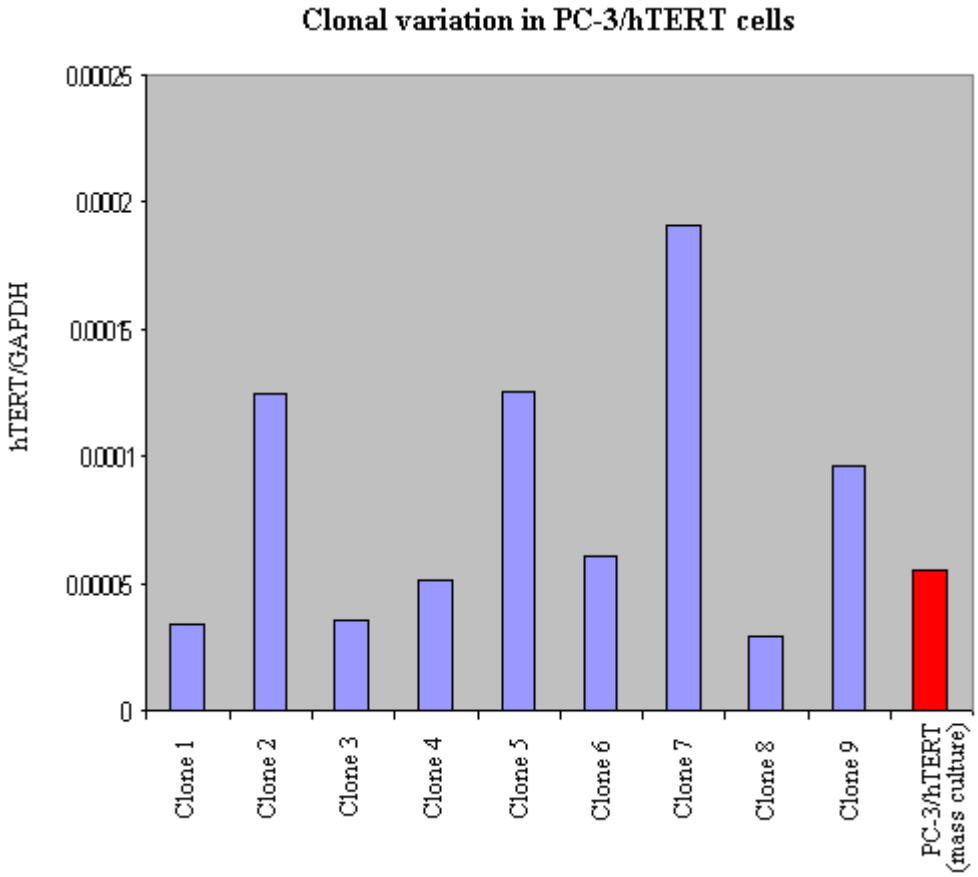



Figure 6.1: Histogram showing clonal variation in the *endogenous hTERT* mRNA levels observed in PC-3/hTERT cells. The last bar represents *hTERT* level in PC-3/hTERT mass cultured cells.

The degree of clonal variation was evaluated from the control PC-3/hTERT clones. The average normalized *hTERT* and sd values of the 9 PC-3/hTERT clones were $8.33 \times 10^{-5} \pm 5.5 \times 10^{-5}$ and the values ranged between $2.96 \times 10^{-5}$ and $1.91 \times 10^{-4}$) (Fig 6.1). Comparison of *endogenous hTERT* mRNA expression levels between the control PC-3/hTERT clones and hybrid clones generated by the transfer of selected normal human chromosomes revealed chromosome 11 to be most effective in repressing *endogenous hTERT* transcription (Fig.6.2). This finding is consistent with my earlier results when chromosome 11 was identified as the most effective in repressing telomerase activity, measured by TRAP (Fig 4.4).

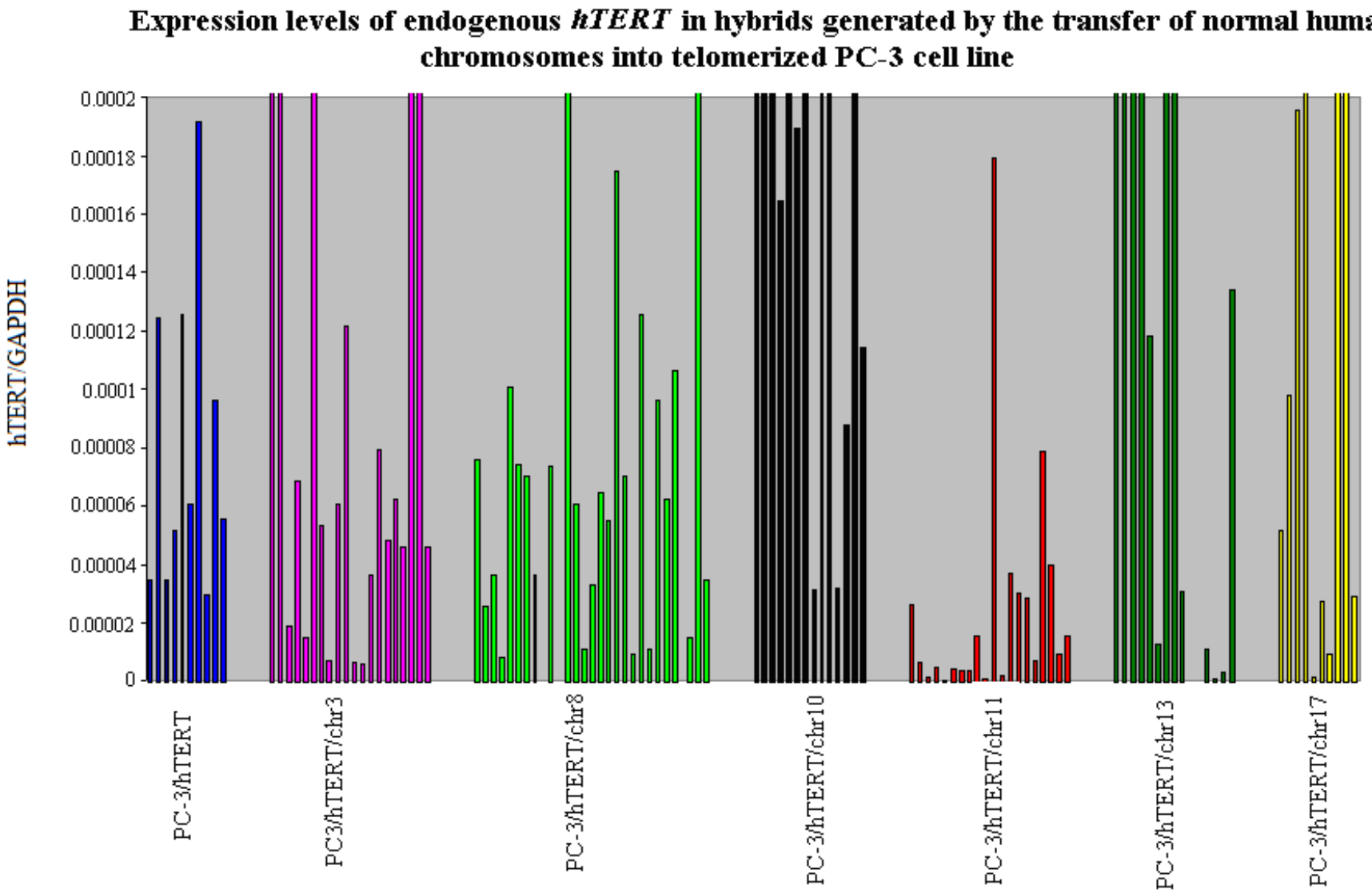



Figure 6.2: Comparison of endogenous hTERT mRNA levels in hybrid clones generated by transferring normal human chromosomes 3, 8, 10, 11, 13 and 17 into PC-3/hTERT cell line.

**Table** 6.1: A summary table showing; (i) the number of hybrid clones that expressed less than 50% or 10% endogenous *hTERT* transcript compared with the average *hTERT* expressed by the control PC-3/hTERT clones, (ii) the normalized mean and sd values of *hTERT* levels expressed by PC-3/hTERT chromosome 3, 8, 10, 11, 13 and 17 hybrids and (iii) comparison of *hTERT* levels expressed by the hybrids with control PC-3/hTERT clones by unpaired "t" test.

| **Hybrids** | **Number of hybrids that expressed less than 50% of the average *hTERT* transcript expressed by PC-3/hTERT clones** | **Number of hybrids that expressed less than 10% of the average *hTERT* transcript expressed by PC-3/hTERT clones** | **Normalized *hTERT* level (mean and sd of clones)** | **No of clones assayed** | **Comparison of *hTERT* levels between the hybrids and PC-3/hTERT clones by unpaired "t" test. Column showing the p values** |
|---|---|---|---|---|---|
| PC-3/hTERT | 3/9 (33.3%) | 0/9 (0%) | $8.33x10^{-5}\pm 5.55x10^{-5}$ | 9 | - |
| PC-3/hTERT/chr3 | 6/20 (30%) | 3/20 (15%) | $2.13x10^{-4}\pm 4.06x10^{-4}$ | 20 | 0.176 |
| PC-3/hTERT/chr8 | 14/30 (46.7%) | 4/30 (13.3%) | $1.29x10^{-4}\pm 3.15x10^{-4}$ | 30 | 0.336 |
| PC-3/hTERT/chr10 | 2/14 (14.3%) | 0/14 (0%) | $5.46x10^{-4}\pm 5.91x10^{-4}$ | 14 | 0.015 |
| PC-3/hTERT/chr11 | 18/20 (90%) | 10/20 (50%) | $2.31x10^{-5}\pm 4.4910^{-5}$ | 20 | 0.0039 |
| PC-3/hTERT/chr13 | 7/15 (46.7%) | 4/15 (26.7%) | $6.28x10^{-4}\pm 1.17x10^{-3}$ | 15 | 0.090 |
| PC-3/hTERT/chr17 | 4/10 (40%) | 1/10 (10%) | $1.41x10^{-4}\pm 1.55x10^{-4}$ | 10 | 0.150 |

In order to determine the level of endogenous *hTERT* repression by different sets of hybrids, I compared the *hTERT* expression levels of PC-3 chromosome 3, 8, 10, 11, 13 and 17 hybrids with the control PC-3/hTERT clones. I found 90% (18/20) of chromosome 11 hybrids expressed lower *hTERT* than 50% of the average *hTERT* level expressed by PC-3/hTERT clones, i.e. the average *hTERT* level of 9 PC-3/hTERT clones was $8.33 \times 10^{-5}$ and 18/20 chromosome 11 clones expressed less than $4.16 \times x10^{-5}$ *hTERT* transcripts (Table 6.1, column 2). Furthermore, 50% (10/20) of chromosome 11 hybrids expressed lower *hTERT* than 10% of the average PC-3/hTERT clones. None of the other hybrids showed such strong repression of *hTERT* transcription as chromosome 11 hybrids. When the *hTERT* expression levels in PC-3/hTERT chromosome 3, 8, 10, 11, 13 and 17 hybrids were compared statistically

with the control PC-3/hTERT clones by the unpaired t test, only chromosome 11 hybrids showed a significant difference i.e. p=0.0039. Consistent with my earlier studies on telomerase activity, hybrid clones generated by the transfer of normal human chromosome 11 into the telomerized PC-3 cell line showed the most significant extent of apparent *endogenous hTERT* transcriptional repression compared with hybrid clones generated by the transfer of chromosomes 3, 8, 10, 13 and 17 (Fig 6.2 and Table 6.1).

Some clones generated by MMCT of chromosomes 3, 8, 10, 13, and 17 into PC-3/hTERT cells expressed much higher levels of *endogenous hTERT* mRNA compared with any of the parent (PC-3/hTERT) clones. However, I did not observe such elevated levels of *endogenous hTERT* expression in any chromosome 11 PC-3/hTERT hybrid clones (Fig 6.2), except for one clone that expressed higher *hTERT*. Similarly, TRAP analysis also revealed that some of the hybrid clones generated by the individual transfer of chromosome 3 (Fig 4.1), chromosome 10 (Fig 4.3) and chromosome 21 (Fig 4.6) into PC-3 possessed higher telomerase activity than the parent PC-3 cells. Currently, I cannot explain why these hybrid clones expressed such high levels of *endogenous hTERT*. There is a remote possibility that these chromosomes possess gene(s) that upregulate *hTERT* transcription.

Next, transfer of chromosome 11 into PC-3/hTERT cell line was repeated 3 times to confirm this high level of *hTERT* repression. The *hTERT* levels in the hybrid clones were compared with the mean *hTERT* level ($8.33 \times 10^{-5} \pm 5.5 \times 10^{-5}$) expressed by the control PC-3/hTERT clones. It is shown as a red dotted line in figure 6.3.

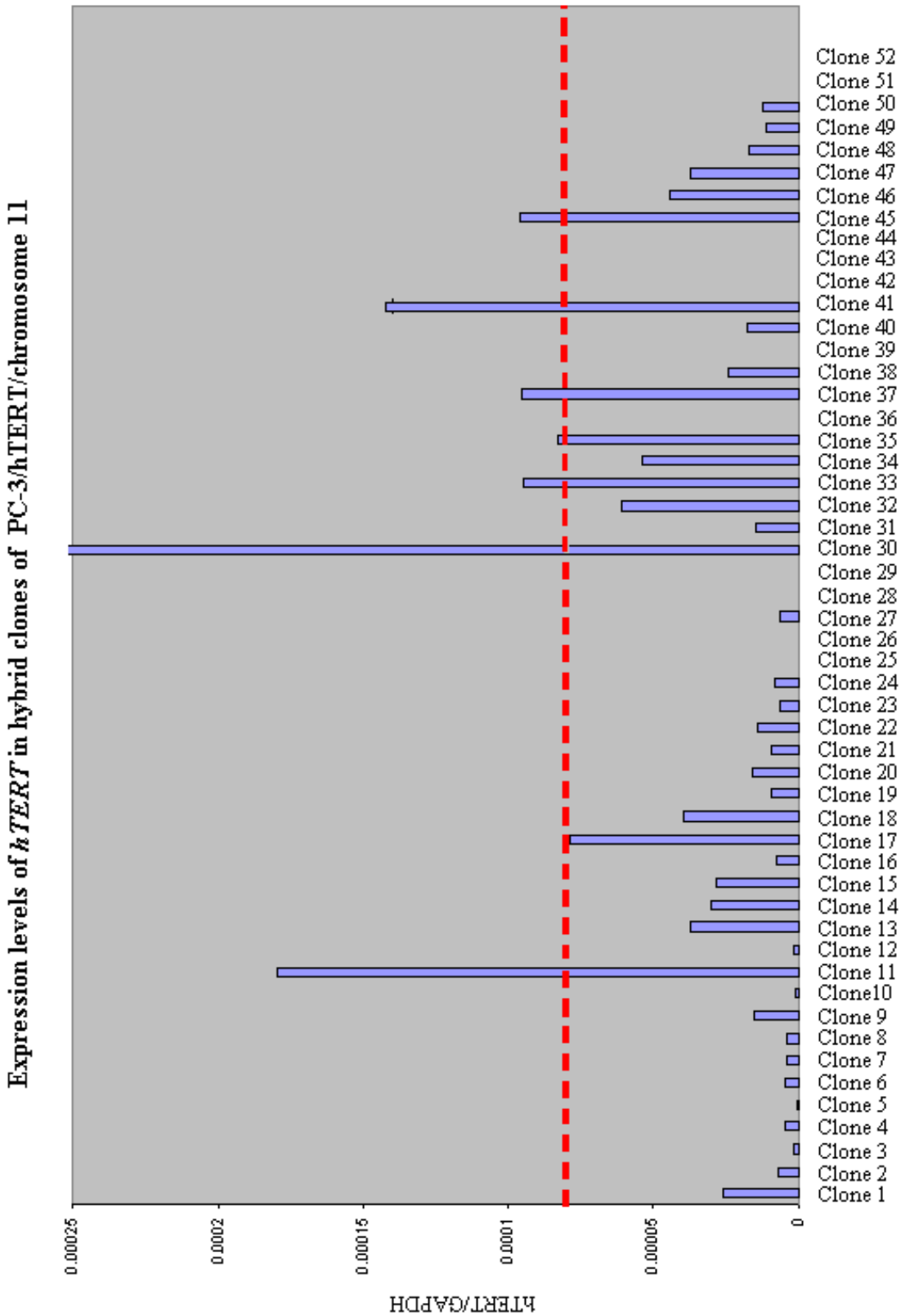



Figure 6.3 : Hybrid clones of chromosome 11 were collected from 3 separate MMCT experiments and assayed for *hTERT* expression levels. The red dotted line incidates the average *hTERT* expression level in PC-3/hTERT clones.

A total of 52 PC-3/hTERT chromosome 11 hybrids were generated from three MMCT; out of which 11/52 (21%) clones had no detectable *hTERT* transcription. When the *hTERT* transcriptional levels of the average PC-3/hTERT clones (denoted as a red dotted line in Fig 6.3) were compared with PC-3/hTERT chromosome 11 hybrids, forty six percent (24/52) of chromosome 11 clones displayed *hTERT* transcription level below 12.5% (hTERT/GAPDH value of 0.00001) of the control mean and in 79% (41/52) clones *hTERT* transcription was less than 50% of the control mean. Therefore the results so far strengthen my earlier hypothesis (Chapter 4) that an *hTERT* repressor sequence is located on chromosome 11.

## 6.4 Discussion

I found hybrids generated only by the transfer of normal human chromosome 11 into the telomerized PC-3/hTERT cell line (that was also ectopically expressing the *hTERT* cDNA) appeared repressed with respect to *endogenous hTERT* mRNA expression levels, compared with monochromosomal hybrids constructed between PC-3/hTERT and chromosomes 3, 8, 10, 13, and 17. Only the difference between the control PC-3/hTERT clones and PC-3/hTERT chromosome 11 hybrids was statistically significant, p=0.0039.

The advantage of measuring *hTERT* transcripts over telomerase activity is that the former is measured quantitatively using real time RT-PCR, whereas the latter is measured semi-quantitatively using the commercially available TRAP kit. However, the drawback with the *hTERT* transcription assay is the low number of *hTERT* transcripts in telomerase-positive cells. Ducrest et al (2001) reported less than 6

spliced *hTERT* RNA molecules per cell in telomerase-positive cells, while as expected these transcripts were below the detection limit in telomerase-negative cells.

To my knowledge there has only been one published study of a direct comparison between telomerase activity and *hTERT* transcription in prostate tumour samples. Kamradt et al (2003) found no correlation existed between the two. Earlier, Liu et al (2001) showed transcriptional upregulation of *hTERT* expression in 94.4% of neoplastic cells obtained from prostate carcinoma tissue by laser capture microdissection which gave a purer population of cells. Kamradt et al (2003) used prostate cancer tissue samples that consisted of a mixture of different cell types. They claimed at least 70% were tumour cells. Therefore, the lack of correlation between telomerase activity and *hTERT* expression could have been due to the heterogeneity of the prostate tissue that they examined.

In my study, I was unable to establish a direct correlation between telomerase activity and *hTERT* mRNA expression levels, because the hybrids in which the endogenous *hTERT* mRNA levels were measured were generated by the transfer of normal human chromosomes into an experimentally manipulated telomerized PC-3 cell line that was ectopically expressing *hTERT* cDNA. However, I found the percentage of telomerase-repressed hybrids (constructed earlier-see Chapter 4, by the transfer of normal human chromosome 11 into PC-3 cells) was comparable with the percentage of hybrids that repressed *endogenous hTERT* mRNA transcripts measured by quantitative real time RT-PCR in hybrids generated by the transfer of

chromosome 11 into a telomerized PC-3 cell line. Eighty percent of PC-3 chromosome 11 hybrids had lower telomerase activity compared with the parent PC-3 cell line (see Table 4.1). Similarly, 85% of chromosome 11 hybrids expressed lower levels of *endogenous hTERT* mRNA transcripts compared with the average *hTERT* expression level in the parent PC-3/hTERT cell line (see Fig 6.3).

The most interesting aspect of my study so far is that telomerase activity and *hTERT* expression both appear to be powerfully repressed by normal human chromosome 11. This strongly suggests that telomerase activity is regulated at the transcriptional level by a gene or genes on normal human chromosome 11 and that this may be damaged in prostate cancer development.

# Chapter 7

# Mapping a candidate *hTERT* transcriptional repressor sequence on human chromosome 11 using irradiation MMCT

## 7.1 Introduction

In Chapter 6, I used the standard MMCT technique to transfer individually whole human chromosomes into the PC-3/hTERT cell line and identified chromosome 11 to be the most effective in down-regulating transcription of *endogenous hTERT*. This standard MMCT technique confirmed my earlier results (Chapter 4) that a gene of interest is present on the transferred normal copy of chromosome 11, but does not identify the region where the sequence is located. In order to identify the region on chromosome 11 carrying the *hTERT* repressor sequence, I employed an irradiation microcell-mediated chromosome transfer (XMMCT) technique. The reason for using XMMCT was to reduce the region of interest on chromosome 11 by randomly fragmenting the whole normal human chromosome prior to fusion with the recipient cells. In this way clones would be generated that contain smaller fragments of the transferred chromosome, aiding the identification of the *hTERT* repressor sequence.

## 7.2 Materials and Methods

### 7.2.1 Irradiation Microcell Mediated Chromosome Transfer (XMMCT)

Microcells were prepared as described in section 3.2.1 from donor cells seeded in 24x25cm$^2$ flasks. In order to generate radiation reduced monochromosome-11 hybrids, these microcells were resuspended in 20ml of complete medium into a 50ml sterile conical flask. A small magnetic stirring-bar was placed into the cell suspension so that the microcells could be agitated whilst being exposed to 25 Grays of γ-radiation. The length of exposure was calculated from the data sheet for the cobalt-60 source. Thereafter, the microcells were centrifuged at 4500rpm for 5min and the cell pellet resuspended in 3ml of serum free Ham's F-12 medium containing 100μg/ml of PHA-P. The microcells were co-incubated with the recipient cells for 25min at 37$^o$C prior to fusion in the usual way.

### 7.2.2 Reverse selection of the HyTk marker with ganciclovir

Hybrids that were to be treated with ganciclovir (GCV) were cultured without hygromycin B for 16-24hr to allow a substantial number of cells to lose the chromosome carrying the HyTK selectable marker. The cells were trypsinized and plated out at 1x10$^6$ cells per P-100 dish and cultured in complete medium containing 2μM GCV. Colonies were picked and cultured in complete medium without GCV.

### 7.2.3 Chromosome painting

Chromosomes can be labelled with fluorophores in a way that permits individual identification. Sequences specific for a chromosome are converted into probes that are labelled with a fluorescent dye. Chromosomes can be detected by hybridizing them under FISH (Fluorescence In Situ Hybridization) conditions with chromosome paint probes, which results in specific labelling of that chromosome. The p- and q-arms of a specific chromosome can be distinguished using arm-specific “paints”. Chromosomal translocations, breakages and other anomalies can be detected with this technique.

#### 7.2.3.1 Slide preparation

In order to arrest the cells in metaphase 0.2µg/ml of colcemid was added to a plate of P-100 cells growing in logarithmic phase and incubated for 1hr in complete culture medium. Cells were trypsinized in 15ml conical tubes as normal and the supernatant aspirated off leaving behind 0.5ml. The pellet was flicked gently and 10ml of 75mM KCl (potassium chloride) pre-warmed to 37°C was added to the cells. The first millilitre of KCl was added very slowly onto the side of the tube whilst the content of the tube was being mixed. The cells were incubated at 37°C for 15min. One millilitre of cold fresh fixative, Methanol: Acetic acid, 3:1 was added drop-wise to the cells prior to spinning them at 1000rpm for 10min. The KCl was aspirated off leaving 0.5ml in the tube. The loose pellet was flicked gently and 10ml of freshly prepared fixative was added drop-wise to the cells. The cells were incubated on ice for a minimum of 10min before spinning at 1000rpm for 10min. The fixative was pipetted

off and the cells washed three more times to remove all the proteins and lipids. Approximately 14µl of the cell suspension was dropped onto a slide that had been washed with deionized water. Slides were used within a couple of days; for longer storage (not more than 2 weeks) they were kept at -20°C.

#### 7.2.3.2 Fluorescence In Situ Hybridization (FISH)

##### 7.2.3.2.i Probe denaturation

Ready prepared chromosome 11 p-arm specific paint probe directly labelled with FITC and q-arm specific paint probe directly labelled with TexasRed were purchased from XCAP-MetaSystems (Germany). Preparing hybridization mixture for a single slide involved pipetting 5µl each of the p-and q-arm specific probe into a sterile microcentrifuge tube. The probe was denatured by incubating at 75$^{o}$C for 5min and then allowed to cool by briefly placing the tube on ice prior to incubating at 37$^{o}$C for 30min. The probe mixture was spun down briefly before hybridization.

##### 7.2.3.2.ii Denaturation of chromosome slides

The slide to be denatured was immersed into denaturation solution (70% formamide in 2xSSC, pH 7.0, pre-warmed to 70$^{o}$C) and incubated for 3min. The slide was transferred into ice cold 70% ethanol for 2min and then immersed into 90% and 100% ethanol for 2min each. The slide was air dried prior to hybridization. It is crucial for the probe mixture and the slide to be denatured ready for hybridization within a couple of minutes of each other. The probe was carefully pipetted onto the slide and overlaid with a glass coverslip (22x22mm$^{2}$). Rubber cement was used to

seal the edges of the coverslip before incubating the slide overnight in a humidified chamber at 37$^{o}$C.

#### 7.2.3.2.iii Post-hybridization treatment

The rubber cement was carefully removed and the slide placed in 1xSSC (pH 7.0-7.5) pre-warmed to 75$^{o}$C for 2min. The slide was washed twice in 2xSSC containing 0.01% Tween20 (pH 7.0-7.5) for 1min at room temperature and then transferred into PBS for 2min at room temperature. Excess fluid was drained off and the slide allowed to air dry. Twenty microlitres of Fluorescence DAPI (Vector, Vectashield) was pipetted onto the slide and overlaid with a coverslip (22x22mm$^{2}$). The coverslip was sealed with rubber cement, and the slide was examined under a fluorescence microscope (Axioplan 2 Imaging).

### 7.2.4 Single copy FISH

Metaphase slides were prepared as for chromosome paints (see section 7.2.3.1).

#### 7.2.4.1 Cutting the HyTK plasmid vector with restriction enzyme-Hae III

In a 100µl reaction volume, 5µg of HyTK plasmid DNA was digested by adding 5U of Hae III restriction enzyme per microgram of DNA and 10µl of X10 buffer. The reaction mixture was incubated at 37$^{o}$C for 2hr. The digested DNA was precipitated by adding 3M sodium acetate followed by ice cold 100% ethanol. The DNA pellet was resuspended in 10µl of DEPC water and the absorbance read at 260/280ηm to calculate the total amount of DNA recovered.

### 7.2.4.2 Labelling the digested DNA with Cy3 dUTP

The digested and undigested plasmid DNA (250ηg DNA in 29µl volume) was mixed with 10µl of random 9-mer primers (Prime-It$^R$ Fluor Fluorescence labelling kit-Stratagene) and the mixture was incubated at 98$^o$C for 5min in a heating block. The tubes were spun briefly before cooling the samples down on ice. 10µl reaction buffer (prepared by adding 1µl of Cy3 dUTP +11.5µl of 5X nucleotide buffer per sample) and 2µl (10U) of endonuclease free klenow was added to the tubes and the samples were mixed by pipetting. The tubes were incubated at 37$^o$C for 20-30min. The reaction was stopped by adding 2µl of stop mix and the samples were stored in the dark at 4$^o$C. To remove the unincorporated nucleotides by precipitation, Herring sperm DNA (15µg) (used as a carrier DNA to spin down with the probe as the concentration of the probe is too low to sediment by itself) was then added to all the samples. The DNA was precipitated by adding 1/10 volume of 3M sodium acetate followed by overnight incubation in ice cold 100% ethanol at -80$^o$C. The samples were spun at 13,000rpm for 20min at 4$^o$C. The pellet was washed with 70% ethanol, dried and resuspended in 10µl of hybridization buffer. The probes were stored at -20$^o$C in the dark and were used as soon as possible for a strong signal.

### 7.2.4.3 Fluorescence In-Situ Hybridization (FISH)

The DNA probes were denatured at 72$^o$C for 5min followed by incubation at 37$^o$C for 20min in the incubator. The slides were denatured in 70% formamide (4ml 20xSSC, 8ml dd$H_2O$, 28ml formamide) at 72$^o$C for 2min followed by dehydration in 70% ice cold IMS for 2min, then serially dehydrated in 80% , 90% and 100% IMS at room temperature for 2min each. The slides were air dried before 5µl each of the

probe and chromosome 11 p-arm specific paint/18mmx18mm were mixed and applied onto each slide. The slide was incubated overnight at 37$^{o}$C in a dark humidified chamber. The cover slip was taken off and the slide was washed with 50% formamide (4ml 20xSSC, 16ml dd$H_2O$, 20ml formamide) for 8min at 42$^{o}$C followed by a wash in 2xSSC at 37$^{o}$C for 5min and ST buffer (800ml dd$H_2O$, 200ml 20xSSC, 500µl Tween) at room temperature for at least 2min.

## 7.3 Results

### 7.3.1 Transfer of fragmented chromosome 11 by employing irradiation microcell-mediated chromosome transfer (XMMCT) technique

In a similar gene transfer study using our MMCT panel, our collaborators Cosma et al (2003) exposed microcells to 50Grays (5krad) radiation to fragment the chromosomes; however, I was unable to produce any hybrid colonies at this dose. Experience from the breast (chromosome 3) project in our laboratory showed that exposure of microcells to 25Grays successfully generated colonies and this dose completely fragmented chromosome 3. Therefore, I used the same dose of radiation to fragment chromosome 11. I prepared microcells from A9HyTK 11 donor cells seeded in 24 x 25cm$^2$ flasks, compared with 12 x 25cm$^2$ flasks for whole chromosome transfers, to increase the number of colony formation. The colonies were selected in hygromycin B and the resistant clones were picked and their *hTERT* expression levels were measured. The XMMCT technique was expected to generate a mixture of hybrid clones that are *hTERT* repressed or non-repressed, depending on the chromosome 11 fragment that it acquires.

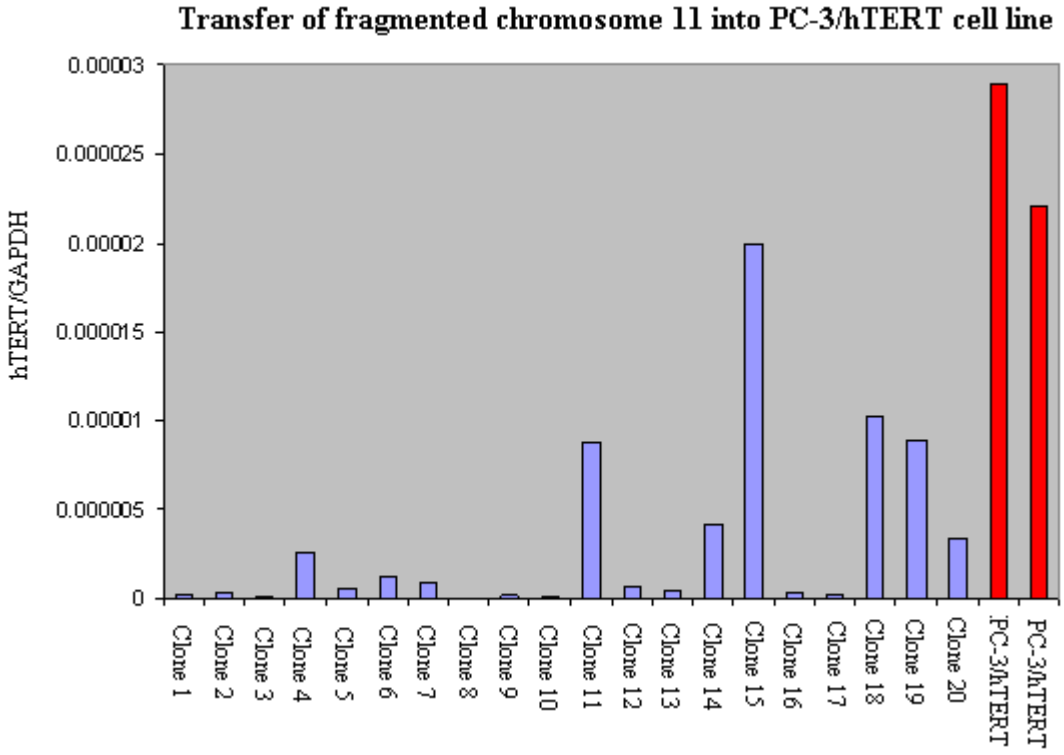



Figure 7.1: XMMCT (irradiation microcell mediated chromosome transfer). Microcells from A9HyTK11 donors were exposed to 25G gamma radiation prior to transfer into PC-3/hTERT cells. Endogenous *hTERT* mRNA expression levels were measured in the resulting clones.

The majority of hybrid clones (14/20) expressed very low levels of *endogenous hTERT* mRNA levels compared with the parent PC-3/hTERT cell line (Fig 7.1). Thirteen out of twenty clones had almost completely silenced *hTERT* expression strongly indicating that the *hTERT* repressor sequence was still present on these fragments. I next embarked on chromosome painting using FISH (Fluorescence In Situ Hybridization) to determine whether the transferred chromosome 11 fragment was a discrete entity or, alternatively, had translocated onto a host chromosome or possibly a donor mouse chromosome during XMMCT. The FISH analysis was also performed to determine the number of chromosome 11 fragments that had been fused into each PC-3/hTERT hybrid clone.

### 7.3.2 Chromosome paints of microcell hybrids illustrating the presence of transferred fragment of chromosome 11.

Metaphase spreads were prepared from the thirteen hybrid clones containing fragmented chromosome 11 that had strongly repressed *endogenous hTERT* mRNA expression levels. The chromosomes in the metaphase spreads were analysed by FISH using chromosome 11 arm-specific paints. Chromosome 11p was painted with FITC (green), 11q was painted with TexasRed (red) and DAPI (4', 6-diamidino-2-phenylindole) painted all the chromosomes blue.

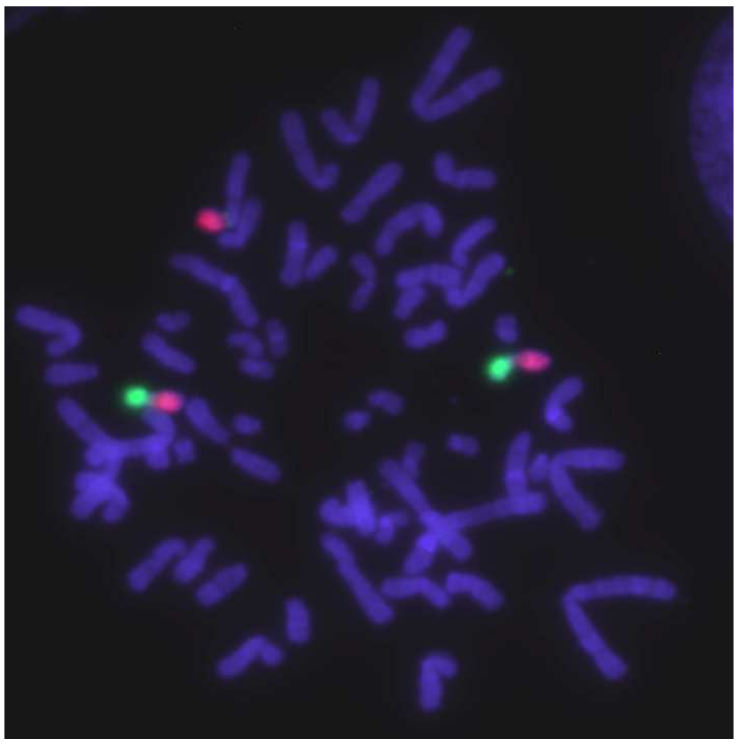


Figure 7.2: PC-3/hTERT, the recipient cell line has 2 whole chromosomes 11, plus an additional copy of an 11q-arm translocated onto another chromosome.

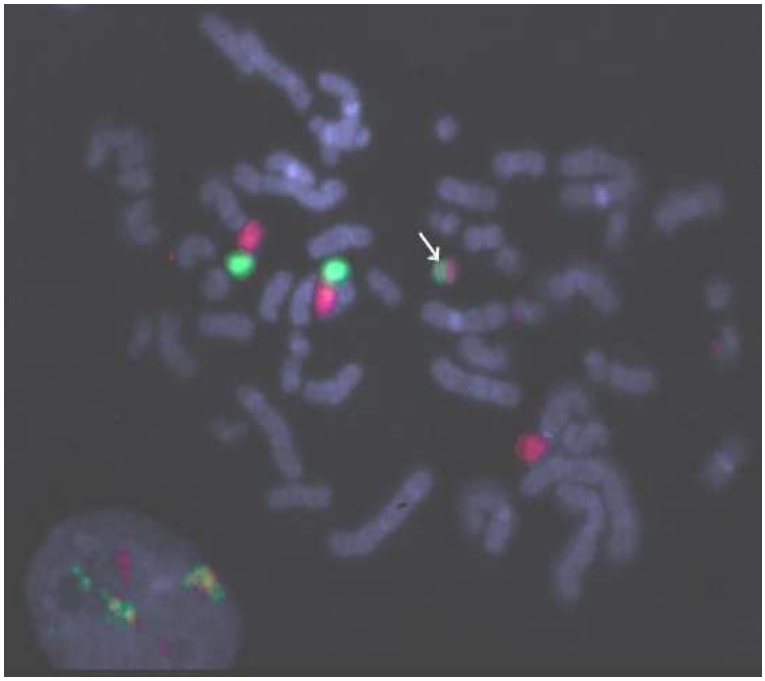


Figure 7.3: A PC-3/hTERT cell line with the transferred fragment of chromosome 11 (PC-/hTERT/fchr11, clone 1). The arrow is pointing at the fragment. Clone 1 was chosen randomly for further studies.

Strongly repressed (*hTERT* mRNA) hybrid clones 1, 2, 3, 5, 7, 8, 9, 10, 12, 13, 16 and 17 were found to possess a single, similar-sized fragment of chromosome 11 (images not shown). All of these clones had a discrete fragment that possessed both the p- and the q-arms of chromosome 11. In fact the fragment was, in effect, a "mini-chromosome" in all the *hTERT*-repressed clones that were examined. The probability that a discrete fragment of this kind was formed in all the 12 independently derived clones is low, as underscored by the fact that when microcells prepared from chromosome 3 were exposed to 25G gamma radiation prior to fusion into a telomerized breast cancer cell line (21NT/hTERT), chromosome 3 was found to be completely "shattered" and translocated onto other human chromosomes. Out of the 54 clones of 21NT/hTERT containing fragmented chromosomes 3 that were collected, none of the clones possessed a discrete independent chromosome 3

fragment. This is in marked contrast to my findings in all the powerfully *hTERT* repressed PC-3/hTERT chromosome 11 hybrid clones.

The similarities found between the 12 clones analysed by FISH may suggest that all these clones were derived from a single MMCT event. However, the possibility of these clones being satellites of a single colony is improbable because a P-100 dish of the recipient PC-3/hTERT cells that were fused with the irradiated microcells was split into 10 x P-100 dishes for selection with hyg B and on average only 2 to 3 colonies per one P-100 dish were picked (see section 3.3.1). I do not have an explanation for the similarities observed between the clones at this stage but from the STS map (see Fig 8.2) it can be seen that regions of high gene densities (http://genecards.weizmann.ac.il/geneloc-bin/gene_densities.pl?chr=11&gc_id=GC11P065251) on chromosome 11 have been retained to form a mini chromosome. Since I did not observe any obvious differences in the fragment between the clones from the FISH analysis (i.e. total size of the fragment, differences in the length of p and q arms) clone 1 was selected randomly to be studied further.

### 7.3.3 Reverse selection of the transferred fragment with ganciclovir

The HyTK selectable marker on normal human chromosomes used in this study allowed for positive or negative selection (see section 3.1). Positive selection of transferred chromosome 11 in the recipient cells was carried out by culturing the cells in hygromycin B and negative selection was achieved by culturing the cells in ganciclovir (see section 3.1). Reverse selection of the HyTK marker, and hence the chromosome, should be accompanied by the loss of the *hTERT* repressed phenotype.

Clone 1, generated by the transfer of fragmented chromosome 11 into PC-3/hTERT cell line, was subjected to reverse (GCV) selection of the transferred fragment as it expressed very low levels of *endogenous hTERT*. Reverse selection of the fragment was achieved by culturing the cells without hygromycin B for 16hr allowing a proportion of the cells to lose the fragment. Thereafter, treatment of these cells with 2µM ganciclovir permitted selection of cells that had lost the chromosome fragment. The clones that appeared were picked and assayed for *hTERT* expression.

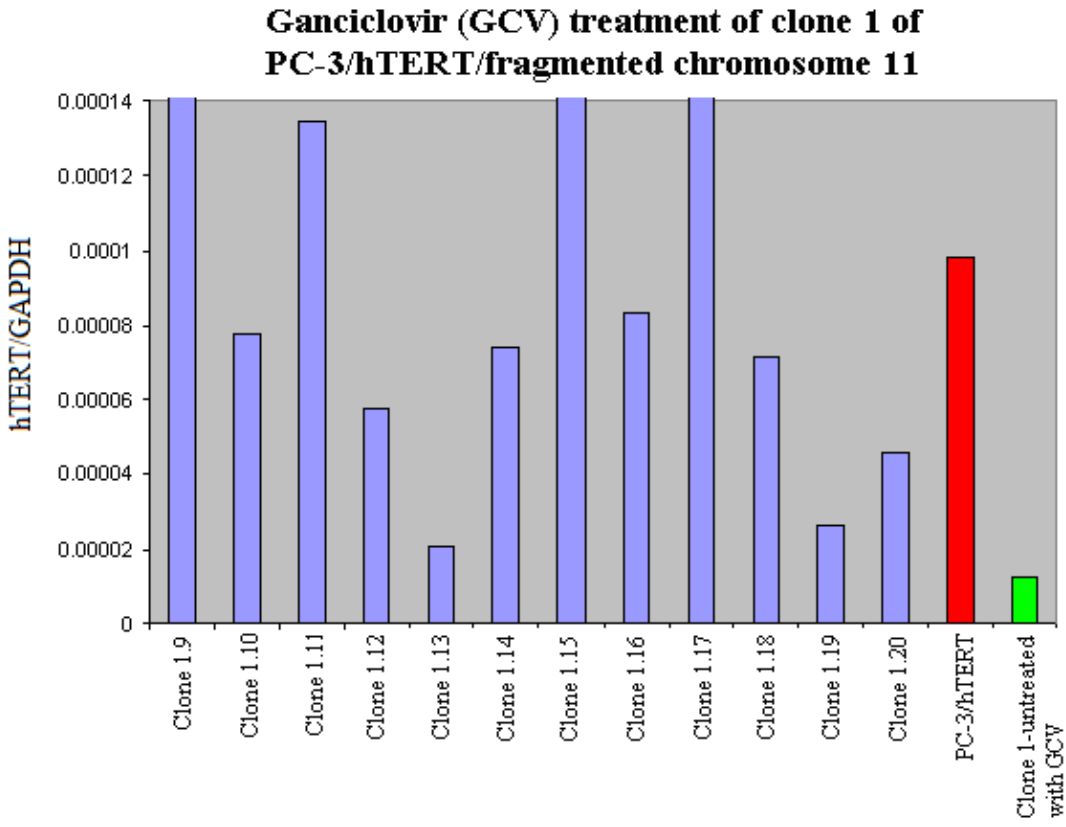



Figure 7.4: Reverse selection of the fragment from clone 1 of PC-3/hTERT/fragmented chr11 was achieved by treating clone 1 with 2µM GCV. Graph showing the generated clones assayed for endogenous *hTERT* mRNA expression levels. C-1 (green bar) untreated with GCV retains the fragment.

The majority (8/12) of the clones assayed had *hTERT* mRNA expression levels approximately equivalent to or higher than those observed in the parent PC-3/hTERT cell line. Two out of 12 clones remained low (Fig 7.4). The results provide independent confirmation that the repression of *hTERT* observed was due to the chromosome 11 fragment and its loss reverted the cells to their original endogenous *hTERT* phenotype. Loss of the fragment accomplished with ganciclovir treatment was confirmed by chromosome painting of metaphase spreads prepared from clone 1 of PC-3/hTERT/fchr11 (Fig 7.5).

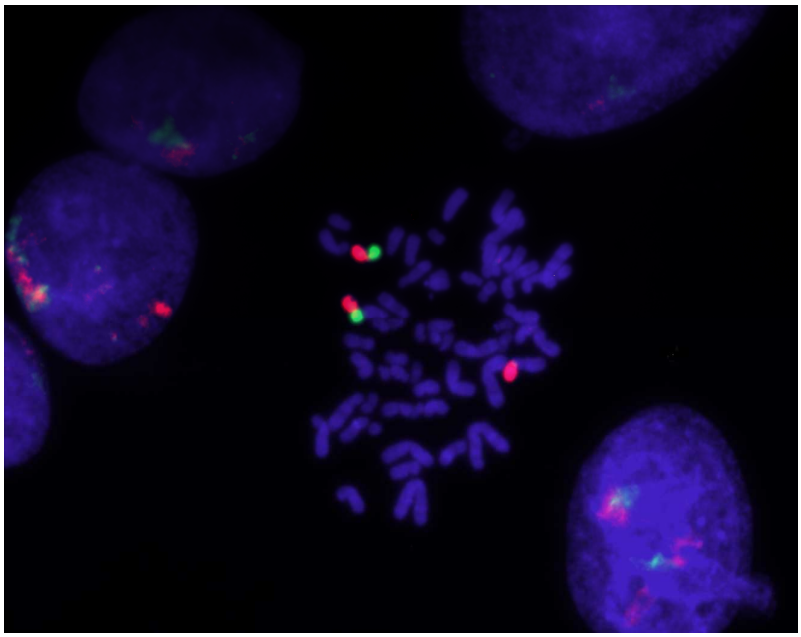


Figure 7.5: Chromosome paint confirmed the loss of the fragment after GCV treatment.

The loss of chromosome 11 fragment (shown by chromosome paints) with GCV treatment resulted in high levels of endogenous *hTERT*, however, a possible shortcoming in this part of my study is that I did not investigate the effect of GCV treatment on *hTERT* transcription in the parent PC-3/hTERT cells.

### 7.3.4 Localization of the HyTK marker on chromosome 11 fragment by single copy FISH

Next, I wanted to show using direct visualization that the HyTK selectable marker was present on the transferred fragment. The HyTK plasmid DNA was digested with Hae III and labelled with Cy3 probe (red) prior to co-hybridization onto a metaphase spread with 11p arm specific (FITC-green) paint.

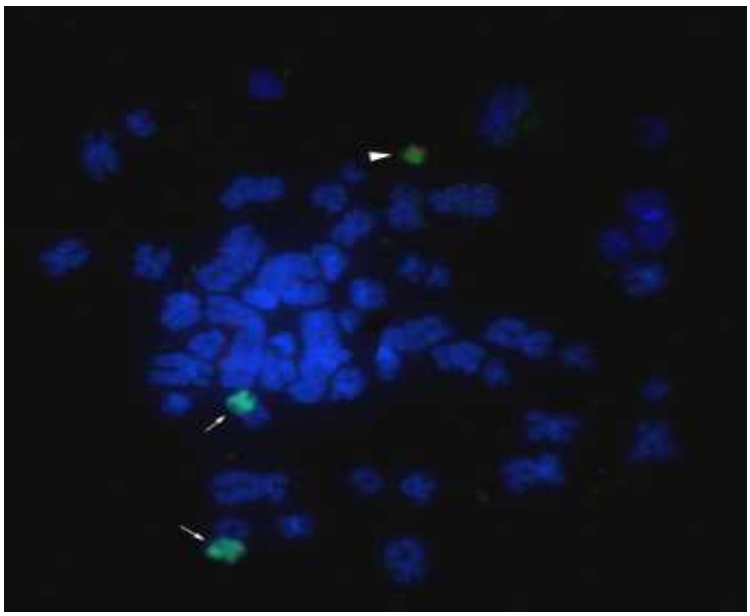


Figure 7.6: The HyTK selectable marker labelled with Cy3 (painted red) can be identified on chromosome 11 fragment, in a metaphase spread prepared from clone 7 of PC-3/hTERT/fchr11 cells. The two chromosome 11p arms (normally present in PC-3/hTERT cells) are painted green (Image captured by Dr. Julio Masabanda, BICGP).

From the above image the HyTK marker labelled with Cy3 (red) was clearly visible on the transferred chromosome 11 fragment. The Cy3 dye did not seem to co-localize with the green FITC paint which is specific for chromosome 11p suggesting that the HyTK selectable marker is probably localized on the q arm of chromosome 11. In our laboratory, the arm-specific paint available for chromosome 11q was

TexasRed and since the HyTK marker was labelled with Cy3, (both painting red), a decision not to paint chromosome 11q was taken in order to see the small HyTK signal on the fragment.

## 7.4 Discussion

Transfer of human sub-chromosomal fragments has been used successfully by the Brunel team, in collaboration, to identify several human disease genes (e.g. Zhu et al., 1998; Cosma et al., 2003; Coenen et al., 2004; Coelho et al., 2008). I used the XMMCT technique developed by Dowdy et al (1990) to investigate further and identify the region(s) on chromosome 11 that may be involved in *hTERT* regulation. One of the principal strengths and advantages of the MMCT technique is the capacity to generate segregant hybrids that have lost the DNA sequence of interest. Unfortunately, I was unable to isolate any spontaneous segregants in my system (all the hybrids collected were repressed with respect to endogenous *hTERT* transcription), possibly due to the HyTK selectable marker's (close) proximity to the *hTERT* repressor sequence.

It has been reported that exposure of microcells to gamma radiation prior to fusion into a recipient cell line may result in random deletions which can range from simple interstitial deletions to complex rearrangements. The deleted region and the size of the chromosomal fragments depend on the strength of the radiation dose (Hernandez et al., 1999). I chose to use a gamma irradiation dose of 25 Grays, which falls between the exposure strength of 50 Grays used by Cosma et al (2003) and 10 to 20 Grays used by Cody et al (2007). In hindsight, a higher dose of 50 Grays should have

been used to obtain segregants which would have made fine mapping of the repressor region far easier. Due to a lower frequency of colony formation at 50 Grays, a lower dose of 25 Grays was selected for this study.

Professor Newbold's choice of tagging the normal human chromosome with the HyTK selectable marker proved extremely important to demonstrate clearly the presence of an *hTERT* transcriptional repressor sequence on a chromosome 11 fragment. I believe I have convincingly demonstrated that reverse selection of the marker (hence the fragment) with GCV treatment was accompanied by elevated levels of endogenous *hTERT* expression, was comparable to that in or exceeding the parent cell line. This confirms the presence of the *hTERT* repressor sequence on chromosome 11 fragment. Szutorisz et al (2003) reported that only a proportion of GCV segregants which had lost the copy of transferred normal human chromosome 3 into 21NT had re-expressed the *hTERT* gene. These authors suggested that additional changes were required for the re-expression of endogenous *hTERT* apart from the loss of the *hTERT* repressor sequence. However, I have successfully demonstrated the re-expression of *hTERT* transcription in more than 80% of my GCV-treated segregants, and these were confirmed to have lost the transferred fragment by FISH analyses.

# Chapter 8

# Genetic and functional characterization of reduced chromosome 11 fragments to fine-map the *hTERT* repressor

## 8.1 Introduction

The next step in the project required the characterization of the DNA content of the normal human chromosome 11 fragment followed by the identification of regions where the *hTERT* transcriptional repressor may be located. Several studies have used polymorphic microsatellite markers to characterize the content of the transferred chromosome in human monochromosomal hybrids (Cuthbert et al., 1999; Cody et al., 2007). This procedure requires the assessment of the markers for informativeness by defining the allelotypes of both the donor hybrids and the recipient cells. I took a different approach; I characterized the fragment by transferring it from the human background into mouse A9 cells. The advantage of this transfer was that the resulting hybrids were heterospecific allowing much more effortless fine mapping of the fragment with STS markers and, since there are no other alleles, all STS markers could be used. In this study, I used a combination of MMCT, molecular and cytogenetic techniques to enable me to identify regions (by eliminating deleted regions) on the fragment that may carry the *hTERT* transcriptional repressor.

## 8.2 Materials and Methods

### 8.2.1 Transfer of PC-3/hTERT fragmented chromosome 11 into A9

Fragmented clone 1 of chromosome 11 (PC-3/hTERT/fchr11.1) that was selected to be studied further was transferred into A9 (recipient) cells by seeding Clone 1 cells at $1.5x10^6$ in $24x25cm^2$ flasks in Ham's F-12 medium containing 20% FBS and 100U/ml of hygB. The cells were cultured at 37°C and 6.5% $CO_2$ in a fully humidified incubator for 24hr, after which 0.08μg/ml of colcemid was added to the cells. The cells were allowed to incubate for 48hr for micro-nucleation to occur. The fragment was transferred following the standard MMCT protocol and the hybrids were selected with 800U/ml of hyg B. The colonies that appeared were picked and thereafter cultured in half the dose of hyg B.

### 8.2.2 Transfer of A9 containing fragmented chromosome 11 back into PC-3/hTERT to confirm retention of the *hTERT* repressor sequence

A9 cells containing the fragmented chromosome 11 were seeded at $1.25x10^6$ cells into $48x25cm^2$ flasks in DMEM medium containing 20% FBS and 400U/ml of hygB. The cells were cultured at 37°C and 10% $CO_2$ for 24hr prior to adding 0.05μg/ml of colcemid to the cultures for micro-nucleation to occur. The fragment in A9 background was transferred back into PC-3/hTERT cells as above. Hybrids were gradually selected with 100U/ml of hygB for the first 3 days then the dose increased to 200U/ml until colonies appeared. Chromosome 11 fragments were maintained in the PC-3/hTERT hybrid clones with 100U/ml of hygB.

### 8.2.3 Identification of human chromosome by STS (Sequence Tagged Site) markers

Human: rodent hybrids in which the transferred chromosome had to be identified were cultured in P-60 dishes as normal and harvested when 70 to 80% confluent. The culture medium was aspirated off and the cells were detached and spun at 1500rpm for 5min. The cellular pellets were resuspended in 1ml of PBS and transferred into a 1.5ml microcentrifuge tubes. The pellets were washed twice in warm PBS and stored at -20$^{o}$C until required for DNA isolation. DNA was isolated using the manufacture's protocol (Promega Wizard DNA isolation kit). DNA was quantified using an Eppendorf BioPhotometer and diluted to 100ηg per µl in DEPC water. Polymorphic DNA markers were selected from the NCBI database and PCR conditions optimized on DNA prepared from 21NT, a breast cancer cell line. Twenty microlitre reaction mixtures were set up for PCR. They consisted of 1µl of 100ηg/µl of DNA, 1µl of 10pmol of primer pair (Sigma, Dorset) and 18µl of ReddyMix (*AB*Gene). The standard PCR cycle used was 94$^{o}$C for 5min for initial denaturation, followed by 35 amplification cycles of 94$^{o}$C for 45 sec denaturation, 55$^{o}$C for 45sec annealing and 72$^{o}$C for 45sec extension. A final extension step of 72$^{o}$C for 10min was added. PCR amplification products were resolved on 2.0% agarose gel for approximately 1.5h at 50V constant voltage in TBE (Tris borate EDTA) buffer. The gel was visualized on an Alpha Imager (Alpha Innotech Corporation).

### 8.2.4 Extraction of cytoplasmic RNA

Cells growing in log phase were harvested from 6 x P-100 dishes in 6ml of PBS. The cells were aliquoted into sterile 1.5ml microcentrifuge tubes and spun at 13000rpm for 1min. The supernatant was removed and 200µl of lysis buffer (140mM NaCl,

2mM MgCl, 0.5% nonidet P40 (IGEPAL; Sigma), 200mM Tris pH 8.5) was added on ice. The cell pellet was resuspended by pipetting and spun down immediately at 13000rpm for 1min at 4$^{o}$C. The cytoplasmic supernatant was transferred into a fresh microcentrifuge tube and the nuclear pellet discarded. TSE buffer, 300µl (0.5% SDS, 5mM EDTA pH 8.0, 10mM Tris 8.5) was added to the supernatant and mixed thoroughly by shaking. Tris-equilibrated Phenol (500µl) was then added to the supernatant and mixed again before spinning at 13000rpm for 1min at 4$^{o}$C. The phenol extraction on the aqueous phase was repeated two more times. Chloroform, 500µl was added to the aqueous phase in fresh tubes and mixed well by vortexing. The tubes were spun at 13000rpm for 1min at 4$^{o}$C. The aqueous phase from all the microcentrifuge tubes was combined in a sterile 13ml tube and 2.5 volume of ice cold ethanol was added. The RNA was left to precipitate overnight at -20$^{o}$C. The sample was aliquoted into 1.5ml microcentrifuge tubes and spun at 13000rpm for 20min at 4$^{o}$C. The pellets were resuspended on ice in 10µl DEPC water and combined and stored at -20$^{o}$C.

## 8.3 Results

### 8.3.1 Characterization of the transferred fragment of chromosome 11 by STS markers

The next step towards identification of the *hTERT* repressor was to accurately map the gene sequence content of all the fragments in A9 hybrid clones in order to eliminate as much of the chromosomal gene-content as possible. For this purpose, we had adopted the rationale that transfer of this human fragment into a mouse background would enable us to use human STS (sequence tagged sites) markers of

high densities and hence facilitate accurate mapping. Therefore, I transferred PC-3/hTERT/fchr11.1 (Clone 1) back into A9, a mouse fibroblast cell line, to map the fragment. Because the results of chromosome paints suggested the presence of both the p and q arms on the fragment, STS markers were selected (from the NCBI website) starting at the centromere region and radiating distally along both arms of chromosome 11. Twenty-two human: A9 hybrids were generated and STS mapped (initially only the first ten clones were mapped). All the hybrids were positive for the polymorphic marker D11S2365 except for clone 13. D11S2365 is the closest marker to the centromere at map position 53.5Mb. D11S2002 had a map position of 77.3 Mb and its sequence was absent in 5 of the 22 hybrids (Fig 8.1).

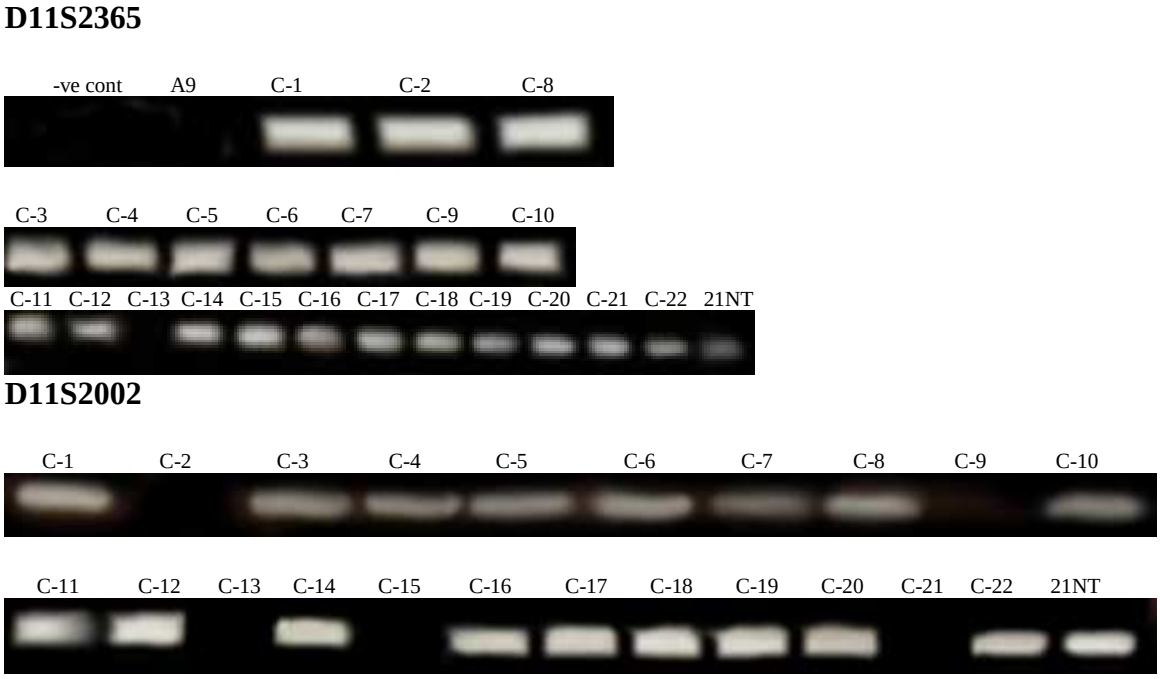



Figure 8.1: Structural analysis of the *hTERT* repressor on chromosome 11 fragments in the human: A9 hybrids. Example of analysis with STS markers D11S2365 and D11S2002 is shown.

The STS map (Fig 8.2) confirmed the presence of the heterochromatin rich centromere in all the hybrid clones except for Clone 13 (A9/fchr11.1.13). The STS

map shows the presence of telomeres on both the p and q-arms in most of the clones. Clone 9 (A9/fchr11.1.9) was the only clone without telomeres and Clone 13 had lost the telomeric region on the p-arm. Clone 5 (A9/fchr11.1.5) had some deleted loci on the p-arm at the telomeres. The map showed the presence of a much larger region of chromosome 11 on the fragment than I had anticipated from the roughly estimated size of the fragment from the FISH images. It suggests that complex rearrangement had occurred in the original Clone 1 (PC-3/hTERT/fchr 11.1) after the 25G gamma radiation exposure. The larger similar-sized deletions on both the p and q arms of the fragment in most of the twenty-two mouse: human hybrid clones were most probably due to the initial radiation, since they were all derived from a single clone (PC-3/hTERT/fchr11.1). Smaller deletions in the fragment were possibly acquired by the MMCT of the fragment from the human PC-3/hTERT cell line into the mouse A9 cells. The combined effects of gamma radiation exposure of the microcells followed by the MMCT of the resulting fragment probably resulted in broad variation in the marker content (and therefore gene presence) on these fragments. This was considered to be advantageous for narrowing down the region carrying our *hTERT* repressor sequence.

The selection of STS markers from the NCBI website was random along the whole length of chromosome 11. However, I used expression primers to look for the presence of loci for specific genes of interest which have included the following *HNRPUL*, *PHF21A*, *CREBL1*, *C11orf80*, *WT*, *NAT10*, *ZFPL1*, *SPDYC*, *KAT5*, *FOSL1*, *BRMS1*, *DRAP1*, *SART*, *KDM2A* and *EST1* (Fig 8.2). We were provided with a list of genes (as suggested by microarray data) which were differentially

expressed in my panel of *hTERT* repressed and non-repressed clones by Professor Colin Cooper (ICR UK) (see section 8.3.3 below). From this list, genes that had the potential to be involved in *hTERT* regulation were selected, i.e. genes involved in cell proliferation, senescence, differentiation, transformation etc. If the locus for the chosen gene was present on the human fragment in the mouse background then the same set of primers was used to determine its expression in my panel of *hTERT* repressed and non-repressed clones (see section 8.3.3 below) by quantitative real time RT-PCR.

It should be emphasized that the construction of Figure 8.2 constituted a major part of the work in the latter part of my PhD project. Figure 8.2 is an extensive high-density STS map of chromosome 11 fragments from irradiated hybrid Clone 1 (PC-3/hTERT/fragmented chromosome 11.1) that was transferred into mouse A9 cells.

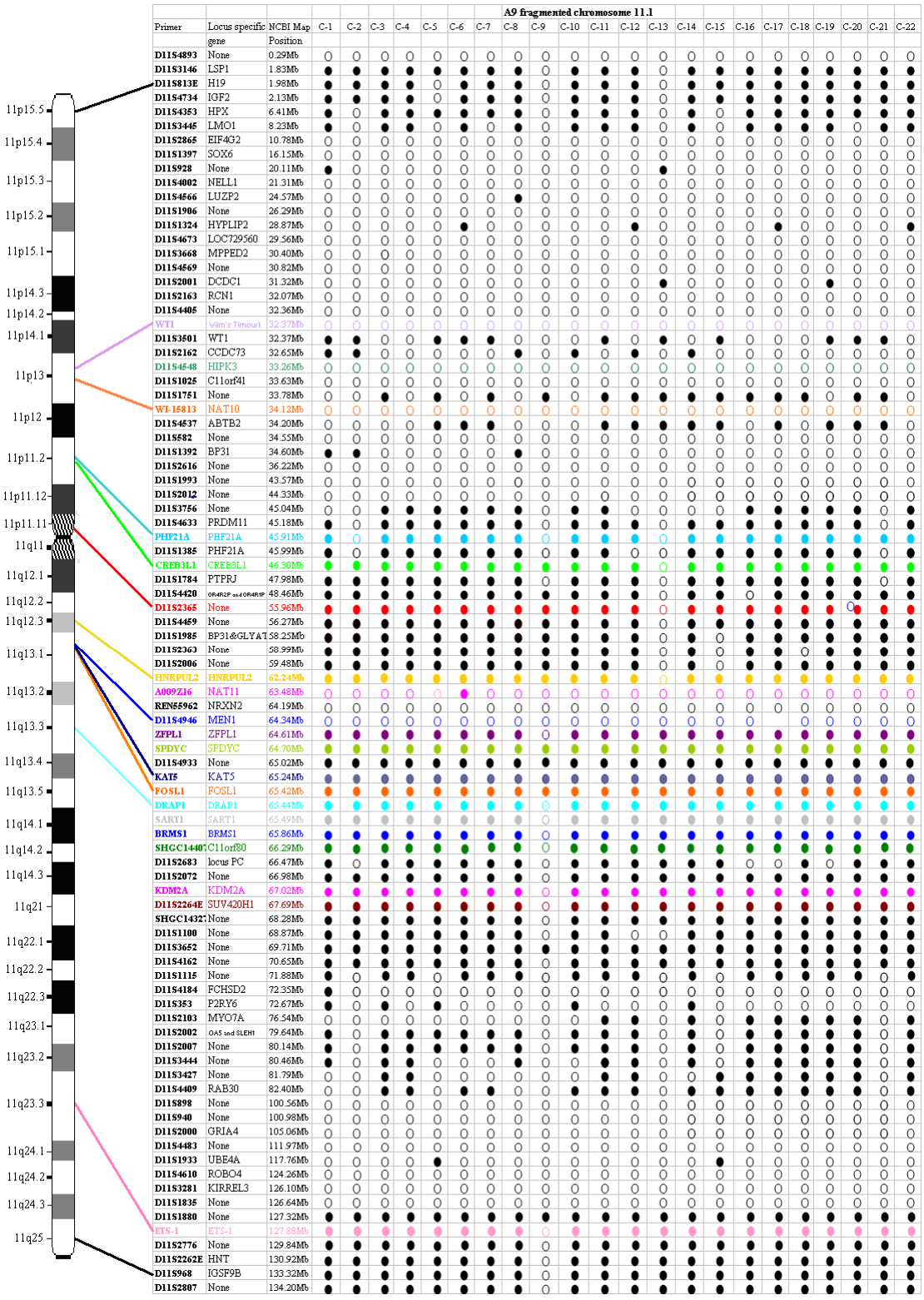



Figure 8.2: STS map of the 22 human: rodent hybrids selected from the transfer of clone 1 of PC-3/hTERT/fchromosome11.1 into the mouse A9 cell line. D11S2365 (red) is the closest marker to the centromere. Filled circles indicate the presence of the locus and open circles indicate its absence. Expression primers were used for the following genes of interest; *WT*, *NAT10*, *PHF21A*, *CREBL1*, *HNRPUL2*, *ZFPL1*, *SPDYC*, *KAT5*, *FOSL1*, *DRAP1*, BRMS1, KDM2A and *ETS-1*.

### 8.3.2 Transfer of chromosome 11 fragment back into the PC-3/hTERT cell line to determine the presence of *hTERT* transcriptional repressor sequence

The aim of individually transferring selected chromosome 11 fragments from the mouse A9 cells back into the human PC-3/hTERT cell line was to confirm that the *hTERT* repressor sequence on the fragment had been retained during the transfer. When I started fine mapping the fragment I did not expect to see such extensive variations between the fragments that was eventually observed in the 22 clones. I had initially only fine-mapped the first 10 clones (see section 8.3.1) and randomly selected A9/fchr11.1.8 and A9/fchr11.1.2 to be transferred first into the PC-3/hTERT cell line.

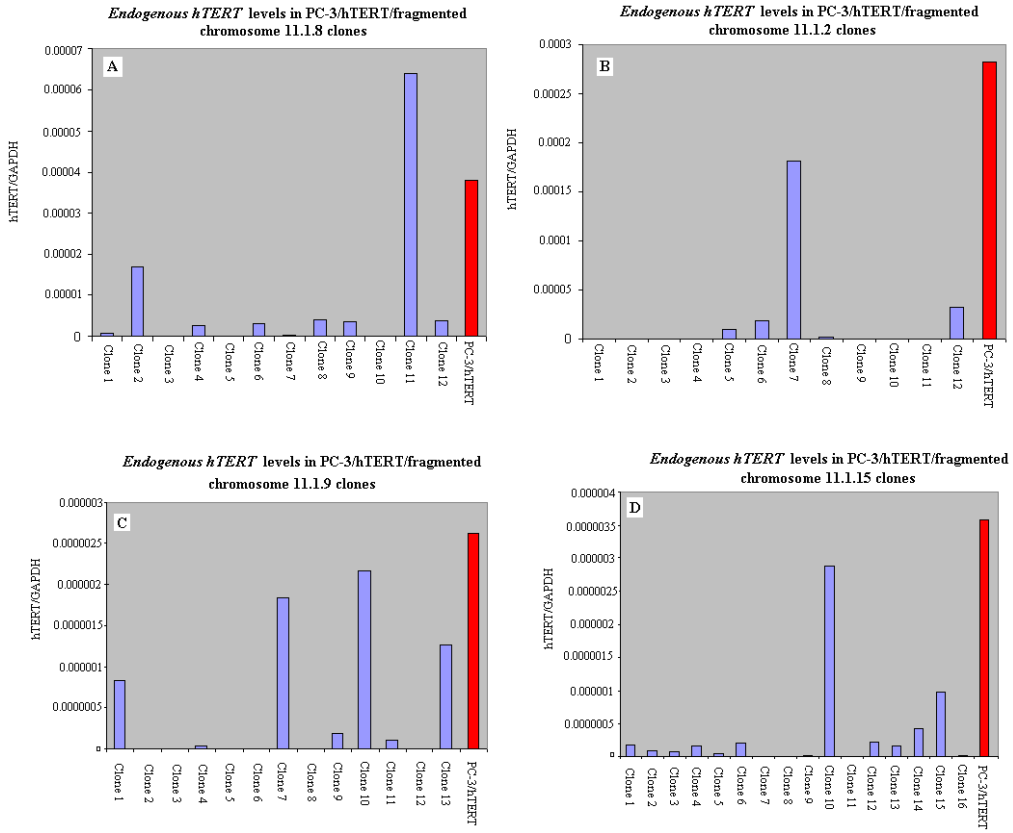



Figure 8.3: Measurement of endogenous *hTERT* mRNA levels in hybrids generated from the individual transfer of (A) A9/chr11.1.8, (B) A9/chr11.1.2, (C) A9/chr11.1.9 and (D) A9/chr11.1.15 fragment into PC-3/hTERT cell line.

Human TERT *(hTERT)* transcription was repressed in 10/12 A9/fchr11.1.8 clones (Fig 8.3; A). Four clones had no detectable *hTERT* expression and only clone 11 expressed higher *hTERT* than the parent PC-3/hTERT cells, suggesting that the *hTERT* repressor had been retained on the fragment carried in the A9/fchr11.1.8 clone. Compared with the A9/fchr11.1.8 results (Fig 8.3; A), I observed an even higher *hTERT* transcriptional repression in 11/12 clones that were generated by the transfer of A9/fchr11.1.2 into PC-3/hTERT cell line (Fig 8.3; B). Eight clones had completely silenced *hTERT* transcription, and only clone 7 had comparable *hTERT* transcription level to that of the parent PC-3/hTERT cells. Results from this transfer indicate that the *hTERT* repressor has been retained in A9/fchr11 1 2 clone.

Development of the STS map revealed clone 9 (A9/fchr11.1.9) to be negative for a number of STS markers (Fig 8.2) and it was found to be considerably smaller than any of the other clones that were mapped thus far. Therefore, I decided to transfer A9/fchr11.1 9 next into PC-3/hTERT cell line. The *hTERT* expression levels were measured in the 13 clones that were generated. Once again, the *hTERT* mRNA expression levels observed were substantially lower in the majority (9/13) of the clones compared with the parent PC-3/hTERT cell line, suggesting that chromosome 11 fragment in A9/fchr11.1.9 hybrid clone had also retained the *hTERT* repressor sequence (Fig 8.3; C). Next, I wanted to confirm the presence of human chromosome 11 fragment in clone 9. Thus, metaphase spreads were prepared from A9/fchr11.1.9 cells and painted with arm-specific chromosome 11 paints to show the presence of the human fragment in the mouse background. A single human fragment amongst the acrocentric mouse chromosomes was observed (Fig 8.4).

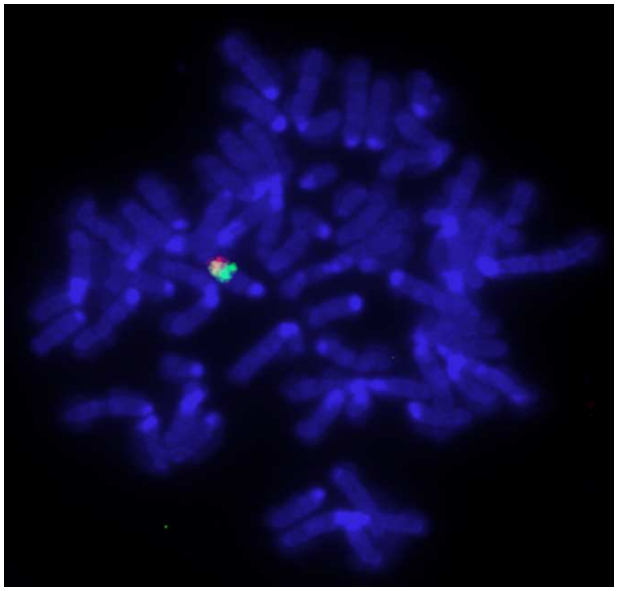


Figure 8.4: Image of A9/fchr11.1.9 metaphase spreads painted with arm-specific chromosome 11 paints, showing the presence of the human chromosome fragment in the mouse A9 background. 11p is painted green and 11q red.

A high level of *hTERT* repression in PC-3/hTERT/fchr11.1.9 clones (Fig 8.3; C) suggested that the *hTERT* repressor sequence may be located in the peri-centromeric region of normal human chromosome 11, a 5.4Mb region flanked by STS polymorphic markers D11S4420 and D11S2006 (Fig 8.2). In order to reduce the area around the centromere I selected clone 15 (A9/fchr11.1.15) to be transferred next into PC-3/hTERT cell line for analysis of repressive function. If positive for repressive activity this would reduce the region of interest by 1.3Mb as most of the q-arm just beneath the centromere has been deleted in this clone.

The *hTERT* mRNA levels were measured in the 16 hybrid clones that were generated. Repression of *hTERT* transcription in 15 out of 16 clones was observed, in which 6 were completely silenced with respect to *hTERT* transcription and the rest expressed very low levels. Again, these results confirmed the presence of the *hTERT*

repressor sequence on this reduced fragment (Fig 8.3; D). The presence of the human chromosome 11 fragment in clone A9/fchr11.1.15 was confirmed by arm specific chromosome 11 paints (Fig 8.5).

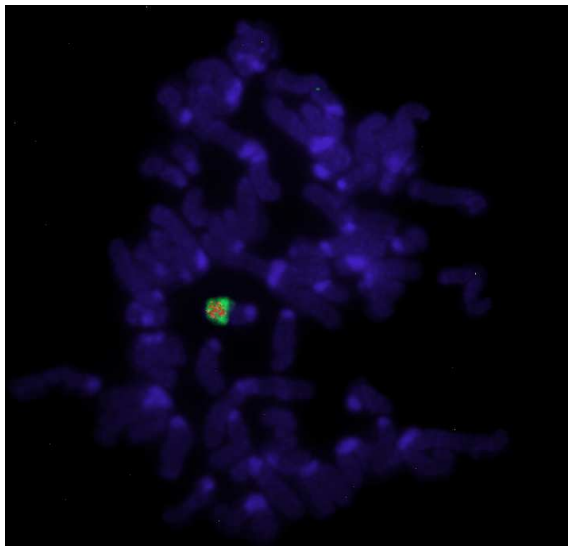


Figure 8.5: Image of the A9/fchr11.1.15 metaphase spread painted with arm-specific chromosome11 paints, showing the presence of human chromosome 11 fragment which seems to have translocated onto a mouse chromosome.

Further development of the STS map (Fig 8.2) revealed clone 13 to possess a deleted centromeric region. Therefore, I selected clone 13 to transfer next into PC-3/hTERT cell line because it would permit me to either include or eliminate the centromeric region as the location of the *hTERT* repressor sequence.

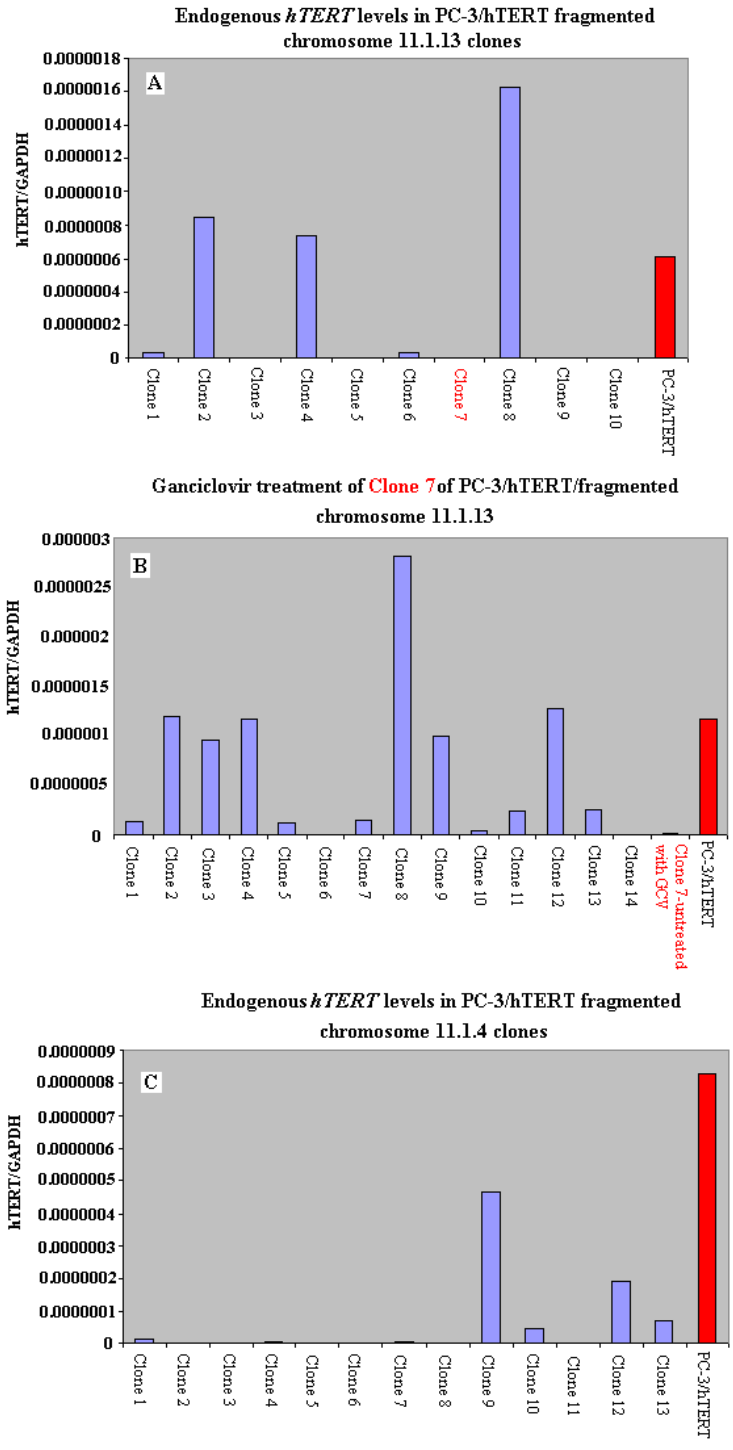



Figure 8.6: Measurement of endogenous *hTERT* mRNA levels in hybrids generated from the individual transfer of (A) A9/chr11.1.13 and (C) A9/chr11.1.4 fragment into PC-3/hTERT cell line. (B) Reverse selection of the fragment from clone 7 of PC-3/hTERT/fragmented chr11.1.3 was achieved by treating clone 7 with 2µM GCV. Graph showing the generated clones assayed for endogenous *hTERT* mRNA expression levels. Clone 7 (second last bar) with the fragment remains repressed.

Transfer of clone 13 into PC-3/hTERT cell line, as with the previously analysed fragments, showed high level of repression in 70% (7/10) of the clones (Fig 8.6; A).

In order to confirm the presence of the *hTERT* repressor sequence on the fragment in clone 13 I randomly selected clone 7, from all the other clones that had undetectable level of *hTERT*, for reverse selection of the fragment. GCV treatment of clone 7 resulted in 6/14 clones having higher or equal levels of *hTERT* compared with the PC-3/hTERT cell line (Fig 8.6; B). Eleven out of fourteen clones had higher *hTERT* than the untreated clone 7 and 3 clones displayed very low undetectable levels. One possible reason for clones 6, 10 and 14 failing to revert is that the HyTK selectable marker on the fragment had been lost or mutated in these clones and the *hTERT* repressor sequence had been retained. These results suggest that reverse selection of the fragment, hence the *hTERT* repressor sequence, in clone 7 with GCV treatment can revert the cells back to the original *endogenous hTERT* expression phenotype as in the parent PC-3/hTERT cell line.

Clone 4 (A9/fchr11.1.4) was selected from the STS map to be transferred next into the PC-3/hTERT cell line with the intention to eliminate the p-arm of chromosome 11 from the possibility of retaining the *hTERT* repressor sequence. High levels of *hTERT* repression were observed in 11 out of 13 clones (Fig 8.6; C) indicating the presence of an *hTERT* repressor sequence on A9fchr11.1.4 hybrid clone.

Table 8.1: Summary of the total number of clones collected from the six microcell transfer (MMCT) experiments of the human chromosome 11 fragment from mouse A9 cells into the human PC-3/hTERT cell line (in the order in which they were performed).

| **From Fig 8.2 (STS map) Human chromosome 11 fragment clone** | **Designation of A9 fragment donor cell line** | **Reason for transfer** | **Number of clones collected** | **Number of highly repressed *hTERT* clones** |
|---|---|---|---|---|
| 8 | A9/fchr11.1.8 | Random | 12 | 10/12 (83%) |
| 2 | A9/fchr11.1.2 | Random | 12 | 11/12 (92%) |
| 9 | A9/fchr11.1.9 | Smallest fragment. Both telomeres missing | 13 | 9/13 (69%) |
| 15 | A9/fchr11.1.15 | Negative between 58.3-59.5Mbp on q-arm | 16 | 14/16 (88%) |
| 13 | A9/fchr11.1.13 | Centromere and the p-telomere missing | 10 | 7/10 (70%) |
| 4 | A9/fchr11.1.4 | No positive STS markers on the p-arm between 10.8-44.3Mbp | 13 | 11/13 (85%) |

I found that individual transfer of chromosome 11 fragments produced higher proportion of clones that showed endogenous *hTERT* repression compared with the clones in which a whole chromosome 11 was transferred (Fig 6.2). This may be due to the formation of a "telescoped" chromosome (when microcells containing whole chromosome 11 were originally exposed to gamma radiation for XMMCT) having the HyTK marker closer to the *hTERT* repressor sequence. Thus far, in these experiments, I have been unable to identify a negative fragment (i.e. a fragment that had lost the *hTERT* repressor sequence). All six transfers produced clones in which the majority repressed endogenous *hTERT* levels (Table 8.1). This result would be

expected if the HyTK marker and the *hTERT* repressor sequence had by chance become closely juxtaposed.

I used the results from individual transfers of chromosome 11 fragments from A9 clones 2, 4, 8, 9, 13 and 15 into PC-3/hTERT cell line (i.e. that had all retained the *hTERT* transcriptional repressor sequence) to inform a more detailed analysis of the STS map (Fig 8.2) in order to narrow down the region of interest (i.e. *hTERT* repressor) on chromosome 11. The positive results with clone 9 eliminated the possibility that the *hTERT* repressor is located in the telomeric regions of the p and q-arms. Transfer of clone 13 also eliminated the telomeric region of the p-arm, in addition to the centromeric region, and transfer of clone 4 excluded the p-arm of chromosome 11. From these six selected transfers I have identified 3 possible regions of interest on chromosome 11 where the *hTERT* repressor may be located. The first is a 0.72Mb region, map position 64.70Mb to 65.42Mb (11q13.1). The second and third regions are flanked by a single STS marker at 69.71Mb (11q13.3) and 127.32Mb (11q24.3) (Fig 8.7).

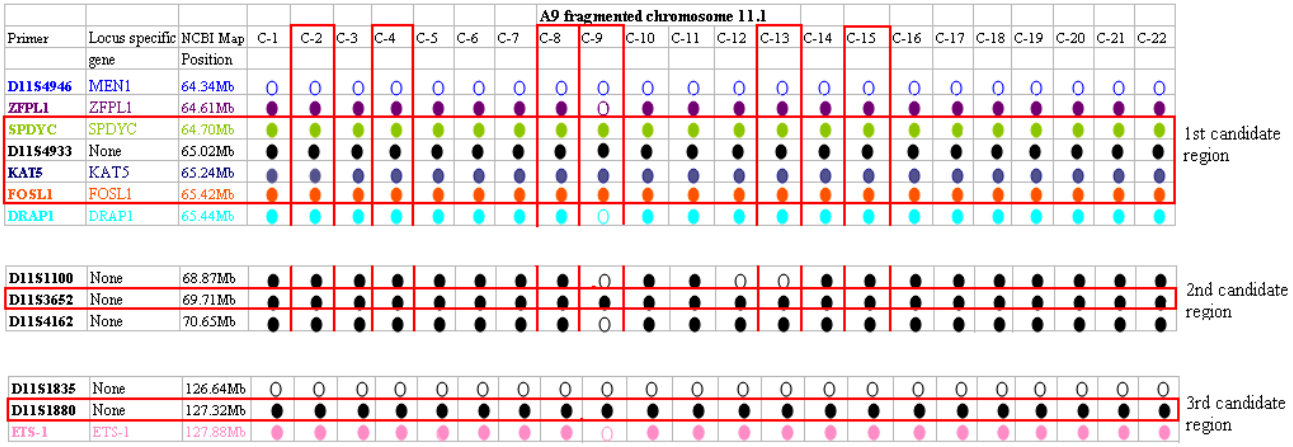



Figure 8.7: Sections of the STS map highlighting the 3 possible candidate regions where the *hTERT* repressor sequence may reside.

### 8.3.3: Selection of *endogenous hTERT* repressed and non-repressed clones to construct a panel against which the genes of interest could be tested

A number of *hTERT* repressed and non-repressed hybrids were selected from the individual transfers of human chromosome 11 fragment in mouse A9 clone 2, 8, 9, and 15 (A9/fchr11.1.2, A9/fchr11.1.8, A9/fchr11.1.9 and A9/fchr11.1.1) into PC-3/hTERT cell line (Fig 8.8), to construct a panel against which the genes of interest could be tested. The panel consisted of eight clones that had highly repressed *hTERT* transcription and six clones that did not show repressed *hTERT*.

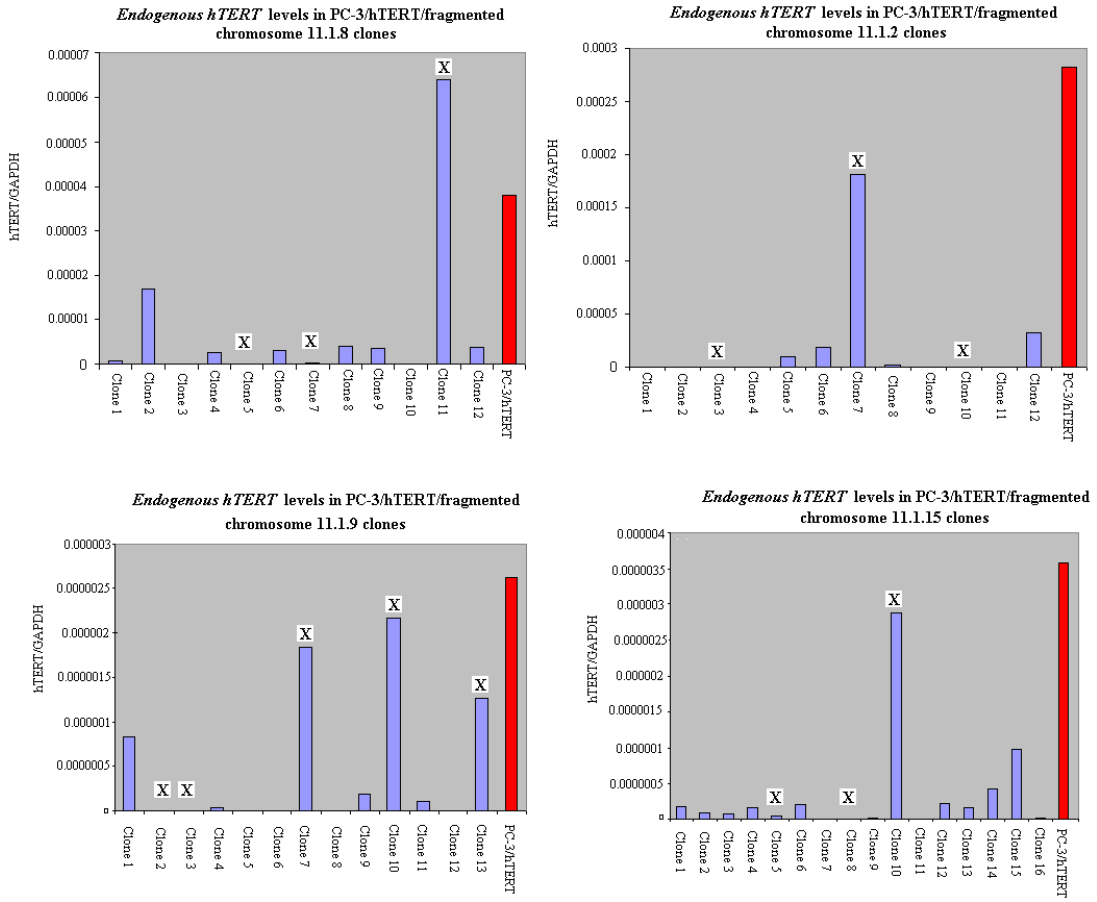



Figure 8.8: Construction of a panel consisting of *hTERT* repressed and non-repressed (labelled with X) hybrid clones selected from MMCT of clones 2, 8, 9 and 15 from mouse A9 cells into human PC-3/hTERT cells.

### 8.3.4: Identification of the differentially expressed genes (by microarray) in the minimal region of clone 13, as defined by CGH

At this stage of the project we collaborated with Professor Colin Cooper's team at the Institute of Cancer Research (ICR) UK to try to identify the *hTERT* repressor sequence on the fragment. They used the comparative genomic hybridization (CGH) technique to analyse the copy number on the DNA fragment in clones 2, 8, 9, 13 and 15 and microarray technology was used in an attempt to identify genes that were differentially expressed in our panel of *hTERT* repressed and non-repressed clones in the regions of the fragment as determined in these five clones by CGH.

I provided genomic DNA from A9 parent cells and from clones 2, 8, 9, 13 and 15 (A9/fchr11.1.2, A9/fchr11.1.8, A9/fchr11.1.9, A9/fchr11.1.13 and A9/fchr11.1.15) which contained the human chromosome 11 fragment in mouse A9 background to Professor Cooper's team for CGH analysis. I also provided the cytoplasmic RNA from the two sets of hybrid clones from the panel of *hTERT* repressed and non-repressed clones and the ICR team synthesized complementary DNA (cDNA) and tagged each set with different colour fluorochromes, namely Cy 3 (red) and FITC (green). These test samples when co-hybridized to arrays spotted with either normal oligonucleotide or cDNA clones (representing specific genes) gave a ratio of the intensity of the fluorochromes. Any abnormal ratios represented a difference in RNA expression levels between the *hTERT* repressed and non-repressed clones. A list of up and down-regulated genes in the two sets of hybrids was generated with the Aglilent microarray and Affymetrix exon microarray using the minimum region on A9/fragmented chromosome 11.1 clone 13. (A9/fchr11.1.13).

The two minimum regions identified by the ICR team were both on the q-arm of chromosome 11. The first region was between 61037739-70771300bp and the second was the q telomeric region between 126851379-134340191bp (Fig 8.9).

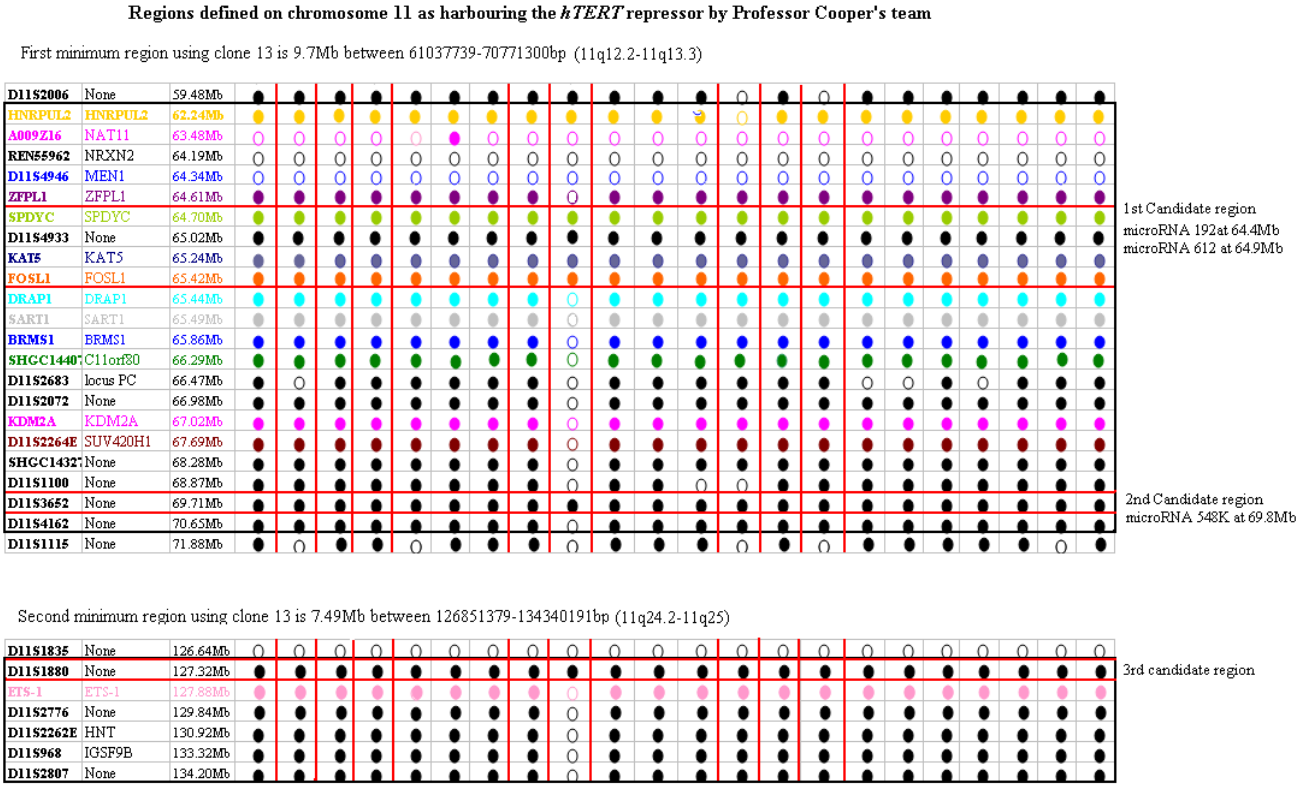



Figure 8.9: The two minimum candidate regions for harbouring the *hTERT* repressor, as defined by CGH by Professor Cooper's team are highlighted in black. The three candidate regions I have identified by STS mapping, highlighted in red, all fall within these two regions.

Noticeably, the three much smaller regions I had independently identified all fall within the two minimum regions identified by Prof Cooper's team. They also identified six microRNAs, namely has-mi-612, has-mi-1237, has-mi-192, has-mi-548k, has-mi-194-2 and has-mi-611 in the minimum regions. Two out of the six miRNAs were in my first candidate region and a third was just outside my second candidate region (Fig 8.9).

## 8.4 Discussion

The MMCT transfers of carefully selected A9: human chromosome 11 XMMCT clones (that showed variations in DNA content in the human fragment) back into PC-3/hTERT cells allowed three regions of interest to be identified, all on the q-arm of chromosome 11 (Fig 8.7). Individual transfers of clones 4, 9, and 13 from this set have been invaluable in identifying the long arm of chromosome 11 as carrying the *hTERT* transcriptional repressor sequence. The largest of the three regions that has been identified is 0.72Mb, map position 64.70Mb to 65.42Mb (11q13.1). The second and third regions were flanked by a single STS marker at 69.71Mb (11q13.3) and 127.32Mb (11q24.3). The genes in these regions are listed in Fig 8.4.1.

One of the three regions that have been identified as the possible location for the *hTERT* repressor sequence on the chromosome 11 fragment will contain the HyTK selectable marker. I have previously shown the presences of the HyTK on the q-arm of chromosome 11 by single copy FISH (see section 7.3.4).

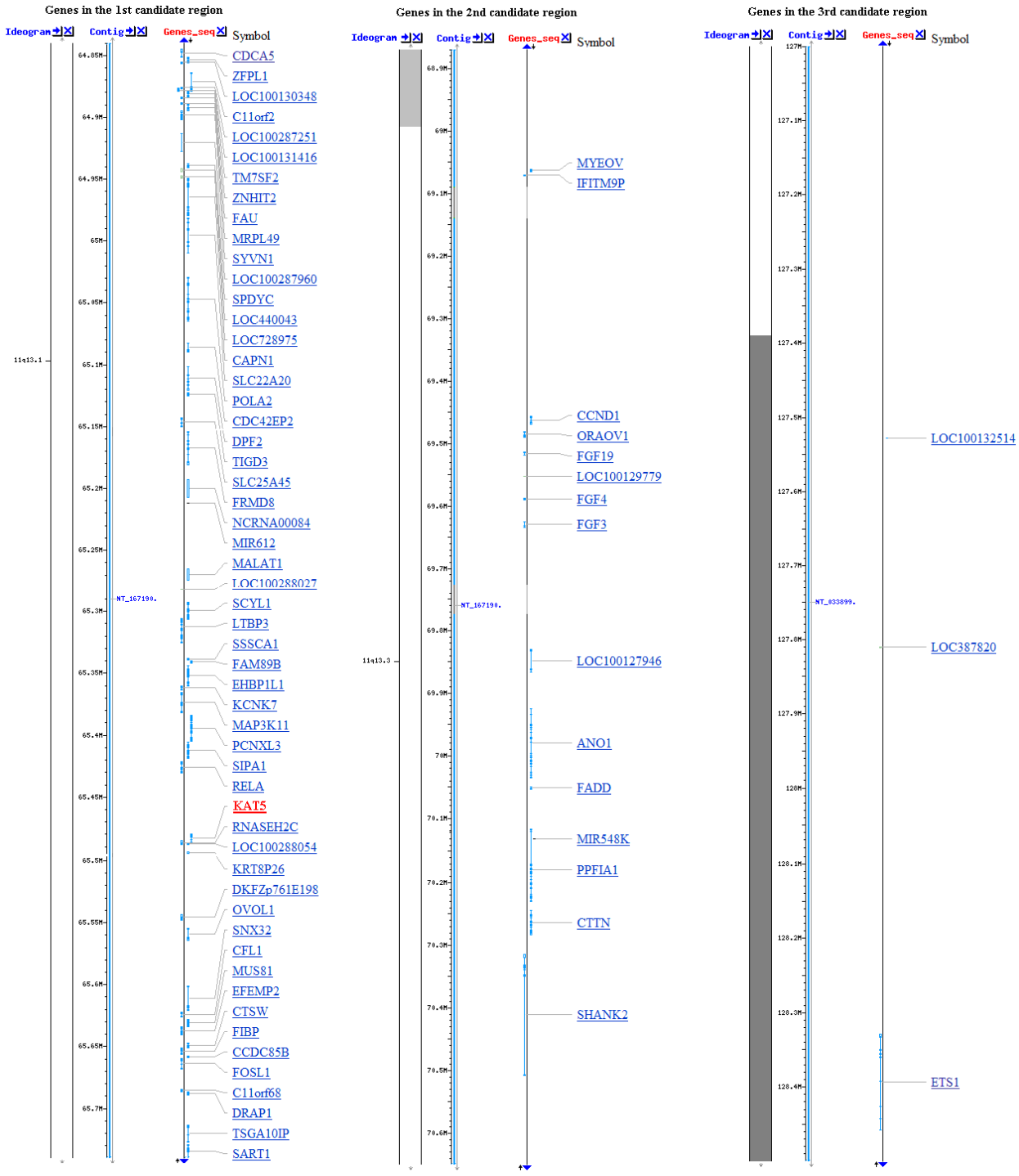



Figure 8.10: The list of genes in the three candidate regions obtained from the NCBI website.

Independent verification of our very original findings was corroborated (in collaboration with ICR) by Professor Colin Cooper's team. They performed CGH (comparative genomic hybridization) analysis on the A9/fchr11.1.13 clone and their

data confirmed our STS observations that both the centromeric region and the telomeric region on the p-arm are deleted in Clone 13. Using this clone allowed them to define two minimum regions which may carry the *hTERT* repressor. The first minimal region is between 6107739-70771300bp and the second is between 126851379-134340191bp on the q-arm of chromosome 11. Interestingly, my first candidate region between 64.70Mb-65.42Mb and the second region flanked by a single STS marker at 69.71Mb fell into Prof. Cooper's first region and my third candidate region fell into their second region (see Fig 8.9). Professor Cooper's team also identified six microRNAs (miRNA) within their defined regions. The miRNA-612 (map position 64.9Mb) falls within my candidate region. Two of the miRNAs, namely miRNA-192 (map position 64.4Mb) and miRNA-548K (map position 69.8Mb) fall just outside my first and second candidate regions respectively.

More than 50% of miRNA genes have recently been identified in genomic regions associated with cancer. These non-protein coding RNAs that are approximately 20-25 nucleotides long can inhibit gene expression at the post-transcriptional level by either cleaving the target mRNA or having a high degree of complementation to the mRNA at the 3' untranslated end (reviewed in Zhang et al., 2007; Pasquinelli et al., 2005). However, in my study I have been trying to identify a repressor that functions at the transcriptional level. Therefore, there will probably be no urgency to examine the expression profiles of the three miRNA in our panel of *hTERT* repressed and non-repressed clones.

# Chapter 9

# Identification of potential *hTERT* repressor genes from the minimal chromosome 11 region of interest: *KAT5* as an attractive candidate

## 9.1 Introduction

In this section of the study, genes that could possibly repress *hTERT* expression were identified by two means. First, I used the NCBI website to select plausible genes (from the total number of genes listed in my three candidate regions) i.e. that were either known or predicted to be involved in regulating cell proliferation, differentiation, transformation etc and hence had the potential to mediate *hTERT* repression. The expression levels of these genes were measured in my panel of *hTERT* repressed and non-repressed hybrids (see Chapter 8), using the real time qRT-PCR relative quantification method. Second, a list of differentially expressed genes, as determined by microarray data, in my *hTERT* panel was provided by Professor Cooper. Those genes that showed the most significant difference between the repressed and non-repressed clones were selected for measurement of *hTERT* expression levels by qRT-PCR.

## 9.2 Materials and Methods

### 9.2.1 Real-time qRT-PCR-relative quantification

The real-time qRT-PCR relative quantification method was used to measure the expression levels of the selected genes in the panel of *hTERT* repressed and non-repressed clones. This method compares the Ct value of a target gene with an internal housekeeping gene in a single sample. I used this method to analyse changes in gene expression in my panel of *hTERT* repressed and non-repressed clones relative to the parent PC-3/hTERT cell line.

The Mastermix for the target gene (gene of interest) was prepared by adding an optimized concentration of the forward and reverse primer probes to 1xSybr Green (Applied Biosystems) and the Mastermix for the endogenous gene, *GAPDH* was prepared by adding 0.4M forward primer probe 1457 (5'GAAGGTGAAGGTCGGAGT 3' sequence localized at exon 1) and 0.4µM reverse primer probe 3407 (5'GAAGATGGTGATGGGATTTC 3' sequence localized at exon 3) to 1XSybr Green. To 24µl of the prepared mastermix (containing the primers for the target gene or the endogenous GAPDH) 1.0µl of cDNA was added in each well of a microtiter plate in triplicate. Thermal cycling conditions consisted of a step at 50$^{o}$C for 2min, a denaturing step at 95°C for 10min followed by 45 cycles at 95°C for 15s and 60°C for 1min (ABI PRISM 7900 HT Sequence Detection System). The results were analysed by the relative quantification (ΔΔ Ct) study assay using the SDS 2.1 software. The expression level of the target gene in the

*hTERT* repressed and non-repressed clones was plotted relative to the level measured in PC-3/hTERT cells (Fig 9.1).

## 9.3 Results

The NCBI website (http://www.ncbi.nlm.nih.gov/mapview/maps.cgi?taxid=9606&chr=11) listed 50, 14 and 3 genes in the first, second and third candidate regions respectively. A list of all the genes (and their known or predicted functions) in the three candidate regions is outlined in Table 9.1.

Table 9.1: A list of all the genes and their functions as published on the NCBI and GeneCards (http://www.genecards.org/) websites. The genes in bold text have been tested against the *hTERT* panel (see section 8.8).
First candidate region at 11q13.1; map position 64.70Mb to 65.42Mb (0.72Mb).

| **Gene** | **Known or predicted function of the gene** |
|---|---|
| ***ZFPL1*** | Required for cis-Golgi integrity and efficient ER to Golgi transport. Involved in the maintainance of the integrity of the cis-Golgi. |
| *LOC100130348* | Not known |
| *C11orf2* | Required for both Golgi structure and vesicular trafficking, and ultimately lipid transport. |
| *LOC100287251* | Not known |
| *LOC100131416* | Not known |
| *TM7SF2* | Involved in the conversion of lanosterol to cholesterol. |
| *ZNHIT2* | Not known |
| *FAU* | This gene encodes a fusion protein consisting of the ubiquitin-like protein fubi at the N terminus and ribosomal protein S30 at the C terminus. It has been proposed that the fusion protein is post-translationally processed to generate free fubi and free ribosomal protein S30. Fubi is a member of the ubiquitin family, and ribosomal protein S30 belongs to the S30E family of ribosomal proteins. The function of fubi is currently unknown and ribosomal protein S30 is a component of the 40S subunit of the cytoplasmic ribosome. |
| *MRPL49* | Mammalian mitochondrial ribosomal proteins are encoded by nuclear genes and help in protein synthesis within the mitochondrion. |
| *LOC100287960* | Not known |
| ***SPDYC*** | Promotes progression through the cell cycle via binding and activation of CDC2 and CDK2. |
| *CAPN1* | Calcium-regulated non-lysosomal thiol-protease which catalyze limited proteolysis of substrates involved in cytoskeletal remodelling and signal |

| | |
|---|---|
| | transduction. |
| *SLC22A20* | Organic anion transporter that mediates the uptake of estrone sulfate. May act as an odorant transporter. |
| *POLA2* | May play an essential role at the early stage of chromosomal DNA replication by coupling the polymerase alpha/primase complex to the cellular replication machinery. |
| *CDC42EP2* | May act downstream of CDC42 to induce actin filament assembly leading to cell shape changes. |
| *DPF2* | May be a transcription factor required for the apoptosis response following survival factor withdrawal from myeloid cells. Might also have a role in the development and maturation of lymphoid cells. |
| *TIGD3* | The exact function of this gene is not known. |
| *SLC25A45* | SLC25A45 belongs to the SLC25 family of mitochondrial carrier proteins. |
| *FRMD8* | Not known |
| *NCRNA00084* | Not known |
| *MALAT1* | Not known |
| *SCYL1* | Regulates COPI-mediated retrograde traffic. Has no detectable kinase activity in vitro. Isoform 6 acts as transcriptional activator. It binds to three different types of GC-rich DNA binding sites (box-A, -B and -C) in the beta-polymerase promoter region. It also binds to the TERT promoter region. |
| *LTBP3* | May play critical roles in controlling and directing the activity of TGFB1. |
| *SSSCA1* | Might play a role in mitosis. Could be a centromere-associated protein. May induce anti-centromere antibodies. |
| *FAM89B* | Not known |
| *EHBP1L1* | Not known |
| *KCNK7* | Probable potassium channel subunit. No channel activity observed in vitro as protein remains in the endoplasmic reticulum. May need to associate with an as yet unknown partner in order to reach the plasma membrane. |
| *MAP3K11* | Activates the JUN N-terminal pathway. Required for serum-stimulated cell proliferation and for mitogen and cytokine activation of MAPK14 (p38), MAPK3 (ERK) and MAPK8 (JNK1). Plays a role in mitogen-stimulated phosphorylation and activation of BRAF, but does not phosphorylate BRAF directly. Influences microtubule organization during the cell cycle. |
| *PCNXL3* | Not known |
| *SIPA1* | GTPase activator for the nuclear Ras-related regulatory proteins Rap1 and Rap2 in vitro, converting it to the putatively inactive GDP-bound state. |
| *RELA* | NF-kappa-B is a pleiotropic transcription factor which is present in almost all cell types and is involved in many biological processes such as inflammation, immunity, differentiation, cell growth, tumorigenesis and apoptosis. NF-kappa-B is a homo- or heterodimeric complex formed by the Rel-like domain-containing proteins RELA/p65, RELB, NFKB1/p105, NFKB1/p50, REL and NFKB2/p52. The dimers bind at kappa-B sites in the DNA of their target genes and the individual dimers have distinct preferences for different kappa-B sites that they can bind with distinguishable affinity and specificity. Different dimer combinations act as transcriptional activators or repressors, respectively. |
| *KAT5* | Catalytic subunit of the NuA4 histone acetyltransferase complex which is involved in transcriptional activation of select genes principally by acetylation of nucleosomal histones H4 and H2A. This modification may both alter nucleosome-DNA interactions and promote interaction of the modified histones with other proteins which positively regulate transcription. This complex may be required for the activation of transcriptional programs associated with |

| | |
|---|---|
| | oncogene and proto-oncogene mediated growth induction, tumor suppressor mediated growth arrest and replicative senescence, apoptosis, and DNA repair. NuA4 may also play a direct role in DNA repair when recruited to sites of DNA damage. Directly acetylates and activates ATM. In case of HIV-1 infection, interaction with the viral Tat protein leads to KAT5 polyubiquitination and targets it to degradation. |
| *RNASEH2C* | Non catalytic subunit of RNase H2, an endonuclease that specifically degrades the RNA of RNA: DNA hybrids. Participates in DNA replication, possibly by mediating the removal of lagging-strand Okazaki fragment RNA primers during DNA replication. Mediates the excision of single ribonucleotides from DNA: RNA duplexes. |
| *LOC100288054* | Not known |
| *KRT8P26* | Not known |
| *DKFZp761E198* | Not known |
| *OVOL1* | Putative transcription factor. Involved in hair formation and spermatogenesis. May function in the differentiation and/or maintenance of the urogenital system. |
| *SNX32* | Not known |
| *CFL1* | Controls reversibly actin polymerization and depolymerization in a pH-sensitive manner. It is the major component of intranuclear and cytoplasmic actin rods. |
| *MUS81* | May be required in mitosis for the processing of stalled or collapsed replication forks. |
| *EFEMP2* | The protein encoded by this gene contains four EGF2 domains and six calcium-binding EGF2 domains. This gene is necessary for elastic fiber formation and connective tissue development. Defects in this gene are cause of an autosomal recessive cutis laxa syndrome. |
| *CTSW* | May have a specific function in the mechanism or regulation of T-cell cytolytic activity. |
| *FIBP* | May be involved in mitogenic function of FGF1. |
| *CCDC85B* | Functions as a transcriptional repressor. May inhibit the activity of CTNNB1 in a TP53-dependent manner and thus regulate cell growth. May function in adipocyte differentiation, negatively regulating mitotic clonal expansion. |
| ***FOSL1*** | The Fos gene family consists of 4 members: FOS, FOSB, FOSL1**,** and FOSL2. These genes encode leucine zipper proteins that can dimerize with proteins of the JUN family, thereby forming the transcription factor complex AP-1. As such, the FOS proteins have been implicated as regulators of cell proliferation, differentiation, and transformation. |
| *C11orf68* | Not known |
| ***DRAP1*** | The association of the DR1/DRAP1 heterodimer with TBP results in functional repression of both activated and basal transcription of class II genes. This interaction precludes the formation of a transcription-competent complex by inhibiting the association of TFIIA and/or TFIIB with TBP. Can bind to DNA on its own. |
| *TSGA10IP* | Not known |
| ***SART1*** | May play a role in mRNA splicing. May also bind to DNA. |

Second candidate region flanked by a single STS marker at 69.71Mb (11q13.2)

| **Gene** | **Function** |
|---|---|

| | |
|---|---|
| *CCND1* | Essential for the control of the cell cycle at the G1/S (start) transition. |
| *FLJ42258* | Not known |
| *ORAOV1* | Not known |
| *FGF19* | May be involved in brain development during embryogenesis. |
| *LOC100129779* | Not known |
| *FGF4* | Can transform NIH 3T3 cells from a human stomach tumour (hst) and from karposi's sarcoma (KS3). It has a mitogenic activity. |
| *FGF3* | Could be involved in ear development. |
| *LOC100127946* | Not known |
| *TMEM16A* | Acts as a calcium-activated chloride channel. Required for normal tracheal development. |
| *FADD* | Apoptotic adaptor molecule that recruits caspase-8 or caspase-10 to the activated Fas (CD95) or TNFR-1 receptors. The resulting aggregate called the death-inducing signalling complex (DISC) performs caspase-8 proteolytic activation. Active caspase-8 initiates the subsequent cascade of caspases mediating apoptosis. |
| *PPFIA1* | May regulate the disassembly of focal adhesions. May localize receptor-like tyrosine phosphatases type 2A at specific sites on the plasma membrane, possibly regulating their interaction with the extracellular environment and their association with substrates. |
| *CTN* | May contribute to the organization of cell structure. The SH3 motif may function as a binding region to cytoskeleton. Tyrosine phosphorylation in transformed cells may contribute to cellular growth regulation and transformation. |
| *SHANK2* | May play a role in the structural and functional organization of the dendritic spine and synaptic junction. |
| *C11orf76* | Not known |

Third candidate region flanked by a single STS marker at 127.32Mb (11q24.2-3)

| **Gene** | **Function** |
|---|---|
| *LOC100132514* | Not known |
| *LOC387820* | Not known |
| ***ETS1*** | ETS transcriptions factors, such as ETS1, regulate numerous genes and are involved in stem cell development, cell senescence and death, and tumorigenesis. The conserved ETS domain within these proteins is a winged helix-turn-helix DNA-binding domain that recognizes the core consensus DNA sequence GGAA/T of target genes (Dwyer et al., 2007 ). Transcription factor. |

Next, the expression levels of the above genes needed to be analysed. In the first phase of the investigation, genes that could potentially repress *hTERT* transcription

were selected to be tested against the panel of *hTERT* repressed and non-repressed clones (see section 8.3.3).

I am currently confirming the validity of some of the candidate genes selected from the microarray data. A number of genes that may possibly be involved in transcriptional repression of *hTERT* were tested first against this panel but to date I have been unable to identify a gene that was differentially expressed between the *hTERT* repressed and non-repressed hybrid clones by the real time qRT-PCR method. The genes that have been tested so far include *HNRPUL, CREB3L1, PHF21A, C11orf80, ETS1, WT1, SUV420H1* (variant 1 and 2), *ZFPL1*, *FOSL1*, *DRAP1* and *SART*. An example of the expression level of *PHF21A* in my *hTERT* repressed and non-repressed clones is given in Fig 9.1.

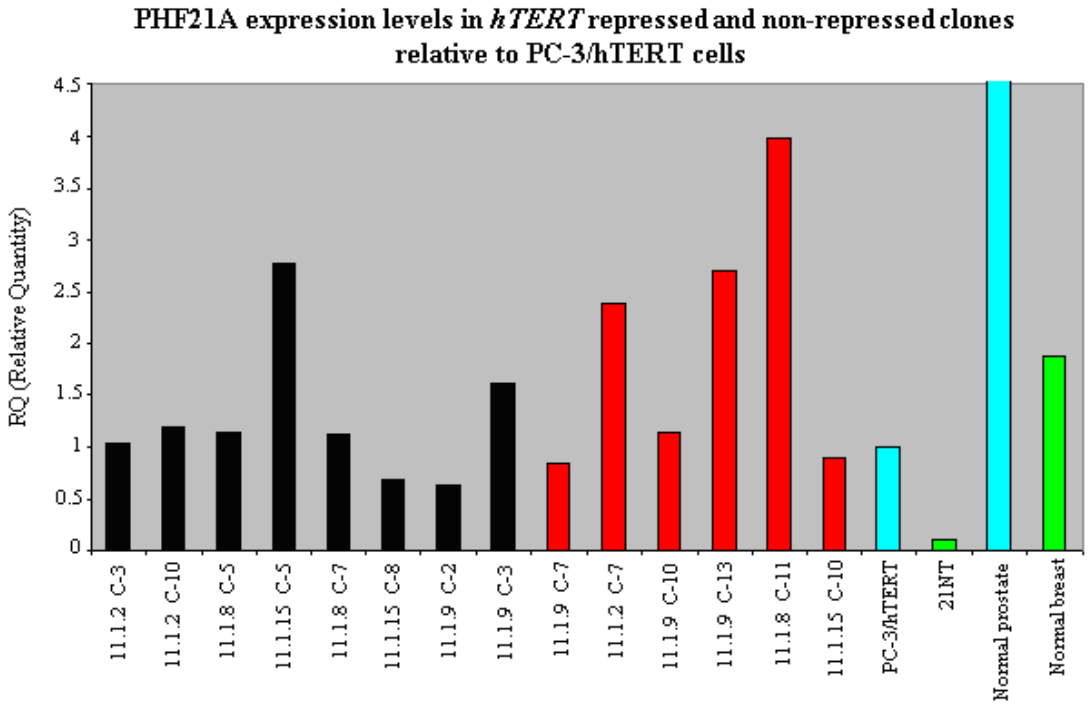



Figure 9.1: Real-time qRT-PCR relative quantification of PHF21A gene in *hTERT* repressed (black) and non-repressed (red) hybrid clones.

## 9.4 Discussion

### 9.4.1 Potential candidate genes for repressing *hTERT* transcription

The candidate regions that have been identified on chromosome 11 as possibly carrying the *hTERT* transcriptional repressor sequence excluded the *MEN1* gene (located at 11q13 map position 64.34Mb). MEN1 encodes menin, a protein that acts as a tumour suppressor in endocrine neoplasia. Menin is thought to suppress *hTERT* by possibly mediating its effects through interaction with JunD and/or NF-κB at the *hTERT* promoter region (Lin and Elledge, 2003). Recently, it has been shown that over-expression of menin decreases *hTERT* transcription in a cell type specific manner (Hashimoto et al., 2008). Therefore, it may be possible that menin does not have any repressive effects on *hTERT* transcription in MMCT experiments with prostate cancer cells. The locus for the *MEN1* gene was absent in all the 22 clones (see Fig 8.2). So menin can be confidently eliminated as the repressor involved in PC-3/hTERT repression.

*ETS1* was another gene (located at 11q23.3, map position 127.88Mb) just outside the third candidate region that had the potential for repressing *hTERT* transcription. The ETS1 protein belongs to a family of transcription factors involved in cellular proliferation, senescence, apoptosis, and tumorigenesis. Interestingly, recent studies have shown that the ETS1 protein inhibits telomerase activity by binding directly to the *hTERT* promoter region in K562 cells, a human myeloid erythroleukemia cell line (Dwyer J et al., 2007). I had initially eliminated *EST1* as a possible candidate for repressing *hTERT* transcription because its locus was missing from clone 9 (see Fig 8.2). However, because of its reported involvement in *hTERT* repression I tested the

expression levels of *EST1* in my panel of *hTERT* repressed and non-repressed clones (see section 8.8) and found no difference in the two sets of samples (data not shown).

### 9.4.2 Search for putative tumour suppressor gene(s) in my candidate regions from the literature

Loss of heterozygosity (LOH) studies has played an essential role in identifying putative tumour suppressor genes on chromosomal regions from where genetic material has been lost in a non-random fashion from cancer tissues. LOH is a very common event in epithelial cancers. A literature survey for LOH on chromosome 11 revealed that both arms are implicated in prostate carcinoma. Dahiya et al (1997) compared LOH on chromosome 11 in microdissected samples from normal prostate epithelium with invasive carcinoma from the same patients. They reported four deleted regions at 11p15, 11p12, 11q22, and 11q23-24. LOH was observed in 25% and 39% of tumours at 11p15 and 11p12 respectively. The percentage of tumours with LOH at 11q22 and 11q23-24 was higher at 66% and 47% respectively.

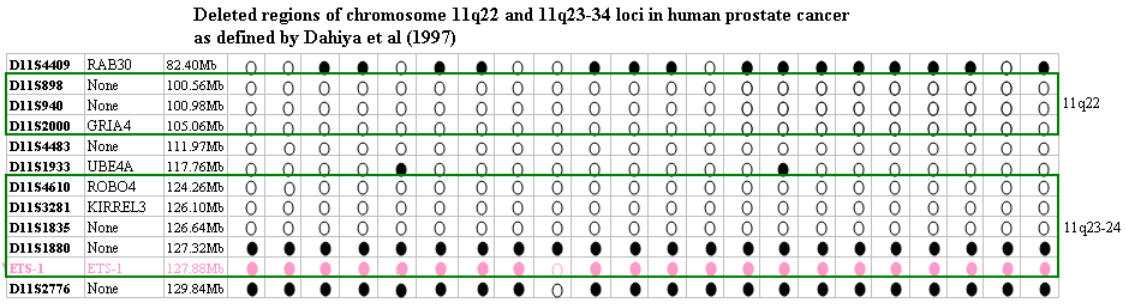



Figure 9.2: Part of my STS map showing the two deleted regions of the q-arm of chromosome 11 in human prostate cancer as defined by Dahiya et al (1997).

I also used the two STS markers, D11S898 and D11S940 at 11q22, used by Dahiya et al (1997). I found both markers to be negative for all of the 22 A9 clones (hybrids containing human chromosome 11 fragment in mouse A9 background, see Fig 8.2). Therefore, I eliminated this region as potentially possessing the *hTERT* repressor sequence (Fig 9.2). D11S1818 (map position 108.78Mb) was another marker used by Dahiya et al (1997), but unfortunately I have a gap in STS markers between 105.06Mb to 111.97Mb. So, it is possible that I may have missed the region around 108Mb. However, Prof. Cooper's team did not identify the 11q22 region as a potential location for the *hTERT* repressor (their second minimum region was between 126.8Mb-134.3Mb). Therefore I can conclude with some degree of confidence that 11q22 is not the region of interest.

Dahiya et al (1997) used three STS markers at 11q23-24, namely D11S924, D11S1336, and D11S912. Map positions for these markers are 119.4Mb, 122.6Mb, and 128.6Mb respectively. D11S912 is the only marker that falls in Prof. Cooper's second minimum region (Fig 8.9) and it is just outside my third candidate region. Therefore, the possibility that our gene of interest may be located at 11q23-24 is slim.

Dumur et al (2003) performed a genome-wide search for LOH in prostate cancer using human SNP microarray technology. They studied a cellular model which consisted of a prostate cell line and its tumourigenic sub-line and they also analysed prostate cancer patients. Their results from the cellular model showed LOH between 11q21.1-11q24.2 in the prostate tumourigenic sub-line. Other deletions were on

chromosomes 3p12-p22.1, 19p13.12, and 19q13.42. The authors observed LOH at 11q23.3 in 33% of the prostate cancer patients. Interestingly, once again the region between 11q21.1-11q24.2 falls in the region defined by both Prof. Cooper's team (by using array CGH) and mine (by using STS mapping). The 11q23.3 locus, deleted in 33% of prostate cancer patients is the location of the *ETS1* gene which has already been eliminated (see above).

Admittedly, there are gaps in my STS markers in the region between 11q21-11q24.2. However, if more STS markers could be tested for the presences of sequence in these gaps then perhaps clone 9 could prove to be even more invaluable than it has already been in further narrowing the region on 11qter, as it possesses the smallest chromosome 11 fragment.

### 9.4.3 *hTERT* repressor - a possible chromatin remodelling factor

Several groups have investigated the nuclease-sensitive sites in the region of the *hTERT* gene in order to identify the mechanisms that regulate *hTERT* expression in normal and cancerous cells. Szutorisz et al (2003), in close collaboration with our group, reported chromosome 3 to transcriptionally repress *hTERT* by remodelling the chromatin structure of the *hTERT* gene at intron 2 in a breast cancer cell line into a more "closed" conformation. Reverse selection of transferred chromosome 3 with GCV treatment resulted in reverting to an open chromatin structure. In another report, Wang and Zhu (2003) identified a DNase I hypersensitive site near the *hTERT* transcription initiation site in telomerase positive cells. The authors also demonstrated that the treatment of telomerase negative cells with histone deacetylase

inhibitor trichostatin A (TSA) induced *hTERT* transcription and increased chromatin sensitivity to DNase I treatment. The authors concluded that the endogenous chromatin environment plays a vital role in the regulation of *hTERT* expression during cellular immortalization. Therefore, gene(s) that may be involved in epigenetic regulation through altering the chromatin structure would make excellent candidates in my study.

### 9.4.4 The *KAT5* gene, a credible candidate?

There are three histone modifying genes listed in Professor Cooper's first region between 61.08Mb-65.42Mb. These are *KAT5*, a histone acetyltransferase (HAT), *BRMS1*, a histone deacetylase (HDAC) and *KDM2A*, a histone demethylase (see Fig 8.9). However, I eliminated the *BRMS1* and *KDM2A* genes on the basis that both loci were deleted in clone 9. Clone 9 has been extremely useful in narrowing the regions of interest as it is the smallest fragment at approximately 16 Mb (Fig 8.2). Therefore, from my data the most likely candidate gene in the three defined regions is *KAT5*.

It is well established that HDACs function as repressors of gene expression by removing the negatively charged acetyl groups from the histone NH2 terminal domains resulting in a tighter chromatin structure. This type of chromatin remodelling makes it difficult for the gene to be transcribed (reviewed by Zhang and Dent, 2005). Therefore, a simple scenario in my study would be that the *hTERT* repressor sequence encodes a HDAC that could catalyse the deacetylation of histones on the *hTERT* regulatory sequence leading to *hTERT* repression. The *BRMS1* gene (encodes a HDAC) exists in very close proximity to my first candidate region, at a

distance of 0.44Mb away, but the *BRMS1* gene has already been eliminated as a possible *hTERT* repressor. The *KAT5* (K(lysine) acetyltransferase 5), encoding a HAT is the only chromatin remodelling gene in my three candidate regions. HATs generally function as activators of gene expression. Acetylation by HATs neutralizes the positive charge on the lysine residues resulting in an open chromatin structure that allows transcription factors to gain access to the regulatory sequence (reviewed by Zhang and Dent, 2005).

Several HATs have been identified since their first discovery in the mid-nineties in *Tetrahymena*. The A-type nuclear HATs are divided into four families based on their conserved sequence within the HAT domain. The *KAT5* gene encodes an A-type nuclear HAT that belongs to the MYST family of protein (reviewed in Marmorstein and Trievel, 2009). In humans, the MYST family consists of five proteins, namely TIP60, MOF, HBO1, MOZ and MORF, all of which contain a highly conserved MYST domain that is composed of an acetyl-CoA binding motif and a zinc finger (reviewed by Avvakumov and Cote, 2007; Yang, 2004). These members of the MYST family also contain various other structural subunits. Consequently, considerable diversity exists within this group of HATs (Yang, 2004).

A great deal of variation exists not only in the structural organisation of MYST proteins but also in their biological function. MYST proteins are involved in a number of nuclear processes such as DNA repair and apoptosis and they are also associated with cancer (reviewed by Avvakumov and Cote, 2007). There is evidence to suggest that the MYST family of HATs function as coregulators of transcription

i.e. TIP60 can act as transcriptional corepressor for *TEL* and *STAT3* and coactivator for androgen receptor and *c-Myc* (reviewed by Yang, 2004). From these published reports of structural and functional complexities in the MYST proteins, it is possible that the HAT protein encoded by the *KAT5* gene may function as a repressor when recruited to the *hTERT* promoter region to regulate transcription.

The credibility of the *KAT5* gene being an *hTERT* repressor comes from the breast cancer project that ran in parallel to my prostate study in our laboratory (Cuthbert et al., 1999). Chromosome 3 was identified as possessing an *hTERT* repressor sequence when transferred into a telomerized breast cancer cell line, 21NT/hTERT (Ducrest et al., 2001). A candidate region, flanked by a single STS marker D3S4066 (map position 47.14Mb) on the p-arm of chromosome 3, was identified as the possible location for the *hTERT* repressor sequence. Seven genes were listed in this region and on examination *SETD2* (SET domain containing protein 2) was the most likely candidate based on the biological function of these genes. Sarakbi et al, (2009), in collaboration with our group recently reported significantly lower levels of *SETD2* expression in breast carcinoma. Furthermore, the *SETD2* transcripts decreased with increasing tumour stage. Recently, using copy number analysis, we have demonstrated allele loss of *SETD2* in breast cancer cell lines, including intragenic microdeletions (Roberts et al., in preparation).

The *SETD2* gene encodes a histone methyltransferase that specifically methylates lysine-36 of histone H3, and methylation of this residue is associated with active chromatin (reviewed in Zhang and Dent, 2005). Precisely how SETD2 is involved in

regulating gene transcription is not known but recently Xie et al (2008) reported that the interaction of SETD2 with p53 could regulate the activity of p53 as a transcription factor. The authors demonstrated that overexpression of *SETD2* upregulated the transcript of p53. Alternatively, knock down of endogenous *SETD2* expression with RNA interference resulted in opposite effects. Therefore, their findings suggest that the interaction of SETD2 with p53 could selectively regulate its downstream effector genes.

The fact that both *KAT5* and *SETD2* genes are identified as potential *hTERT* transcriptional repressors in prostate and breast cancer cell lines respectively is highly interesting, as they are both histone modifying enzymes that transcriptionally regulate gene expression. Even though the exact mechanism of how the products of these genes regulate transcription is unknown, it has been proposed irrespectively that they both interact with p53 to regulate downstream effector genes (Sykes et al, 2006; Xie et al., 2008).

# Chapter 10

## General discussion

Lack of telomerase activity as a result of *hTERT* transcriptional repression in normal human cells suggests the existence of telomerase repressor genes that tightly regulate its activity. Conversely, elevated and dysregulated telomerase activity in cancerous and immortal cells indicates that there are defects in telomerase regulation, possibly resulting from the inactivation of *hTERT* transcriptional repressors. The *hTERT* transcriptional regulation is highly complex and still largely uncharacterized. Hence, identification of *hTERT* repressor(s) would be an essential step in understanding how telomerase is regulated in normal and cancerous cells. In the work described in this thesis, I have tried to locate an *hTERT* transcriptional repressor sequence that regulates telomerase activity in prostate cancer through genetic complementation studies.

Using the MMCT technique, genes located on different chromosomes have been implicated in repressing telomerase activity in tumour cells originating from a range of human tissues. Thus far, there are no reports of a gene that may be involved in repressing telomerase activity in prostate cancer cells. Furthermore, the literature survey performed at the start of this study provided no indication which, if any, chromosome may possibly carry a prostate cancer telomerase repressor gene (or genes). Therefore, I generated hybrids to screen the whole genome for repressive

function via the individual transfer (by MMCT) of normal human chromosomes into PC-3, a prostate cancer cell line. As a result of this work, the data generated has convincingly shown that chromosome 11 harbours a telomerase repressor sequence.

Previous reports demonstrated telomerase repression by MMCT of normal human chromosome 3 into breast, renal and cervical cancer cell lines (Cuthbert et al., 1999; Horikawa et al., 1998; Backsch et al., 2001 respectively). However, chromosome 3 had no repressive effect in the present study when it was transferred into PC-3 cell line. MMCT of chromosome 10 and chromosome 4 into PC-3 cells were also comparatively ineffective in repressing telomerase even though chromosome 10 was reported to have repressed telomerase activity in a hepatocellular carcinoma (Nishimoto et al., 2001) and chromosome 4 in a cervical carcinoma cell line (Backsch et al., 2001). These findings support the theory that not all the tumours have the same defective gene that regulates telomerase activity (Oshimura and Barret., 1997) and that telomerase repressors may be subject to inactivation in a tissue specific manner (Tanaka et al., 2005).

A more forward approach towards identifying telomerase repressor gene(s) located on a normal human chromosome is to measure *hTERT*, the catalytic subunit of telomerase. In order to identify repressor sequences that mediate *hTERT* transcription, I adopted the method reported by our close collaborators Ducrest et al (2001). They had previously shown a substantial number of *hTERT* RNA transcripts that had mainly retained intron 2, were present exclusively in telomerase positive cells and the level of this immature nuclear *hTERT* correlated with telomerase

activity. Furthermore, transfer of normal human chromosome 3 into 21NT, a breast cancer cell line completely abolished the expression of immature endogenous *hTERT* in cells that were ectopically expressing the *hTERT* gene. In my study, I have shown hybrid clones generated by the individual transfer of normal human chromosomes 3, 8, 10, 13 and 17 into PC-3 cells transfected with an *hTERT* cDNA plasmid construct did not effectively repress immature endogenous *hTERT*, in marked contrast to hybrid clones constructed by the transfer of human chromosome 11 into PC-3 cells that were ectopically expressing *hTERT*. These results further substantiate the involvement of genes located on chromosome 11 in telomerase repression.

To further localize the telomerase repressor region on chromosome 11, I employed the XMMCT technique to randomly fragment whole chromosome 11 prior to fusion into the recipient cells (Dowdy et al., 1990). The advantage of this technique is that it can generate a variety of hybrids in which the irradiated transferred chromosome can range from simple interstitial deletions to complex rearrangements resulting in a mixture of repressed and segregant hybrids and therefore I thought it would prove to be highly useful in fine mapping the region of interest. Perhaps an even more effective method of isolating the telomerase repressor region may have been a technique where the donor chromosome is truncated prior to transfer into the recipient cell line, as specific regions on the target chromosome can be removed using homologous DNA sequence (Abe et al., 2010). However, this technique requires previous knowledge of the regions that can be truncated on the target chromosome and since the *hTERT* repressor in my study could be located anywhere along chromosome 11, I could not utilize this technique.

Use of arm-specific chromosome paints identified the transferred chromosome 11 fragment as an independent entity in the highly repressed *hTERT* hybrids. To show that the *hTERT* repressive effect was due to the transferred chromosome 11 fragment, I made use of the HyTK selectable marker on the fragment. Reverse selection of the HyTK marker hence the transferred fragment from a randomly selected *hTERT* repressed hybrid was achieved by treating the cells with ganciclovir (GCV). I have convincingly demonstrated that the loss of the fragment from hybrids, shown by chromosome painting, was accompanied by elevated levels of endogenous *hTERT* expression comparable or exceeding those in the parent PC-3/hTERT cells, this confirmed the presence of an *hTERT* repressor sequence on the fragment.

Using a novel approach in an effort to locate the *hTERT* repressor, I generated heterospecific hybrids by transferring an *hTERT* repressed fragment from human PC-3/hTERT cells back into mouse A9 to allow fine mapping of the fragment with STS markers. From my STS map, I made use of the DNA variations observed in the resulting human: mouse A9 monochromosomal hybrids by performing another round of MMCT of carefully selected clones (fragments) back into PC-3/hTERT cells. DNA sequence, common to the fragments that repressed *hTERT* transcription led to the identification of three small regions on the q-arm of chromosome 11 as the location of *hTERT* repressor sequence. The fact that all the fragments tested for *hTERT* repression were positive was a disappointment. The only plausible explanation for this is that the *hTERT* repressor sequence by chance lies in very close proximity to the HyTK selectable marker.

In my study, the genes of interest on 11q include the *MEN1* and *EST* genes located at 11q13 and 11q23.3 respectively, both implicated in *hTERT* transcriptional repression (Lin and Elledge, 2003; Dwyer et al., 2007), were eliminated because they fall out of my three candidate regions. Dahiya et al (1997) reported four deleted regions on chromosome 11 in microdisected prostate carcinoma tissue. However, it is unlikely that any of the four regions are of interest as two of the deletions are on 11p and the other two are at 11q22 and 11q23-24, both of which also fall outside my candidate regions. There are no known tumour suppressor genes in my candidate regions; therefore possible candidate genes, based on their known or predicted functions as published on the NCBI website, were tested for differential expression in a panel that consisted of a mixture of hybrids that had repressed and non-repressed *hTERT* transcription. Thus far, I have been unable to identify a gene from my list of candidates that is expressed in the repressed but not in the non-repressed hybrids, in my panel.

Studies have shown that remodelling the chromatin structure at either the *hTERT* gene or its promoter may play an important role in its regulation (Szutorisz et al., 2003; Wang and Zhu, 2003). Concurrent work on the breast project in our laboratory has identified *SETD2*, a histone methyltransferase located on chromosome 3p as a strong candidate *hTERT* transcriptional repressor. Similarly, *KAT5*, a histone acetyltransferase in my first candidate region could therefore be a plausible candidate for repressing *hTERT* expression in a prostate cancer cell line.

In our laboratory, recent work performed on the breast cancer cell lines 21NT and its telomerized sub-line, 21NT/hTERT, transfected with *SETD2* plasmid construct, has shown an inverse relationship between *SETD2* and endogenous *hTERT* expression levels i.e. results have shown that hybrids that expressed high levels of *SETD2* expressed low endogenous *hTERT* levels (Roberts et al., in preparation). Further functional analyses are required to confirm the validity of *SETD2* involvement in *hTERT* repression. However, preliminary data from the breast project makes *KAT5* a very credible candidate in my study since the probability of locating histone modifying genes in relatively small regions of both chromosomes 11 and 3 in two completely separate mapping exercises in different cell lines would be remote. Identifying the *hTERT* repressor sequence in either case may provide important clues to the mechanism that regulates telomerase activity through transcriptional repression of *hTERT*.

## Summary and conclusion

I have performed a novel and an in depth study in an effort to identify and locate a telomerase repressor sequence involved in prostate cancer by using genetic complementation. Firstly, I identified chromosome 11 as possessing a telomerase repressor sequence by carrying out TRAP analysis on hybrids generated by the individual microcell transfers of normal human chromosomes into PC-3 cells. Secondly, I identified chromosome 11 as possessing an *hTERT* transcriptional repressor sequence, using the real-time qRT-PCR method, in hybrids generated by the transfer of normal human chromosomes into a PC-3 cell line that was ectopically expressing *hTERT* cDNA.

I have successfully narrowed down the region of interest on chromosome 11 by employing the XMMCT technique. I have convincingly demonstrated that reverse selection of chromosome 11 fragment with GCV treatment of a randomly selected gamma irradiated hybrid, that expressed very low levels of immature *endogenous hTERT*, generated clones in which the *hTERT* mRNA levels were comparable to (or higher than) those observed in the parent cell line. The loss of the fragment, resulting in elevated levels of *hTERT* transcription, strongly suggested the presence of the repressor sequence on the fragment.

The use of molecular and cytogenetic studies has positively identified three small regions on the q-arm of chromosome 11 as being strong candidates where the *hTERT* transcriptional repressor may be located. Our collaborators at ICR, Professor Colin Cooper's team, also independently identified two regions on the q-arm of

chromosome 11 as possessing the *hTERT* repressor sequence by using CGH analysis and microarray technology on my samples. Therefore, from this study I can conclude that the location of the *hTERT* repressor sequence involved in prostate cancer is on the q-arm of chromosome 11.

The NCBI website listed 50, 14 and 3 genes in my first, second and third candidate regions respectively. The most plausible candidate gene that I have identified, in my first region, is *KAT5*, a histone modifier. It's credibility as a potential *hTERT* transcriptional repressor is based on the recent identification of *SETD2*, (another histone modifying gene) as a probable repressor of *hTERT* transcription in breast cancer cell lines in our laboratory.

# Future work

### *KAT5*, a histone modifying gene- a candidate *hTERT* repressor

I have identified *KAT5*, a histone modifying gene belonging to the MYST family of HATs, as a plausible candidate for repressing *hTERT* transcription. If continuing the project, I would measure the expression levels of *KAT5* in my panel of *hTERT* repressed and non-repressed hybrid clones. If I observed differential expression of *KAT5* in the two sets of hybrids then the next step would be to examine the function of *KAT5* by cloning it into an appropriate expression vector and transfecting the gene into the PC-3/hTERT cell line. The expression levels of *KAT5* and endogenous *hTERT* would be measured by real-time qRT-PCR in the resulting hybrid clones. If a significant number of clones expressed high levels of *KAT5* and low levels of *hTERT* then *KAT5* could be investigated further for its involvement in *hTERT* repression. Further confirmation of *KAT5*'s involvement would be verified by knocking down its expression by using small interfering RNAs (siRNAs) that specifically target the mRNA transcript. Finally, expression and the structural integrity of the gene would then be investigated in prostate carcinoma tissue and cell lines.

### If *KAT5* is not an *hTERT* repressor

In the event of not observing repression of *hTERT* in the *KAT5* transfected clones, several different approaches could be employed to identify the *hTERT* repressor sequence. By using the NCBI database I could further investigate the genes in the three candidate regions that I identified (Table 9.1). There are over 50 genes in the first candidate region. Genes that may possibly be involved in telomerase regulation

i.e. cell cycle regulators; senescence and apoptosis etc would be investigated in the first phase of the study. If a selected gene is present on the human chromosome 11 fragment then the expression profile of that gene would be investigated in my panel of *hTERT* repressed and non-repressed clones.

**Transfer of a fragment from an *hTERT* non-repressed clone back into A9**

Another approach that could be adopted in locating the *hTERT* repressor sequence on the fragment would be to transfer the chromosome 11 fragment from one of the non-repressed *hTERT* clones in the human PC-3/hTERT cells back into A9 cells for fine structure mapping. Prior to transfer, I would need to know if the fragment exists as a discrete entity or if it had translocated onto another human chromosome. Transfer of a discrete fragment into A9 would favour fine mapping of the resulting clones with STS markers. Loss of markers (loci) in the clones compared with the original parent clone would together further pinpoint the probable location of the *hTERT* repressor. As a start, I have already used chromosome painting (FISH) to analyse all the non-repressed hybrids and selected a panel clones from which the fragment can be transferred into A9 cells.

**Single-nucleotide polymorphism (SNP) genotyping**

A recently developed technique called single-nucleotide polymorphism (SNP) is increasingly being used for identifying gene(s) associated with a variety of diseases. This technique makes use of the fact that SNPs are the most common form of sequence variation, approximately occurring in one base pair in a thousand when

alleles from two chromosomes are compared. SNPs are stable genetic markers and over a million SNPs are available for genotype studies in humans. It would be possible to use this technique to look for SNPs in our *hTERT* repressed and non-repressed clones. I would initially be interested in analysing only the long arm of chromosome 11 for SNP in the regions independently defined by us and Professor Cooper's team as the region where the *hTERT* repressor may be located. A number of SNP markers spanning the regions on the q-arm of chromosome 11 would be selected and any differences in the marker content between the two sets of samples may again further refine localization of the *hTERT* repressor sequence.

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# Appendix

**The MMCTs performed in this study and the nomenclature used for the generated hybrid clones**

Hybrids generated from the transfer of normal human chromosome 11 in telomerized PC-3 (PC-3/hTERT) cells were symbolized as PC-3/hTERTchr11, followed by the clone number, i.e. PC-3/hTERT/chr11.1, PC-3/hTERT/chr11.2 etc.

Hybrids generated by the transfer of fragmented (by exposing microcells containing whole chromosome 11 to 25G gamma radiation) chromosome 11 prior to fusion in PC-3/hTERT cells were symbolized as PC-3/hTERT/fchr11, followed by the clone number, i.e. PC-3/hTERT/fchr11.1, PC-3/hTERT/fchr11.2 etc.

Clone 1 from PC-3/hTERT/fchr11 (PC-3/hTERT/fchr11.1) hybrid was selected randomly to be treated with GCV.

Reverse selection of the fragment from PC-3/hTERT/fchr11.1 confirmed the presence of an *hTERT* transcriptional repressor sequence by quantitative real time RT-PCR and FISH. Therefore, PC-3/hTERT/fchr11.1 was investigated further.

The PC-3/hTERT/fchr11.1 hybrid was next transferred into A9, to generate hybrid clones for fine STS mapping. The resulting hybrids were symbolized as A9/fchr11.1 followed by the clone number, i.e. A9/fchr11.1.1, A9/fchr11.1.2, A9/fchr11.1.3 etc, in 22 clones that were collected.

Selected hybrids clones from the STS map i.e. A9/fchr11.1.4, A9/fchr11.1.9, A9/fchr11.1.13 etc were individually transferred back into PC-3/hTERT cell line.

The hybrids generated, for example from the transfer of A9/fchr11.1.9 were symbolized as PC-3/hTERT/fchr11.1.9 followed by the clone number, i.e. clone 1 was designated PC-3/hTERT/fchr11.1.9.1, clone 2 was designated PC-3/hTERT/fchr11.1.9.2 etc.

The *hTERT* repressed and non-repressed hybrid clones generated by the selected individual transfers of the fragments from mouse A9 cells back into human cells were used to construct a panel in which the genes of interest could be tested.