INVESTIGATING TELOMERASE REGULATION IN HUMAN BREAST CANCER CELLS: A SEARCH FOR TELOMERASE REPRESSOR SEQUENCES LOCALISED TO CHROMOSOME 3P

A thesis submitted for the degree of Doctor of Philosophy

by

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ABSTRACT

Cellular immortality is one of the ten hallmarks of human cancer and has been shown to be an essential prerequisite for malignant progression (Hanahan and Weinberg., 2011, Newbold *et al.,* 1982, Newbold and Overell., 1983). In contrast, normal human somatic cells proliferate for a limited number of population doublings before entering permanent growth arrest known as replicative senescence. This is thought to be due to the progressive shortening of telomeric sequences with each round of cell division. Over 90% of human tumours, but not the majority of human somatic cells, have been found to express telomerase activity (Kim *et al.,* 1994). The rate-limiting component of the human telomerase enzyme is the telomerase reverse transcriptase subunit, which is encoded by the *hTERT* gene. Transfection of *hTERT* cDNA into normal human fibroblasts and epithelial cells may sometimes be sufficient to confer cellular immortality (Newbold., 2005, Stampfer and Yaswen., 2002). Therefore, de-repression of *hTERT* and telomerase re-activation are thought to be critical events in human carcinogenesis and is the predominant mechanism by which cancer cells maintain their proliferative capacity.

Previously, our group has shown that introduction of a normal, intact copy of human chromosome 3 into the 21NT primary breast cancer cell line by microcell-mediated monochromosome transfer (MMCT), is associated with strong telomerase repression and induction of cell growth arrest within the majority of hybrid clones (Cuthbert *et al.,* 1999). Structural mapping of chromosome 3 within telomerase-positive revertent clones revealed two regions of deletion: 3p21.3-p22 and 3p12-p21.1, thought to harbour the putative telomerase repressor sequence(s). Subsequent studies showed that the chromosome 3p-encoded telomerase repressor sequence(s) mediates its function by means of transcriptional silencing of *hTERT,* in part, through chromatin remodelling of two sites within intron 2 of the *hTERT* gene (Ducrest *et al.,* 2001, Szutorisz *et al.,* 2003). Attempts to achieve positional cloning of *hTERT* repressor sequences on chromosome 3p identified two interesting candidates; the histone methyltransferase *SETD2* and an adjacent long non-coding RNA (lncRNA) sequence known as *FLJ/KIF9-AS1* (Dr. T. Roberts, unpublished data). Through MMCT-mediated introduction of intact chromosomes 3 and 17 into the 21NT cell line, I have demonstrated that at least two as yet unidentified telomerase repressor sequences (one located on each of these two normal chromosomes) may function to repress telomerase activity within the same breast cancer cell line, which suggests that multiple, independent telomerase regulatory pathways may be inactivated within the same cancer type. Furthermore, by examining the consequences of forced *SETD2* and *FLJ* expression within the 21NT cell line, together with siRNA-mediated knockdown of *SETD2* within a single telomerase-repressed 21NT-chromosome 3 hybrid, I have provided evidence to show that neither of these two candidate genes may function as a regulator of *hTERT* transcription. Through interrogation of relevant literature, a set of four candidate

telomerase regulatory genes (*BAP1, RASSF1A, PBRM1* and *PARP-3)* were selected for further investigation based on their location within the 3p21.1-p21.3 region together with their documented role in the epigenetic regulation of target gene expression. Using mammalian expression vectors containing candidate gene cDNA sequences, my colleague Dr. T. Roberts and I demonstrated that forced overexpression of *BAP1* and *PARP-3* within the 21NT cell line is associated with consistent, but not always sustained, repression of *hTERT* transcriptional activity and telomerase activity. It is therefore possible that at least two sequences may exist on chromosome 3p that function collectively to regulate *hTERT* expression within breast cancer cells.

Finally, using an *in vitro* model of human mammary epithelial cell (HMEC) immortalization, involving the targeted abrogation of two pathologically relevant genes, *p16* and *p53* to generate a series of variant clones at different stages of immortal transformation (developed by my colleague Dr. H. Yasaei), I have shown that single copy deletions on chromosome 3p are a frequent, clonal event, specifically associated with *hTERT* de-repression and immortal transformation. Subsequent high-density single nucleotide polymorphism (SNP) array analysis of immortal variants carried out by Dr. H. Yasaei, identified a minimal common region of deletion localized to 3p14.2-p22. Together, these findings provide additional evidence to show that chromosome 3p may harbour critical *hTERT* repressor sequences, that are lost as an early event during breast carcinogenesis.

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AUTHOR'S DECLARATION

All of the work presented in this thesis is my own unless stated. Any work of others is appropriately referenced.

Hannah Louise Linne, August 2015

ABBREVIATIONS

ADH	Atypical Ductal Hyperplasia
ALT	Alternative Lengthening of Telomeres
BAP1	BRCA1 associated protein-1
CADM2	Cell adhesion molecule 2
cDNA	Complementary DNA
CFU	Colony Forming Unit
CMV	Cytomegalovirus
CNV	Copy Number Variation
COSMIC	Catalogue of Somatic Mutations in Cancer
CSC	Cancer stem cell
Ct	Cycle threshold
DCIS	Ductal Carcinoma *in situ*
DNMT	DNA methyltransferase
DSB	Double strand break
EBV	Epstein Barr Virus
ER	Estrogen receptor
FISH	Fluorescence *in situ* hybridization
FLJ/ KIF9-AS1	KIF9 antisense RNA 1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HAT	Histone acetyltransferase OR Hypoxanthine aminopterin thymidine
HDAC	Histone deacetylase
HER2/Neu/ ERBB2	erb-b2 receptor tyrosine kinase 2
HMEC	Human mammary epithelial cell

HPV	Human papillomavirus
HR	Hormone receptor
HRT	Hormone replacement therapy
hTERT	Human telomerase reverse transcriptase
hTR	Human telomerase RNA component
Hytk	Hy, hygromycin phosphotransferase; tk, thymidine kinase
IDC	Invasive ductal carcinoma
ILC	Invasive lobular carcinoma
IS	*in situ*
LCIS	Lobular carcinoma *in situ*
LOH	Loss of heterozygosity
MMCT	Microcell-mediated monochromosome transfer
Neo	Neomycin
NRQ	Normalised relative quantities
OIS	Oncogene-induced senescence
PARP-3	Poly (ADP-ribose) polymerase family, member 3
PBRM1	Polybromo 1
PD	Population doubling
PgR	Progesterone receptor
PI3KCA	Phosphatidylinositol-4, 5-bisphosphate 3-kinase, catalytic subunit alpha
qRT-PCR	Quantitative real time-polymerase chain reaction
RAR-β	Retinoic acid receptor, beta
RASSF1	Ras association (RalGDS/AF-6) domain family member 1
SETD2	SET domain containing 2
SIPS	Stress-induced premature senescence

SUMF1	Sulfatase modifying factor 1
SV40-LT	Simian vacuolating virus 40- large T antigen
TDLU	Terminal ductal-lobular unit
TNM	Tumour/Node/Metastasis
TOP1	Topoisomerase (DNA) 1
TRAP	Telomere repeat amplification protocol
TRF	Telomere restriction fragment
UDH	Usual ductal hyperplasia
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta

CHAPTER 1

1 GENERAL INTRODUCTION

1.1 ANATOMY AND FUNCTION OF THE FEMALE BREAST

The female breast is made up of three main structures (i) milk-producing glands known as lobes and lobules, (ii) Lactiferous ducts, responsible for facilitating the transport of milk to the nipple, and (iii) connective tissue/stroma that surrounds the ducts and lobules, which contains blood, lymphatic vessels and adipose tissue (Figure 1.1) (Pocock and Richards, Human Physiology., 2006). The functional unit responsible for milk production is known as the terminal ductal-lobular unit (TDLU). From these terminal acinar units to the nipple, the ductal system consists of an outer basement membrane, a basal cell layer comprising of contractile myoepithelial cells, progenitor stem cells and an inner secretory luminal epithelial cell layer (Figure 1.1, Gusterson *et al.,* 2005).

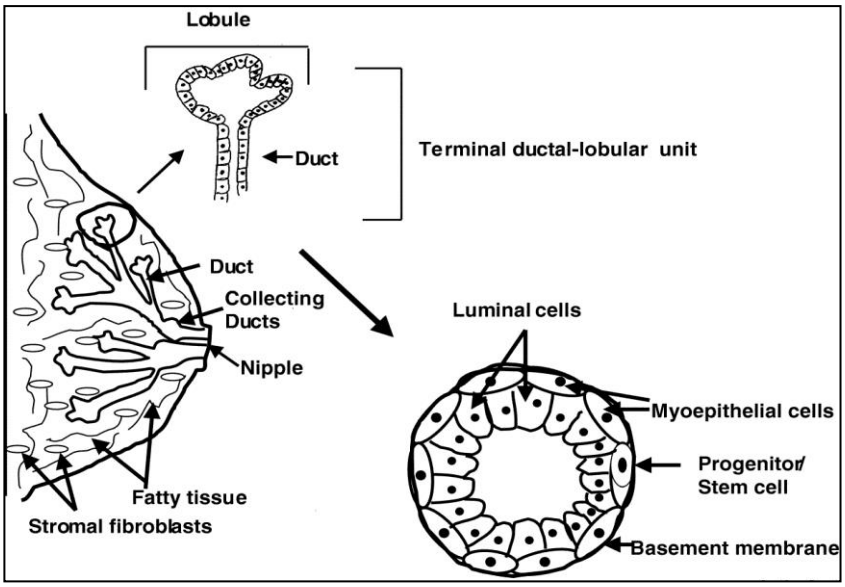


Figure 1.1 - *Female mammary gland structure including the terminal ductal-lobular unit (TDLU) and the multi-layer architecture of the mammary ductal system. Figure obtained from Dimri et al., (2005).*

The primary function of the female breast is to synthesize, secrete and eject milk, known as lactation (reviewed by Ellis and Mahadevan, 2013). During puberty, the production of oestrogen and progesterone causes ductal cell proliferation, which results in the development of a lobular network in order to prepare for lactation. The process of lactation begins during the third trimester of pregnancy, when human placental lactogen (hpL) is released from the placenta. Following gestation, the simultaneous production of prolactin from the anterior

pituitary gland and a reduction of circulating oestrogen, progesterone and hpL in the blood stream, initiates the process of lactation (Ellis and Mahadevan, 2013).

1.2 BREAST CANCER

1.2.1 SUBTYPES AND CLASSIFICATION

Breast cancer is characterized by the abnormal proliferation of cells within the breast ducts, lobules and rarely within the connective tissues (American Cancer Society, Types of Breast Cancers; www.cancer.org/acs/groups/cid/documents/webcontent/003090-pdf.pdf). It is the most common cancer diagnosed in females world-wide and comprises of a large heterogeneous group of diseases that exhibit different pathological, histological and molecular features (Stewart and Wild, World Cancer Report., 2014).

The majority of breast cancers arise from epithelial cells lining the breast ducts and lobes and are known as breast carcinomas or adenocarcinomas (epithelial cells of glandular tissues) (Stewart and Wild., 2014). The development of malignant tumours within connective tissues of the breast such as fat, muscle or blood vessels, are known as sarcomas. According to the recent World Health Organization (WHO) Classification of Tumours of the Breast (Lakhani *et al.,* 2012), there are over 20 different breast cancer subtypes.

1.2.1.1 *In Situ* Breast Lesions

The clonal proliferation of cells with malignant features within the terminal duct-lobular unit of the breast, which have not infiltrated the basement membrane, are known as pre-invasive or *in situ* breast lesions (Beckman *et al.,* 1997). These are sub-divided into lobular carcinoma *in situ* (LCIS) and ductal carcinoma *in situ* (DCIS) depending on their cytological and architectural features (Stewart and Wild., 2014).

DCIS represents around 25% of all diagnosed breast cancer cases and can present clinically as a palpable mass, nipple discharge or mammary Paget's disease (a rare breast cancer type that involves the nipple and the areola) (Virnig *et al.,* 2009, Tillman and Klimberg., 2000). DCIS encompasses a spectrum of diseases that are classified according to cytonuclear differentiation, architectural features and the presence or absence of comedonecrosis (central luminal inflammation with devitalized cells) (Burstein *et al.,* 2004, Fabri *et al.,* 2007). Low-grade DCIS lesions consist of well-differentiated cells with monomorphic nuclei and a clinging, cribiform, papillary or micropapillary growth pattern (Fabri *et al.,* 2007). Cells within intermediate-grade DCIS lesions demonstrate some architectural differentiation, with pleomorphic nuclei and can also display comedonecrosis. Around two-thirds of patients with low and intermediate grade DCIS present with multi-focal disease, with up to 1cm gaps between

individual foci (Burstein *et al.,* 2004). On the other hand, high-grade DCIS commonly presents as a continuous lesion containing cells with pleomorphic nuclei, necrosis and with a micro-papillary, solid or pseudo-cribriform (a particular histological tissue architecture characterised by the presence of multiple perforations) growth pattern. Women diagnosed with high-grade DCIS have an increased risk of developing invasive breast cancer recurrence following surgical removal of the lesion (lumpectomy) compared to women diagnosed with intermediate or low-grade DCIS (Kerlikowske *et al.,* 2003). The presence of comedonecrosis within DCIS lesions is associated with an increased risk of local recurrence following lumpectomy and poorer disease free survival compared to those without comedonecrosis (Poller *et al.,* 1995). The majority of low-grade DCIS lesions have been found to express oestrogen (ER) and progesterone receptors (PgR), whereas high-grade lesions frequently overexpress the epidermal growth factor (EGF) receptor tyrosine kinase 2 (ERBB2 or HER2/neu^{+}) (Burstein *et al.,* 2004). Breast tumours positive for hormone receptors are generally associated with a good response to endocrine therapy and a favourable prognosis (Osborne *et al.,* 1980). On the other hand, patients diagnosed with DCIS lesions overexpressing *HER2* are associated with worse disease prognosis (reviewed by Eccles., 2001).

DCIS lesions of the breast are commonly thought to represent an intermediate stage between atypical hyperplasia and invasive ductal carcinoma (IDC) (Figure 1.2, Stewart and Wild., 2014). High-grade DCIS lesions have been associated with disruption of myoepithelial cell layers and the basal lamina surrounding the breast lumen leading to stromal invasion, which is a typical feature of breast IDC (Figure 1.2, Damiani *et al.,* 1999). Betsill *et al.,* (1979), found that six out of ten (60%) untreated female patients initially diagnosed with low-grade DCIS, were found to develop invasive carcinoma within the same breast after an average of around 10 years. In further support of this model, molecular features of breast tumorigenesis present within invasive breast lesions can be observed within pre-invasive hyperplastic and DCIS lesions (Burstein *et al.,* 2004). According to the Catalogue of Somatic Mutations in Cancer database (COSMIC, Forbes *et al.,* 2015) the two most frequently mutated genes within atypical hyperplasia, DCIS and IDC lesions include the phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (*PIK3CA*) and tumour protein 53 (*TP53/p53*) gene, which demonstrates a clonal evolutionary relationship. From ductal hyperplasia without atypia to IDC of the breast, an increase in the number of chromosomal gains and losses have also been observed with increasing histological grade (Figure 1.2, Aubele *et al.,* 2000).

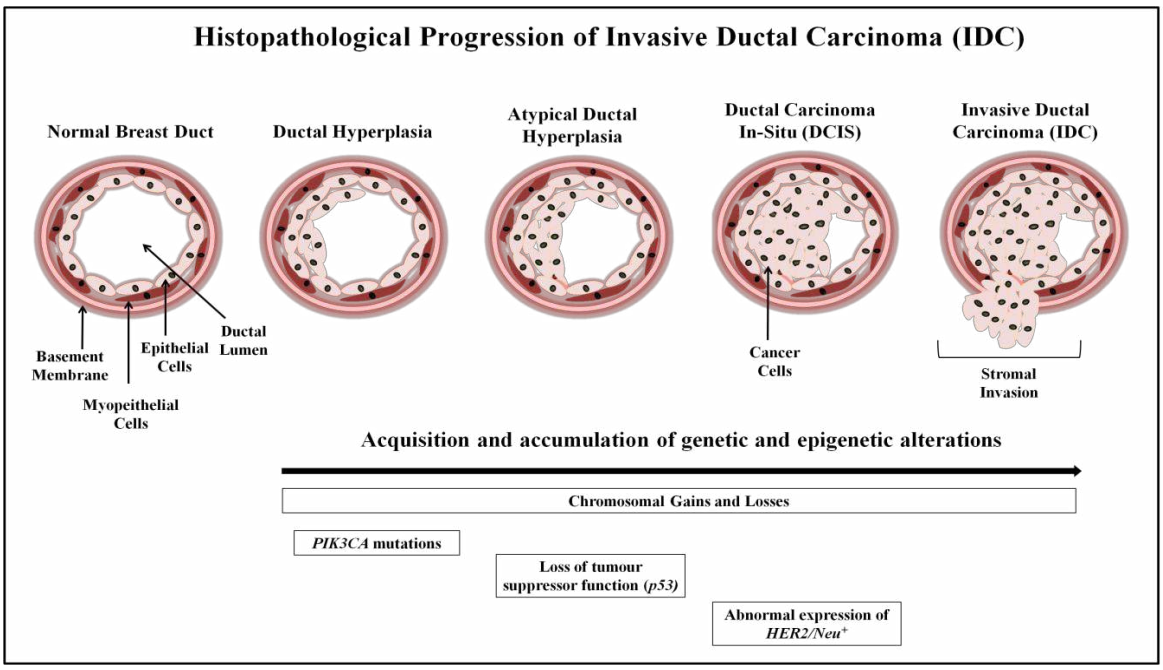


Figure 1.2 - *A cross-sectional view of the breast ductal lumen at various stages during the progression of invasive ductal carcinoma (IDC) and common molecular changes that occur. Adapted from Burstein et al., (2004).*

Lobular carcinoma *in situ* (LCIS) is a rare type of lobular neoplasia (LN) that is usually identified incidentally as a result of a breast biopsy carried out for another reason (Tillman and Klimberg., 2000). The majority of LCIS lesions cannot be detected using mammographic screening methods, and therefore the true incidence of LCIS within the general population is difficult to ascertain. However, institution-based and population-based studies indicate that up to 3.8% of the population may be affected by LCIS (reviewed by Oppong and King., 2012). The majority of LCIS lesions are multi-focal and are characterized by the abnormal proliferation of large, monomorphic cells between the epithelium and basement membrane of the terminal ducto-lobular unit and usually involves multiple lobular acini (Haagensen *et al.,* 1978, Beute *et al.,* 1991, Hussain and Cunnick., 2011). A distinguishing feature of LCIS lesions is complete loss of transmembrane glycoprotein E-cadherin expression, which is present within DCIS and IDC tissue samples (Acs et al 2001). Unlike DCIS, LCIS lesions do not generally exhibit features of necrosis and around 50% of patients have been found to present with bilateral disease (Beute *et al.,* 1991). Furthermore, LCIS is not considered to be an obligate pre-cancerous lesion, but is associated with around a 7% increased risk of developing invasive cancer in either breast, within ten years of diagnosis (Chuba *et al.,* 2005). Patients diagnosed with LCIS rarely require further intervention with chemo-preventative treatments or surgery, but are offered lifelong surveillance by regular mammographic screening and breast examinations (Oppong and King., 2012).

1.2.1.2 Invasive Breast Lesions

Invasive breast carcinoma (IBC) is the most common type of breast cancer and is characterized by the stromal infiltration of malignant epithelial cells and an increased propensity for metastasis to distant sites (Figure 1.2, Fabri *et al.,* 2007). The largest subset of IBC is infiltrating/invasive ductal carcinoma (IDC) 'no special type/not otherwise specified' (NST/NOS), which represents 40-75% of all breast cancer cases (Table 1.1). This heterogeneous group of tumours vary in size and architecture and constituent cells present with no specific morphological features (Stewart and Wild., 2014). The remaining subset of IBC lesions consist of 'special type' carcinomas, which exhibit different histological types and prognoses and are grouped according to morphological features such as lobular, mucinous, medullary, papillary, apocrine and metaplastic (Table 1.1). Infiltrating lobular carcinoma (ILC) is the second most frequent IBC lesion and accounts for 5-15% of all breast cancer cases. Unlike IDC, ILC is frequently multifocal and bilateral and is not commonly associated with the formation of palpable tumours that can be easily detected by mammographic screening methods (Helvie *et al.,* 1993, Lesser *et al.,* 1982). In addition, lobular and mixed ductal/lobular breast lesions are more likely to be hormone receptor positive (ER and PR) than IDC lesions. However, lobular and ductal/lobular lesions are more likely to be diagnosed at an advanced stage and are therefore not associated with a more favourable clinical outcome compared with breast IDC (Li *et al.,* 2005, Arpino *et al.,* 2004). Using Surveillance, Epidemiology and End Results Program data from over 135,000 patients diagnosed with invasive breast cancer between 1992 and 2001, Li *et al.,* (2005) demonstrated that different histological breast cancer subtypes exhibit a marked variation in clinical features and prognoses. Unlike mucinous, papillary and tubular IBC lesions, inflammatory and medullary carcinoma cases were associated with more aggressive clinical features, such as high tumour grade and absence of hormone receptors. Other ER negative tumour subtypes include adenoid cystic and metaplastic carcinomas (Weigelt *et al.,* 2008). However, many 'special type' IBC lesions including lobular, mucinous, medullary, papillary and tubular carcinomas have been associated with an increased survival rate compared to IDC lesions (Li *et al.,* 2003c).

Table 1.1 - The incidence and histopathological features of invasive breast lesions

Type	Growth Pattern	Incidence	Features	Reference(s)
Invasive Ductal Carcinoma No Special Type (NST)/Not Otherwise Specified (NOS)	Mixed	40-75%	IDC NOS accompanied by another morphological subtype e.g. lobular.	Fabri *et al.,* 2007
	Pleomorphic		Usually high-grade, characterized by pleomorphic and giant cells.	Silver and Tavassoli., 2000
	With osteoclastic giant cells		Characterized by pleomorphic malignant giant cells of epithelial cell origin.	Gupta *et al.,* 1996
Special Type	Acinic cell carcinoma	-	Clinicopathological features of acinic cell differentiation seen in salivary gland tumours.	Damiani *et al.,* 2000
	Adenoid cystic carcinoma	0.1%	Typically observed in salivary glands. Present as irregular, heterogeneous masses. Associated with good prognosis and rare metastases.	Glazebrook *et al.,* 2010
	Apocrine carcinoma	0.3-4%	Cells exhibit features of apocrine cells; eosinophillic cytoplasm with prominent nucleoli.	Frable and Kay., 1968
	Glycogen-rich clear cell carcinoma	1-3%	Cells have clear cytoplasm due to the presence of large quantities of particulate glycogen.	Hull *et al.,* 1981
	Inflammatory carcinoma	1-2%	Characterized by a large mass with rapid growth and lymphatic infiltration. Usually associated with poor prognosis.	Yasumura *et al.,* 1997
	Invasive cribiform carcinoma	0.8-3.5%	Small tumour cells with low or intermediate degree of nuclear pleomorphism. Associated with a good prognosis.	Fabri *et al.,* 2007
	Invasive lobular carcinoma (ILC)	5-15%	Small, non-cohesive tumour cells with oval nuclei and a thin layer of cytoplasm.	Fabri *et al.,* 2007
	Lipid-rich carcinoma	-	Around 90% of tumour cells contain cytoplasmic lipids.	Dina and Eusebi., 1997
	Medullary carcinoma	1-7%	Characterized by poorly differentiated cells with pleomorphic nuclei and lymphoplasmacytic stromal infiltrate.	Fabri *et al.,* 2007
	Metaplastic carcinoma	<1%	Heterogeneous mixture of non-glandular, spindle, mesenchymal and squamous cell differentiation.	Greenberg *et al.,* 2004
	Micropapillary carcinoma	<2%	Characterized by mircopapillae separated by fibrocollagenous/fibrovascular stroma.	Siriaunkgul and Tavassoli., 1993
	Mucinous carcinoma	2%	Uniform round tumour cells with eosinophillic cytoplasm, surrounded by mucin.	Fabri *et al.,* 2007
	Neuroendocrine carcinoma	2-5%	Tumour cells express neuroendocrine markers. Associated with poor prognosis	Wei *et al.,* 2010
	Papillary carcinoma	1-2%	Tumours characterized by fibrovascular stalks and a myoepithelial cell layer. Usually associated with IDC.	Fabri *et al.,* 2007
	Tubular Carcinoma	<2%	Small, regular cancer cells with some nuclear pleomorphism. Usually associated with DCIS and LCIS.	Patchefsky *et al.,* 1977

Incidence rates obtained from Fabri et al., (2007) and Wei et al., (2010).

1.2.2 DISEASE CLASSIFICATION

Breast cancer is a heterogeneous disease that displays a wide variety of clinical, histological and molecular features. Therefore, routine clinical management of breast cancer is dependent on a variety of histopathological features including cytological appearance (grade), tumour size, presence/absence of hormone receptors, HER2/neu^{+} status and anatomical features such as lymph node stage and presence/absence of metastases (Stewart and Wild., 2014). Some of these factors alone or in combination have been shown to be valuable prognostic determinants and can be used to facilitate the selection of suitable therapeutic options and assess treatment progress.

1.2.2.1 Histopathological and Biological

The most widely used histological grading system employed for the clinical management of breast cancer patients in Europe is the semi-quantitative Elson-Ellis modification of the Scarff-Bloom-Richardson grading system (Bloom and Richardson., 1957, Elston and Ellis., 1991). This involves the numerical scoring (1-3) of three cytological parameters: (i) architectural differentiation (tubule and gland formation), (ii) nuclear pleomorphism and (iii) proliferation (mitoses). Overall tumour grade is calculated by adding each score together. Tumours are then classified as grade 1 (3-5, low), grade 2 (6-7, intermediate) or grade 3 (8-9, high). This method of tumour grading was shown to be a useful indicator of patient survival in a retrospective study of 630 breast cancer patients, with a high tumour grade associated with worse patient prognosis (Sundquist *et al.,* 1999).

Tumour histologic grade is also combined with pathological primary tumour size and lymph node stage (the number of tumour cell-positive nodes and degree of nodal involvement), to calculate the Nottingham Prognostic Index (NPI, Galea *et al.,* 1992). As with histologic grade alone, each parameter is assigned an appropriate value and entered into the formula: NPI = [0.2 x tumour size(cm)] + lymph node stage (1 -tumour free, 2 -up to three auxiliary nodes, or 3-four or more nodes/apical node involvement) + grade. The score is positively correlated with patient survival. This classification system combines time-dependent (lymph node and tumour size) and biological (tumour grade) factors and has been shown to provide a reliable estimation of patient prognosis in a prospective study of 320 patients diagnosed with primary breast cancer (Galea *et al.,* 1992, Todd *et al.,* 1985).

The Union for International Cancer Control (UICC) Tumour/Node/Metastasis (TNM) classification system for malignant tumours is the current global standard for breast cancer staging (Sobin *et al.,* 2011). As shown in Table 1.2, this system involves the clinical and histopathological assessment of the anatomic stage of disease by grading three main components: (i) extent of primary tumour (T), (ii) presence/absence and extent of lymph node metastases (N) and (iii) presence/absence of distant metastases (M). TNM staging is used to

provide an indication of patient prognosis, establish suitable treatment options, evaluate the effects of treatment and provide an internationally recognised system that enables the exchange of information between different treatment centres (Sobin *et al*., 2011).

Table 1.2 - Current Union for International Cancer Control (UICC) criteria for clinical and pathological Tumour/Node/Metastasis (TNM) classification system of breast tumours

Classification	Subgroup	Clinical (pre-treatment)	Pathological (post-surgery)
Tumour	TX	Primary tumour cannot be assessed	
	T0	No evidence of primary tumour	
	Tis	DCIS/LCIS/Paget's disease	
	T1	Tumour is <2cm in greatest dimensions; T1mi (Microinvasion, ≤0.1cm), T1a (>0.1 to 0.5cm), T1b (>0.5 to 1cm), T1c (>1 to 2cm).	
	T2	Tumour is >2 to 5cm in greatest dimension	
	T3	Tumour is >5cm in greatest dimension	
	T4	T4a (extension to chest wall), T4b (ulceration, ipsilateral satellite skin nodules, skin oedema), T4c (both 4a and 4b), T4d (inflammatory carcinoma).	
Node	NX	Cannot be assessed	
	N0	No lymph node metastases	
	N1	Metastasis in movable ipsilateral level 1, 2 ALN	pN1mi (micrometastasis >0.2 to 2mm in greatest dimensions or >200 cells), pN1a (1-3 ALN including one >2mm in greatest dimesions), pN1b (IMLN), pN1c (1-3 ALN and IMLN).
	N2	N2a (ALN fixed or matted to each other or to another structure), N2b (IMLN and in absence of ALN).	N2a (4-9 ALN including at least one >2mm), N2b (IMLN in absence of ALN).
	N3	N3a (ILN), N3b (IMLN and ALN), N3c (SLN)	N3a (10 or more ALN or ILN), N3b (ipsilateral IMLN and ALN/more than 3 ALN and IMLN).
Metastasis	M0	No distant metastases	N/A
	M1	Distant metastases	Distant metastases confirmed microscopically.

Classification criteria obtained from Sobin et al., (2011). Abbreviations: ALN (auxiliary lymph node), IMLN (internal mammary lymph node), ILN (infraclavicular lymph node), SLN (supraclavicular lymph node).

In addition to histopathological classification, invasive and *in situ* breast tumours are also routinely assessed by immunohistochemical (IHC) and *in situ* hybridization (ISH) for the presence/absence of hormone receptors (ER and PgR) and the over-expression of the epidermal

growth factor receptor *HER2* (Hammond *et al.,* 2010, Wolff *et al.,* 2007). According to the results of a systematic literature review carried out by an expert panel from the American Society for Clinical Oncology (ASCO) and College of American Pathologists (CAP), the presence of both ER and PgR within invasive breast lesions is positively associated with increased overall survival, time to disease recurrence and response to endocrine therapy (Hammond *et al.,* 2010). Over 70% of breast cancers are hormone receptor positive (Barnes *et al.,* 1996). Studies have shown that oestrogen stimulates the growth rate of hormone-responsive breast cancer cells *in vitro*, whereas anti-estrogens can have the opposite effect on cell growth (Katzenellenbogen *et al.,* 1987, Atiken and Lippman., 1985). Therefore, hormone receptor status is a useful prognostic indicator and can be used to assess patient suitability for endocrine therapy. On the other hand, *HER2* is amplified in approximately 20% of human breast cancers (Wolff *et al.,* 2007). In 1987, Slamon *et al.,* demonstrated a strong association between an increase in *HER2* gene copies and lymph node status, tumour size, reduced time to disease relapse and decreased overall survival. Aside from a prognostic indicator, breast tumours over-expressing *HER2* are also associated with resistance or sensitivity to some endocrine therapies and chemotherapeutic agents (Konecny *et al.,* 2003, Pritchard *et al.,* 2006). Furthermore, a greater increase in time to disease progression has been observed within patients with *HER2*-overexpressing tumours than those without, following treatment with trastuzumab (Herceptin) in conjunction with the chemotherapeutic agent docetaxel and platinum salts (Pegram *et al.,* 2004). Therefore *HER2* receptor status is a valuable factor for determining effective patient treatment plans.

1.2.2.2 Molecular Profiling

As determined by the most recent collaborative analysis of critical gaps in current breast cancer research (Eccles *et al.,* 2013), our understanding of the molecular drivers behind breast cancer subtypes and tumour heterogeneity remains incomplete. Independent studies have demonstrated the reliability of histopathological and biological classification systems in estimating disease prognosis (see above). However, it is thought that these methods may not be sufficient to define or understand the vast array of clinical behaviours of breast cancer (Rakha *et al.,* 2010). For example, breast tumours are known to display both inter and intra-tumour heterogeneity, which may not be taken into account using current histological grading methods (Stewart and Wild., 2014). Patients with similar breast tumour grades may have different clinical outcomes or responses to therapy and concerns have been raised about the inter-observer variability of histopathological assessments (Rakha *et al.,* 2010). Over the last fifteen years, microarray, array comparative genomic hybridization (aCGH) and next generation sequencing (NGS) technologies have facilitated the characterization of transcriptomic and genomic landscapes of breast cancer and have enabled the identification of molecular subtypes (Sorlie *et al.,* 2001, Chin *et al.,* 2007, Stephens *et al.,* 2012). It is thought that, by defining new

molecular classification systems we can increase our understanding of the genetic drivers of breast carcinogenesis, identify new therapeutic targets and move towards the development of more personalized treatment plans (Dawson *et al.,* 2013).

Through microarray analysis and hierarchical clustering of gene expression patterns within breast tumour samples, five distinct intrinsic molecular subtypes comprising ER-positive (luminal A and B) and ER-negative (basal-like, *HER2*-overexpressing, and normal breast-like) groups have been identified (Perou *et al.,* 2000, Sorlie *et al.,* 2001, Sorlie *et al.,* 2003). A subsequent study demonstrated that early-stage breast tumours can also be classified into luminal and basal-like subtypes based on their relative gene expression profiles. These studies provide evidence to show that breast tumours comprise distinct biological entities that may originate from different epithelial cell types (luminal or basal) (Sorlie *et al.,* 2006). Biological pathway analyses revealed that basal-like breast tumours exhibit increased expression of genes involved in the G1/S cell cycle transition, cell proliferation and differentiation, and *p21*-mediated pathways, whereas luminal A subtypes display higher expression levels of genes involved in ER-signalling and fatty acid metabolism. These molecular subtypes have also been found to exhibit significant differences in clinical outcome (Sorlie *et al.,* 2001*,* 2003). Both basal-like and *HER2*-overexpressing tumours have been associated with shorter relapse-free and overall survival compared with other subtypes. Other studies have shown that around 11% of all breast cancers and 25% of IDC NOS tumours fall within the basal-like group, which consist of mainly 'triple-negative' (ER/PgR/*HER2* negative) tumour cells (Dent *et al.,* 2007, Banerjee *et al.,* 2006). This particular group was associated with increased likelihood of distant recurrence and death within five years of diagnosis compared with other breast cancer types.

In 2012, The Cancer Genome Atlas (TCGA) network published results from a comprehensive genome, transcriptome and proteome analysis of primary breast tumours using DNA methylation, gene copy number, exome sequencing, mRNA, miRNA and reverse-phase protein arrays (Cancer Genome Atlas Network., 2012). In concordance with the mRNA-subtypes defined by Sortie *et al.,* (2001, 2003), analysis of combined platforms using a multi-platform data matrix, demonstrated that breast cancers can be divided into four molecular subtypes: luminal A, luminal B, basal-like and *HER2*-overexpressing. Over 10% of all breast cancers harboured somatic mutations in *p53, PIK3CA* or *GATA3.* However, gene mutations, protein expression and copy number alterations common to each subtype were also observed, which provides a greater understanding of the genomic drivers underlying the main breast cancer subtypes and identifies therapeutic drug targets. Luminal breast tumours were found to harbour the highest frequency of *PIK3CA* mutations, therefore this group may respond to inhibitors of active *PIK3CA* or signalling pathway members. Basal-like breast tumours were found to exhibit similar genomic aberrations as serous ovarian cancer including mutations in *p53, BRCA1, BRCA2, ATM* and amplification of *MYC.*

1.2.3 EPIDEMIOLOGY AND RISK FACTORS

1.2.3.1 Global Variation in Breast Cancer Incidence and Mortality

In 2012 around 1.7 million new breast cancer cases were diagnosed and an estimated 521,817 women died of breast cancer globally (International Agency for Research on Cancer, IARC, Ferlay *et al.,* 2013). Breast cancer is the second most frequently diagnosed cancer in the world and is the most common cancer diagnosed in women (25% of all female cancers). It is also ranked as the fifth cause of global cancer-related death. For women in less developed regions, breast cancer is the most common cause of cancer-related death but falls second to lung cancer for women living in more developed regions (IARC, Ferlay *et al.,* 2013).

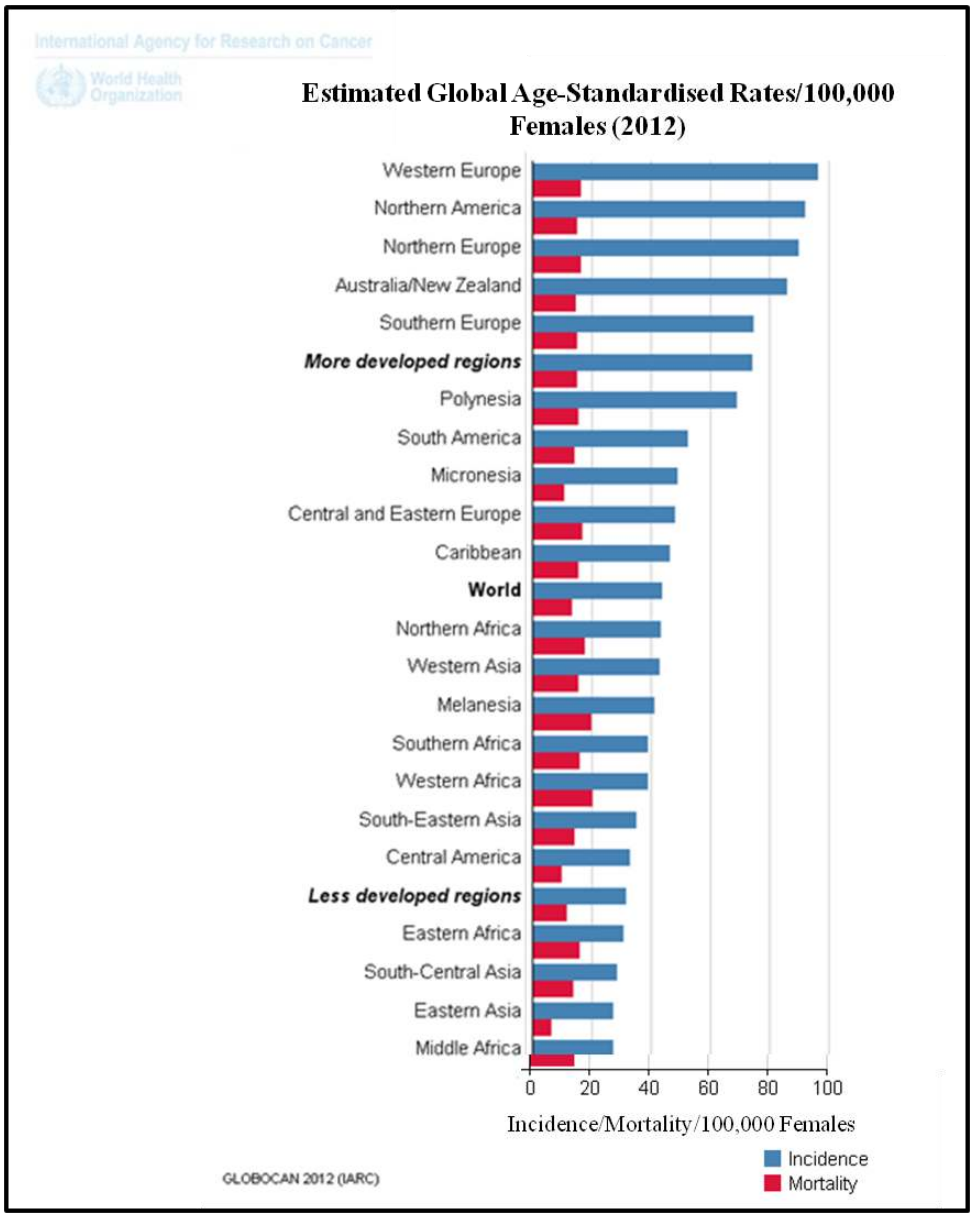


Figure 1.3 - *Age-standardised incidence and mortality rates/100,000 females of breast cancer worldwide in 2012. Data obtained from GLOBOCAN (Ferlay et al., 2013). Image adapted from http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx*

The global age-standardized incidence rates for female breast cancer were 43.3/100,000 female population in 2012 (IARC, Ferlay *et al.,* 2013). There is an approximate 4-fold variation in female breast cancer incidence rates between different regions worldwide, with the highest incidence rates observed within Europe and northern America, and the lowest rates occurring in eastern and central Asia and Africa (Figure 1.3). The highest breast cancer incidence rates worldwide occur in Belgium (111.9/100,000 females), Denmark (105) and France (104.5), while Bhutan (4.6), Lesotho (9), and Mongolia (9.4) have the lowest incidence rates (IARC, Ferlay *et al.,* 2013). The large variation in global breast cancer incidence rates is reportedly due to differences in hormonal and reproductive factors together with the availability of mammographic screening services (Jemal *et al.,* 2010, 2011). Reproductive factors that have been associated with increased breast cancer incidence rates in more developed regions include null parity, late child-bearing, early menarche, or exposure to external estrogens through the use of menopausal hormone therapy and oral contraceptives. The binding of estrodiol to oestrogen receptors is thought to promote breast carcinogenesis by initiating mitogenic signalling pathways leading to increased mammary cell proliferation (Cavalieri and Rogan., 2006). It is also thought that cellular metabolism of estrodiol results in the production of electrophilic metabolites, which react with genomic DNA causing the formation of adducts.

International variations in female breast cancer incidence rates are also thought to be due to differences in diet and alcohol consumption (Althius *et al.,* 2005). A meta-analysis of cohort and case-control studies revealed that a positive correlation exists between alcohol consumption and breast cancer risk (Longnecker., 1993). An increase in plasma estrogens such as estradiol and estrone sulphate in pre- and post-menopausal women following alcohol consumption is thought to be responsible for the increased risk of breast cancer development (Hankinson *et al.,* 1995, Willet., 2001). A positive correlation between global economic development and fat consumption has been observed (Willet., 2001). An association between per capita fat intake and mortality from breast cancer has been observed, which may explain why more developed regions have the highest breast cancer incidence rates (Armstrong and Doll., 1975). However, a review of prospective studies has revealed that high energy intake, but not fat consumption alone, is associated with increased breast cancer risk (Willet., 2001). High energy intake is thought to contribute to early onset of menstruation and increased attained height and weight, which have also been found to be positively correlated with increased breast cancer risk. A high body mass index (BMI) is associated with elevated serum estrodiol, which is thought to promote breast carcinogenesis (Lamar *et al.,* 2003). Interestingly, high body weight has only been associated with increased breast cancer risk in post-menopausal women but an inverse relationship exists between body weight and breast cancer risk in pre-menopausal women (Willet., 2001). This is thought to be due to the observation that heavier pre-menopausal

women are more likely to have irregular menstrual cycles, which confers less exposure to endogenous estrogens (Rich-Edwards *et al.,* 1994).

Female breast cancer incidence rates in European countries, Australia and the USA have increased since late 1980 and have remained stable or decreased since early 2000 (Jemal *et al.,* 2010, 2011). The observed decline in incidence rates within these countries since the beginning of the millennia is reportedly due to a reduction in the use of menopausal hormone therapy known as hormone replacement therapy (HRT) (Parkin., 2009, Cronin *et al.,* 2008). Elevated serum levels of oestrogen and androgen sex hormones within post-menopausal women, have been associated with an increased risk in breast cancer development (Lamar *et al.,* 2003). Randomized studies have shown that use of combined oestrogen and progesterone hormone therapy in post-menopausal women is associated with a significant increase in breast cancer risk, which also increases with duration of treatment (reviewed by Cogliano *et al.,* 2005). Since 2002, the use of HRT in the USA alone was reduced by 50%, which is thought to be responsible for the observed decline in breast cancer incidence rates.

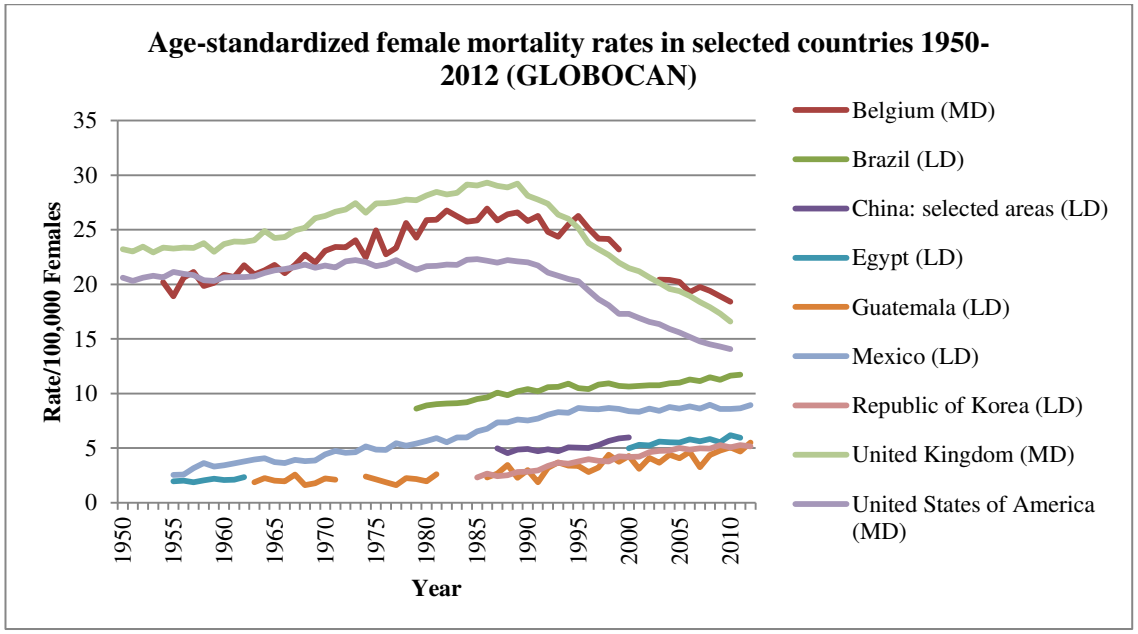


Figure 1.4 - *Age-standardized mortality rates (per 100,000 females) for women in more developed (MD) and less developed (LD) regions between 1950-2012. Data obtained from IARC, GLOBOCAN cancer mortality database (http://www-dep.iarc.fr/WHOdb/WHOdb.htm).*

Global, age-standardized female mortality rates for breast cancer in 2012 were 12.9/100,000 females (IARC, Ferlay *et al.,* 2013). As shown in Figure 1.3, global mortality rates vary much less than incidence rates, which is due to improved survival in more developed regions. Regions within Melanesia and western Africa including Fiji (28.4/100,000 women), Nigeria (25.9) and the Solomon Islands (24.4) were found to display the highest global female breast cancer mortality rates. The lowest rates of breast cancer-associated death in women

worldwide were observed in eastern Asia and central America including Bhutan (1.8/100,000 females), Guatemala (5) and China (5.4). In contrast to the USA and northern European countries, breast cancer incidence and mortality rates within Asian, African and Central American countries such as China, Korea, Egypt, Mexico and Guatemala, have been rising (Figure 1.4, Jemal *et al.,* 2011). The observed reduction in mortality rates in the USA and northern Europe since the late 1980's is likely to be due to earlier detection and improved treatment strategies. It is thought that rising incidence rates within Asian, African and central American countries may be due to the adoption of traits associated with 'westernization' such as physical inactivity, obesity and cultural changes such as late child-bearing or having fewer children. An obesity pandemic is reportedly spreading from the USA and Europe to urban areas of less-developed countries, which are unable to cope with the rise of associated non-communicable diseases due to inadequate health-care services (Prentice., 2005). These factors, together with the lack of early detection services and treatment strategies is thought to underlie the increasing breast cancer mortality rates within less developed countries (Figure 1.4, Jemal., 2010).

Similarly to incidence rates, a large variation in global female breast cancer survival rates have been observed, with five-year relative survival rates of around 12% within African regions to around 90% in the USA and UK (Youlden *et al.,* 2012). Disparities in global survival rates are largely thought to be due to differences in access to treatment services and early detection. Over the last forty years, five-year breast cancer survival rates in the UK have increased from around 50% in 1970 to over 80% in 2011 (CRUK). Similar trends have been observed in more developed countries due to the introduction of mammographic screening programs and the use of adjuvant therapies (Youlden *et al.,* 2012). Breast cancer survival is dependent on both age and stage of disease at diagnosis (CRUK). According to five-year net survival rates of women in the UK between 2007-2011, women aged 40-69 years have the best survival rates of around 90%, compared with 84.8% and 64.1% for women aged 15-39 and 80-99 respectively (CRUK). As shown in Figure 1.5, women diagnosed with localized disease or regional lymph node metastases have the highest five-year relative survival rates compared with patients diagnosed with distant metastases or unknown tumour stage (Survival, Epidemiology, and End Results Program, SEER).

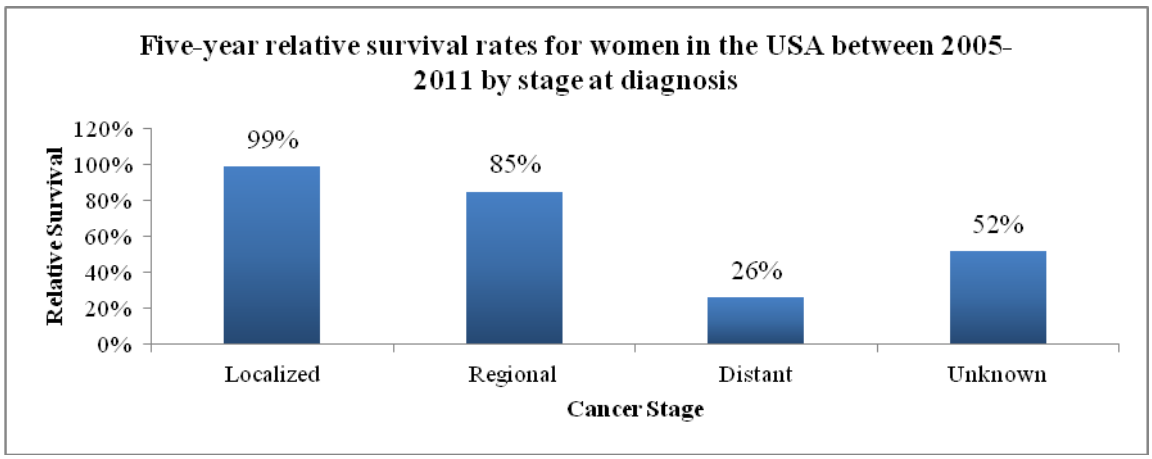


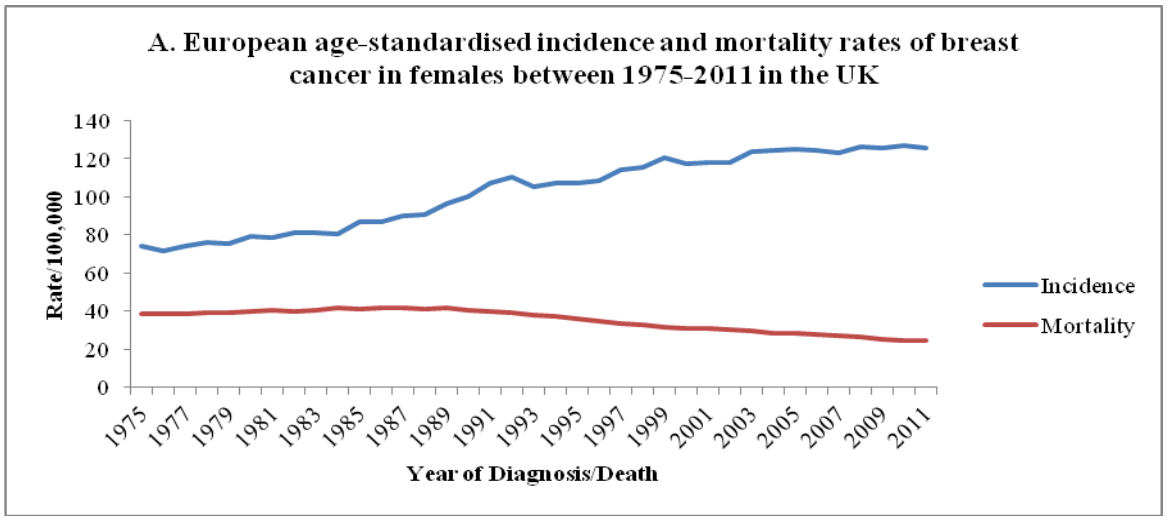
Figure 1.5 - *Breast cancer five-year relative survival rates for women in the USA between 2005-2011 according to stage of disease at diagnosis. Data obtained from Surveillance, Epidemiology, and End Results Program (SEER) of the National Cancer Institute (http://seer.cancer.gov/statfacts/html/breast.html).*

In contrast to females, male breast cancer constitutes less than 1% of all cancers diagnosed in men and incidence rates are around 1-1.2/100,000 male population in Western countries (Rizzolo *et al.,* 2013, Speirs and Shaaban., 2008). Between 1988-2002, the highest incidence rates of male breast cancer were observed in Israel (1.24/100,000 males), the Philippines (0.99), Italy (0.8) and France (0.75) (Ly *et al.,* 2012). Similarly to female breast cancer, regions within eastern Asia such as Thailand (0.14), Japan (0.17) and Singapore (0.19) were found to display the lowest male breast cancer incidence rates within the same time-period. The high incidence of male breast cancer in Israel is thought to be due to the increased frequency of mutations within breast cancer susceptibility genes *BRCA1* and *BRCA2* observed within Ashkenazi Jewish populations (Struewing *et al.,* 1997).

Similarly to females, male age-standardized breast cancer incidence rates have increased from the early 1970's in many regions worldwide, but have reached a plateau since the beginning of the millennia (Anderson *et al.,* 2010, Ly *et al.,* 2012). The observed similarity to female incidence trends could suggest that men and women may be exposed to common breast cancer risk factors. For example, obesity and physical inactivity have been associated with an increased risk of breast cancer for both males and females (Ly *et al.,* 2012). However, male breast cancer incidence rates in European countries have remained fairly stable since the early 1970s (Miao *et al.,* 2011). An example of this can be seen in Figure 1.6A and B, which shows a 72% increase in age-standardized female breast cancer incidence rates in the UK from 1975-2011, but a relatively stable male breast cancer incidence rate of less than 1/100,000 male population within the same time-period (Cancer Research UK, CRUK). Therefore, sex-specific risk factors such as hormonal and reproductive factors, are responsible for the observed differences in breast cancer incidence trends for males and females. It is also thought that these

differences may also reflect changes in the availability of mammographic screening services for women, especially within more developed regions (Ly *et al.,* 2012). A review of SEER population data revealed that both male and female breast cancer mortality rates in the USA have declined between 1973 and 2005. As shown in Figure 1.6A and B, a similar trend was observed in the UK. It is thought that improved adjuvant systemic therapy such as Tamoxifen, may have contributed to the decline in male breast cancer mortality rates within these countries (Anderson *et al.,* 2010).

Breast cancer incidence in men and women is strongly associated with age, with around 24% and 36% of breast cancer cases diagnosed in females and males aged over 75 years between 2009-2011 in the UK respectively (Figure 1.4C and D, CRUK). For men, age-specific incidence rates increase steadily from the age of 35, whereas incidence rates for women increase from the age of 30 until 50 years, where they plateau until the age of 60 when they begin to increase. Female breast cancer incidence rates then decrease slightly between the ages of 70-74, but increase again at ages 75 and over (Figure 1.4C and D, CRUK). A bi-modal age distribution in female breast cancer incidence patterns have also been observed following analysis of SEER program data, with peak incidences at around age 50 (early-onset, premenopausal) and 70 years (late-onset, post-menopausal) (Anderson *et al.,* 2006). This study also found that early-onset breast cancers were associated with more aggressive molecular subtypes including basal-like and *HER2*-overexpressing, while late-onset breast cancers were more likely to be ER-positive (luminal A and B). In contrast, age-dependent incidence frequencies for male breast cancer are uni-modal, with the highest incidence rates observed at age 71 (Anderson *et al.,* 2004). Interestingly, male breast cancers have been found to share common molecular features with late-onset female breast cancers such as ER and PR-positivity and low nuclear grade (Anderson *et al.,* 2004). These findings suggest that late-onset female and male breast cancers may share a common aetiology and risk exposures that are distinct from early-onset female breast cancers.



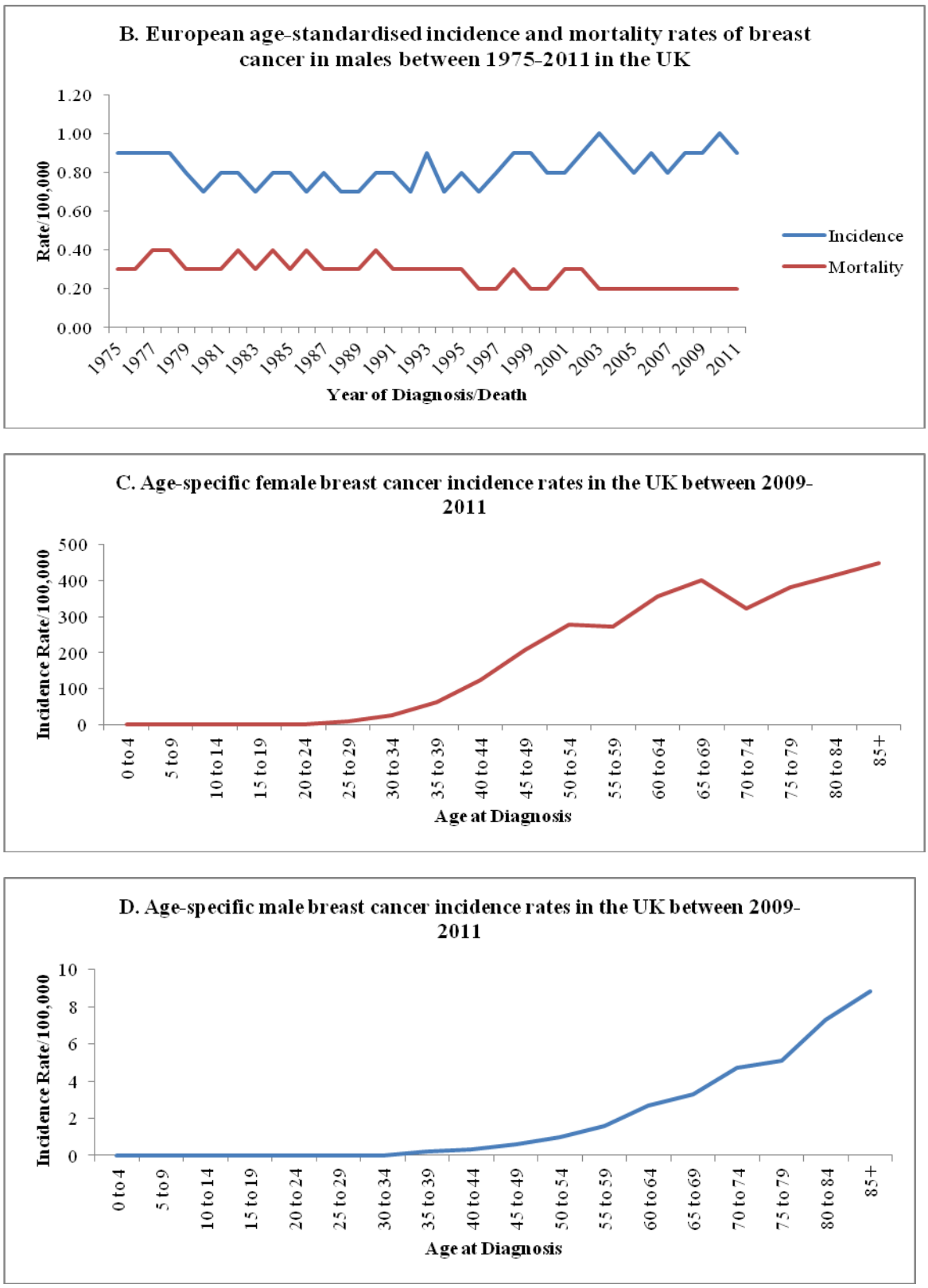


Figure 1.6 - *European age-standardized incidence and mortality rates/100,000 for* ***(A)*** *females and* ***(B)*** *males in the UK between 1975 and 2011. Average age-specific incidence rates for* ***(C)*** *females and* ***(D)*** *males in the UK between 2009 and 2011. Data obtained from Cancer Research UK (CRUK: http://www.cancerresearchuk.org/cancer-info/cancerstats/types/breast/?script=true).*

1.2.3.2 Familial Predisposition

Women with one or more first degree relatives diagnosed with breast cancer are at an increased risk of also developing the disease (Collaborative Group on Hormonal Factors in Breast Cancer., 2001). The assessment of breast cancer risk in groups of monozygotic and dizygotic twins has demonstrated that familial clustering of breast cancer cases is due to genetic factors and not common environmental exposures (Lichtenstein *et al.,* 2000). Through genome-wide association studies (GWAS), linkage analysis and mutational candidate gene screening studies, multiple breast cancer susceptibility genes have been identified (reviewed by Turnbull and Rahman., 2008 and Zhang *et al.,* 2011). Susceptibility genes are currently grouped by their associated risk profiles, which include high (>10% risk), intermediate (2-4% risk) and low (<1.5% risk) penetrance (Table 1.3). The breast cancer susceptibility genes identified to date are estimated to account for only 50% of inherited breast cancer cases; therefore more have yet to be uncovered (Cybulski *et al.,* 2015).

Table 1.3 - Known genes/genomic loci associated with increased breast cancer risk

High Penetrance	Intermediate Penetrance	Low Penetrance Alleles (Putative Causal Genes)		Unknown Penetrance
17q21 - *BRCA1*	**22q12 -** *CHEK2*	**2q33** - *CASP8, TRAK2, ALS2CR12, ALS2CR2, ALS2CR11,*	**10p15** - *ANKRD16, FBXO18***10q21** - *ZNF365*	**19p13 -** *STK11* (Peutz-Jehgers Syndrome)
13q12 - *BRCA2*	**11q22-q23 -** *ATM*	*LOC389286, LOC729191*	**10q22** - *ZMIZ1*	
		2q35	**10q26** - *FGFR2*	**16q22 -** *CDH1* (Hereditary diffuse gastric cancer syndrome)
17p13 - *p53* (Li Fraumeni Syndrome)	**17q22 -** *BRIP1*	**3p24** - *SLC4A7, NEK10*	**11p15** - *LSP1, TNNT3, MRPL23, H19, LOC728008*	
		5p12 - *MRPS30*	**11q13**	
	16p12.2 - *PALB2*	**5q11** - *MAP3K1, MGC33648, MIER3*	**12p12 -** *RECQL*	
10q23 - *PTEN* (Cowden Syndrome)		**6q22** - *ECHDC1, RNF146*	**14q24** - *RAD51L1*	
			16q12 - *TNRC9, LOC643714*	
		6q25 - *ESF1, C60rf97*	**17q22** - *COX11*	
		8q24	**19p13** - *ABHD8, ANKLE1, C19orf62*	
		9p21 - *CDKN2A/2B*		

Data obtained from Turnbull and Rahman., (2008), Zhang et al., (2011) and Cybulski et al., (2015).

The *BRCA1* and *BRCA2* high penetrance breast and ovarian cancer susceptibility genes were identified by linkage analysis and positional cloning during the early 1990's (Hall *et al.,* 1990, Miki *et al.,* 1994, Wooster *et al.,* 1995). Only 0.11 and 0.12% of women in the general population carry germline mutations in *BRCA1* and *BRCA2* respectively (Peto *et al.,* 1999). However, *BRCA1/2* mutation carriers confer a 65% and 45% average cumulative risk of developing breast cancer by the age of 70 years respectively (Antoniou *et al.,* 2003). The relative risk of breast cancer development in women carrying *BRCA1* mutations reduces with advancing age, with the highest relative risk of >30-fold at under 40 years, which declines to around 14-fold at over 60 years of age. On the other hand, a positive correlation between

relative breast cancer risk and age exists within females harbouring germline *BRCA2* mutations (Antoniou *et al.,* 2003). These findings are consistent with those presented by Krainer *et al.,* (1997), demonstrating that mutations in *BRCA1* confer a greater risk of developing early-onset breast cancer than *BRCA2* mutations. Overall, *BRCA1/2* mutations account for approximately 20% of all familial breast cancer cases (Turnbull and Rahman., 2008). In contrast to females, males carrying germline *BRCA2* mutations have a higher average cumulative risk of developing breast cancer by 70 years (6.8%) compared with those that carry *BRCA1* mutations (1.2%) (Tai *et al.,* 2007). The majority of breast cancer-associated *BRCA1* and *BRCA2* mutations involve insertions, deletions, and nonsense mutations that lead to loss of protein function through premature protein truncation (Gudmundsdottir and Ashworth., 2006). However, founder mutations have been observed at a higher frequency within certain ethnic groups such as *BRCA1*-185delAG and *BRCA2*-6174delT within Ashkenazi Jewish populations (Tonin *et al.,* 1996).

Li Fraumeni syndrome is a rare autosomal dominant disorder that is associated with the development of multiple early-onset malignancies including breast carcinoma, osteosarcoma, soft tissue sarcomas, adrenocortical carcinomas, brain tumours, Wilms' tumour, and phyllodes tumour (Birch *et al.,* 2001). Around 50% of affected individuals have been found to carry germline mutations in the *p53* tumour suppressor gene (Hisada *et al.,* 1998). *p53* is a transcription factor that plays an important functional role in mediating the cellular response to DNA damage by regulating the activity of genes involved in cell cycle arrest (Kastan *et al.,* 1991). Interestingly, intermediate penetrance breast cancer genes (*CHEK2, ATM, BRIP1, PALB2*), which account for around 2.3% of inherited breast cancer risk, are involved in the p53 and/or BRCA1/2-mediated DNA damage response pathway (Turnbull and Rahman., 2008). In response to irradiation-induced DNA damage, both ATM and CHK2 protein kinases phosphorylate and activate BRCA1 and p53 proteins to initiate DNA DSB repair pathways and cell cycle arrest (Gudmundsdottir and Ashworth., 2006). Individuals carrying germline, monoallelic, ataxia-telangiectasia-associated mutations in *ATM*, have been found to be at an increased risk of developing breast cancer, with an estimated relative risk of around 2.37 (Renwick *et al.,* 2006). A review of ten case-control studies involving over 10,800 breast cancer patients, revealed that those harbouring the protein-truncating *CHEK2*-1100delC mutation are at an increased risk (estimated odds ratio of 2.34) of developing breast cancer (CHEK2 Breast Cancer Case-Control Consortium., 2004). Monoallelic truncating mutations within the *BRIP1* gene, which encodes a BRCA1-interacting helicase enzyme, have been observed within 0.74% of breast cancer patients with families negative for *BRCA1/2* mutations and were associated with an increased relative risk of breast cancer of 2.0 (Seal *et al.,* 2006). Furthermore, the relative risk of breast cancer within individuals harbouring monoallelic germline mutations in

PALB2, which encodes a protein that interacts with and stabilizes BRCA2, is estimated to be 2.3 (Rhaman *et al.,* 2007).

Low-penetrance breast cancer susceptibility genes, which confer a <1.5 relative risk have so far been identified through genome wide association studies (GWAS) and candidate gene approaches (Turnbull and Rahman., 2008). For example, Easton *et al.,* (2009) screened 227,876 single nucleotide polymorphisms (SNPs) in around 22,000 invasive and *in situ* breast carcinoma patients and controls from 22 case-control studies and identified five loci and four putative causal genes including 5q11(*MAP3K1)*, 8q24, 10q26 (*FGFR2*), 11p15 (*LSP1*), 16q12 (*TNRC9*) (Table 1.3). A recent population-based, whole-exome sequencing study involving breast cancer patients from Poland and Quebec with a family history of the disease, identified rare recurrent mutations in the *RECQL* gene (Cybulski *et al.,* 2015). Interestingly, this gene encodes the RECQL helicase enzyme that plays important functional roles in gene expression, DNA recombination and, similarly to other breast cancer susceptibility genes, promotes genome stability through stabilization and re-initiation of stalled DNA replication forks thereby preventing DSB breaks (Berti *et al.,* 2013).

1.2.4 SYMPTOMS, SCREENING AND DIAGNOSIS

The most common symptoms associated with breast cancer include a palpable lump(s), swelling or thickening of the breast, a change in shape of the breast or nipple (inversion or sinking into the breast), haematic nipple discharge, breast pain, auxiliary adenopathy or spontaneous skin retraction (CRUK, Di Maggio., 2007). Inflammatory breast cancer symptoms may include swelling of the breast and a porous-like breast skin texture. The development of a red, eczematous rash around the nipple is usually associated with Paget's disease.

Since the 1980s, over thirty countries worldwide have implemented national or regional mammographic screening programs for women between the ages of 40 and 75 years (Youlden *et al.,* 2012). The overall aim of breast screening is to detect cancer at a early stage within asymptomatic women, in order to improve prognosis and survival and decrease mortality. As shown in Figure 1.5, an important factor for breast cancer survival is stage at diagnosis, with localized and regional disease stages associated with better survival rates. For many breast cancers there is a positive correlation between tumour size and lymph node involvement (Foulkes *et al.,* 2010). Therefore, early diagnosis is a critical factor in improving survival outcomes for breast cancer patients (Youlden *et al.,* 2012). Mammography is currently the most effective and sensitive method of identifying early-stage breast cancer (Di Maggio., 2007). This particular screening modality can be used to obtain whole-breast images and identify features associated with breast neoplasia including, structural distortions, nodular opacities and

microcalcifications. In this way, non-palpable breast lesions can be discovered and the location(s) and dimensions of breast tumours can be recorded for downstream treatment purposes. However, some breast lesions, such as lobular breast carcinomas, present with minimal stromal involvement and a largely intact glandular architecture, which are difficult to detect using mammographic screening methods. Furthermore, due to the incidence of false-negative readings, the use of mammography for women with dense breast tissue may be less sensitive and other imaging techniques such as ultrasound or magnetic resonance imaging (MRI), may be used to identify breast lesions (Crystal *et al.,* 2002). A review of the NHS Breast Screening Program conducted in 2012, found that an estimated 1,300 breast cancer-associated deaths may be prevented each year as a result of the screening program (Independent UK Panel on Breast Screening., 2012). Similarly, studies carried out in Australia, Denmark, the Netherlands and Sweden have reported a 19%-32% decrease in mortality rates due to mammographic screening programs (reviewed by Youlden *et al.,* 2012). However, a major drawback is the over-diagnosis and treatment of breast cancers that would not have otherwise progressed or become clinically apparent throughout the women's lifetime. As a consequence of this, women undergo unnecessary surgical procedures, radiotherapy or drug regimens (Independent UK Panel on Breast Screening., 2012). The magnitude of over-diagnosis is difficult to ascertain, however, population-based studies estimate that between 1%-54% of patients may be over-diagnosed as a result of screening (de Gelder *et al.,* 2011). Despite these observations, there is a general consensus that the survival benefits of mammographic screening programs outweigh the potential harms (Youlden *et al.,* 2012). However, whether the benefits of mammographic screening outweigh the harms is still under debate. A recent review of Swedish randomised mammographic screening trials, demonstrated that the risk reduction in breast cancer death due to mammographic screening was overestimated due to the use of unconventional statistical methods (Autier *et al.,* 2015).

The diagnosis of breast cancer typically involves a combination of clinical tests, imaging procedures and pathological assessments (Senkus *et al.,* 2013). A medical professional will first examine the breasts for typical extramammary signs of breast cancer and also determine the presence of loco-regional lesions through bimanual palpation of the breasts, surrounding lymph nodes and assess for distant metastases within the bones, brain, liver and lungs. A complete personal medical history is also taken to determine familial risk factors and to assess menopausal status. The principal imaging modality used to determine the presence, location and size of breast tumours is a bilateral mammography (Mariani and Gianni., 2007). To determine the degree of regional lymph node involvement an ultrasound may also be undertaken. Magnetic resonance imaging (MRI) is employed only if conventional imaging methods produce inconclusive results, if individuals have a family history of the disease or if multi-centric or multi-focal lesions are suspected from breast mammography (Schnall and Orel.,

2006). Based on results of imaging procedures, pathological assessment of the breast lesion(s) is then carried out (Senkus *et al.,* 2013). To do this, a manual or ultrasound-guided core needle biopsy or fine need aspirate (FNA) of the primary tumour and suspicious lymph nodes (sentinel lymph node biopsy) is undertaken. Tumour samples obtained from biopsies or surgical specimens are then classified according to histopathological criteria (Chapter 1.2.2.1), including histological sub-type, tumour grade and the assessment of ER, PgR and *HER2* receptor status by immunohistochemistry (IHC) or *in situ* hybridization (ISH). The WHO tumour, node, metastasis (TNM) classification system (Table 1.2 and Lakhani *et al.,* 2012) is used to characterize the stage of disease within each patient following clinical (cTNM) or post-operative (pTNM) pathological assessment of the primary tumour and lymph nodes. If a patient is found to have a large tumour(s) (>5cm in greatest dimensions), auxiliary lymph node involvement or clinical symptoms indicative of distant metastases, additional imaging modalities such as chest computed tomography (CT), bone scans, abdominal and liver ultrasounds or a fluorodeoxyglucose positron emission tomography (FDG-PET) scan may be used to confirm the sites and extent of metastatic spread (Senkus *et al.,* 2013).

1.2.5 TREATMENT

The choice of breast cancer treatment strategy depends on multiple factors including (i) anatomical features such as the number of lesions, tumour size, location and the number and extent of lymph node infiltration (ii) biological factors such as histopathological classification and the presence/absence of hormone receptors (HR) and *HER2* overexpression, (iii) the age and general health of the patient and (iv) patient preferences.

1.2.5.1 Surgery

There are two main surgical options for patients with invasive or *in situ* breast lesions; breast conserving surgery (BCS) and complete mastectomy. Independent trials have shown that in certain instances, BCS followed by radiotherapy for patients with infiltrating breast carcinoma confers similar survival, loco-regional and distant disease recurrence rates, to radical or modified radical mastectomy (Sarrazin *et al.,* 1989, reviewed by Guarneri *et al.,* 2007). An estimated 60%-80% of breast cancers are therefore considered to be treatable by BCS and radiation therapy (Senkus *et al.,* 2013). However, a mastectomy is usually recommended for patients that present with multi-focal disease, a large tumour(s), have positive tumour surgical margins as a result of BCS or due to patient preferences. In addition, a prophylactic bilateral mastectomy is generally offered to high risk patients, such as *BRCA1/BRCA2* mutation carriers, which has been associated with a 95% reduction in breast cancer risk (Rebbeck *et al.,* 2004). Patients presenting with a large uni-focal breast tumour can undergo neoadjuvant systemic therapy prior to breast surgery to reduce tumour size, avoid a mastectomy and improve cosmetic

outcomes following BCS (Senkus *et al.,* 2013). BCS generally involves a wide local excision of the tumour with a minimum 1mm histological margin for invasive lesions and a >2mm margin for *in situ* lesions. Oncoplastic procedures during BCS aim to promote acceptable cosmesis for all patients, particularly those with large breasts or high tumour-to-breast size ratios. In addition, the insertion of a clip during BCS can be carried out to mark the cancer site and direct post-operative radiotherapy treatment.

Following mastectomy procedures, patients can either undergo immediate, delayed or no breast reconstructive surgery depending on cancer type or patient preferences. Immediate reconstructive surgery is not recommended for inflammatory breast cancer as some studies have shown that this may limit radiation coverage to the affected breast tissue and, where appropriate, regional lymph nodes (Dawood *et al.,* 2011). Out of the two major reconstructive surgery techniques, autologous tissue flaps have been associated with more favourable cosmesis and fewer complications following post-surgical radiotherapy when compared with prosthesis (Senkus-Konefka *et al.,* 2004). Sources of autologous tissue flaps include the transverse rectus abdominis and latissimus dorsi muscles from the patients abdomen and back respectively (Senkus *et al.,* 2013).

As part of the disease staging process and to determine long-term patient prognosis, assessment of regional lymph node metastases by auxiliary lymph node dissection can be carried out during breast surgery (Senkus *et al.,* 2013). However, a major contraindication associated with auxiliary clearance and post-operative radiotherapy is disruption of the lymphatic drainage system within the breast leading to lymphoedema (Guarneri *et al.,* 2007). Therefore, in order to reduce the extent of breast surgery, a preceding sentinel lymph node biopsy (SNLB) is usually carried out to determine the degree of regional lymph node involvement and whether auxiliary clearance is required (SinghRanger and Mokbel., 2003).

1.2.5.2 Radiotherapy

Randomized trials have shown that radiation therapy for patients following BCS or mastectomy is associated with a 17%-19% risk reduction of loco-regional recurrence within 5-10 years and a 3.8-5.4% reduction in breast cancer mortality risk within 15 years (Early Breast Cancer Trialists' Collaborative Group., 2006, 2012). Over a period of 3-7 weeks following surgery, patients can undergo local/regional adjuvant external beam radiotherapy treatment which usually involves up to 28 fractions of around 2.0Gy providing a total radiation dose of 45-50Gy (Senkus *et al.,* 2013). In addition to conventional radiation treatment, a boost radiation dose of 10Gy or 16Gy has been associated with a greater risk reduction of ipsilateral breast relapse within patients initially diagnosed with invasive breast carcinoma and treated with local excision and BCS (Romestaing *et al.,* 1997, van Werkhoven *et al.,* 2011). Boost radiation doses may be offered to patients with high-grade tumours, vascular invasion, or patients that have

undergone BCS. Side effects associated with breast radiotherapy include inflammation, reddening of the skin, lymphoedema (commonly associated with auxiliary node clearance) and fatigue (CRUK). Furthermore, a positive correlation between mean radiation dose and risk for ischaemic heart disease has been observed in patients that have received radiotherapy for breast cancer (Darby *et al.,* 2013).

Around 90% of local breast cancer recurrences have been found to occur within the immediate vicinity of the primary breast tumour (Vaiyda *et al.,* 2010). Therefore, in order to reduce harmful side effects and target treatment to the affected area, accelerated partial breast irradiation (APBI) techniques including intra-operative radiotherapy and brachytherapy (see below) have been developed. Intra-operative radiotherapy involves the delivery of a single radiation dose of around 20Gy to the surface of the tumour bed during surgery. Results from the TARGIT-A (targeted intraoperative radiotherapy) randomised phase 3 trial, showed that invasive breast carcinoma patients treated with intra-operative radiotherapy or post-surgical whole-breast external beam radiotherapy, displayed similar 4-year local recurrence rates (Vaiyda *et al.,* 2010). A more recent review of TARGIT-A trial data demonstrated that intra-operative radiotherapy was associated with a 2% higher ipsilateral breast recurrence rate compared with whole breast irradiation (Vaiyda *et al.,* 2012). The brachytherapy form of APBI, which involves the targeted delivery 34Gy of radiation over 10 fractions through interstitial single lumen catheters, has been shown to yield comparable 5-year ipsilateral breast recurrence rates, toxicity levels (fat necrosis, infections and seromas) and cosmesis as whole-breast irradiation (Shah *et al.,* 2013). Long-term local recurrence or survival rates for breast cancer patients treated with brachytherapy compared with whole-breast radiotherapy are currently unknown, but are expected within the next 10 years.

1.2.5.3 Adjuvant Systemic Therapy

Aside from or in addition to surgery, adjuvant systemic treatments such as chemotherapeutic agents and targeted biological agents including endocrine therapies or *HER2*-inhibitors, can be used to improve disease-free survival and overall survival of breast cancer patients (Senkus *et al.,* 2013). The choice of adjuvant systemic therapy treatment for individual patients depends on the intrinsic molecular breast cancer subtype (Table 1.4), as well as age, general health, menopausal status and patient preferences (Goldhirsch *et al.,* 2013, Senkus *et al.,* 2013). Breast tumours positive for ER and PgR receptors (particularly luminal cancers) are treated with endocrine therapies such as selective oestrogen receptor modulators (SERMs, tamoxifen and raloxifane), aromatase inhibitors (letrozole), or gonadotropin-releasing hormone (GnRH) agonists. Tamoxifen is a partial, non-steroidal, oestrogen agonist that binds to the ER causing ligand-dependent conformational changes and disruption of growth factor signalling pathways (Wakeling., 2000). GnRH agonists function to suppress ovarian function, whereas

aromatase inhibitors are a group of steroidal or non-steroidal inhibitors that bind to and disrupt aromatase enzyme function, which leads to reduced serum estrodiol levels (Lumachi *et al.,* 2011). Aromatase is responsible for catalyzing the aromatization of androgens to estrogens. Tamoxifen is considered to be the standard endocrine therapy for pre-menopausal breast cancer patients, but GnRH agonists in combination with aromatase inhibitors may also be considered in the event of tamoxifen-associated contraindications (such as endometrial cancer) (Senkus *et al.,* 2013). A combination of aromatase inhibitors and tamoxifen are usually considered for post-menopausal breast cancer patients. Compared with luminal A, luminal B molecular subtypes have a higher Ki-67 proliferative index, are less endocrine sensitive and are associated with poorer disease prognosis. Therefore, this particular subtype may be treated with a combination of endocrine therapy and chemotherapy (Table 1.4). Multi-gene expression assays, such as the 21-gene recurrence score assay, have been shown to define a particular subgroup of luminal cancers that are associated with higher recurrence rates and an improved response to chemotherapeutic agents (Goldhirsch *et al.,* 2013).

Table 1.4 - Adjuvant systemic treatment recommendations for each intrinsic molecular breast cancer subtype

Clinico-Pathological Surrogate Intrinsic Phenotype	Features	Systemic Treatment
Luminal A-like	ER (+), PgR (+), *HER2* (-), Ki67 (low), MGEA (low risk)	ET
Luminal B-like *HER2* (-)	ER (+) and PgR (-/low) or Ki67 (high), MGEA (high risk)	ET+CT
Luminal B-like *HER2* (+)	ER (+), PgR (+/-), Ki67 (high/low)	ET+CT+Anti-*HER2*
HER2-positive (non-luminal)	ER (-), PgR (-), *HER2* (+)	CT+Anti-*HER2*
Basal-like	ER (-), PgR (-), *HER2* (-)	CT
Special Types A (Cribiform, Mucinous and Tubular)	ER (+), PgR (+)	ET
Special Subtypes B (Metaplastic, Apocrine, Adenoid Cystic, Medullary)	ER (-), PgR (-)	CT

Data obtained from St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer (Goldhirsch et al., 2013). Abbreviations: ER (oestrogen receptor), PgR (Progesterone Receptor) HER2 (Human epidermal growth factor receptor 2), ET (endocrine therapy), CT (cytotoxic therapy), MGEA (Multi-gene expression assay)

HER2-overexpressing breast tumours and metastases are treated with the monoclonal antibody (MAb) trastuzumab (herceptin), directed against the HER-2 protein. Through direct binding to the HER-2 receptor, it is thought that trastuzumab blocks downstream PI3-kinase (PI3K) and MAP-kinase (MAPK) signalling pathways leading to cell cycle arrest in G1 and

induction of apoptosis (Nahta and Esteva., 2005). Trastuzumab has also been found to suppress angiogenesis *in vivo* through downregulation of pro-angiogenic factors such as vascular endothelial growth factor (VEGF) and up-regulation of anti-angiogenic factors such as thrombospondin-1 (TSP-1) (Izumi *et al.,* 2002).

Cytotoxic chemotherapy is prescribed to patients with HR-negative/poor, *HER2*-positive or high grade tumours and those with locally advanced, metastatic disease or recurrence (Senkus *et al.,* 2013). Common chemotherapy regimens for breast cancer usually comprise 4-8 cycles of treatment lasting up to 8 months and generally involve the intravenous administration of a combination of anthracyclines (doxorubicin or epirubicin) with cyclophosphamide and an anti-metabolite (methotrexate or fluorouracil) and in some cases, an additional microtubule agent (docetaxel). Older patients and those with pre-existing heart conditions may be offered regimens that do not contain anthracyclines, such as CMF (cyclophosphamide, methotrexate, fluorouracil), due to the increased risk of cardiomyopathy and congestive heart failure (Cortes-Funes and Coronado., 2007). However, anthracycline-containing chemotherapy regimens are associated with a significant improvement in 10-year recurrence and overall mortality rates for older and younger patients, when compared to CMF regimens (EBCTCG., 2005).

1.2.5.4 Resistance

Around 5% of breast cancer patients present with detectable metastatic disease at initial diagnosis, but up to 40% will go on to develop metastatic disease despite undergoing surgical and/or systemic therapy (Guarneri *et al.,* 2007). In addition, around 30% of patients diagnosed with early-breast cancer will develop recurrent disease, either locally or at distant sites (Gonzalez-Angulo *et al.,* 2007). One of the major contributing factors for disease recurrence in breast cancer patients is resistance to adjuvant systemic agents. Patients with *HER2*-positive breast tumours initially respond to herceptin-containing treatment regimens but undergo disease progression within the first year (Slamon *et al.,* 2001). Furthermore, only around one third of patients diagnosed with ER-positive metastatic breast cancer, have objective tumour regression following endocrine therapy (Osborne and Schiff., 2011). After an initial response, the majority of cancer patients treated with chemotherapeutic agents develop progressive disease (Liu *et al.,* 2009).

1.3 TARGETING CELLULAR IMMORTALITY AS AN ANTI-CANCER MECHANISM

Cancer development and progression is a multi-step process that relies on the accumulation of multiple somatic mutations and epigenetic changes, which confer survival advantages over neighbouring cells through an increased ability to proliferate, migrate and survive within foreign tissues or hostile environments and evade detection by the immune system. This step-wise process is thought to depend on a Darwinian evolutionary system, known as 'clonal evolution,' which is characterized by the continual process of clonal expansion, acquisition of a rare somatic-cell genetic/epigenetic alteration and clonal selection (Greaves and Maley., 2010). At least five or six successive cycles of genetic/epigenetic alteration and clonal expansion are thought to be required for the development of most common human carcinomas (Newbold., 2005). The initiation of cancer by clonal evolution must therefore require an initial step that permits extended cell proliferation over neighbouring cells, a higher level of genetic/epigenetic variability and the capacity for indefinite, unfettered cell growth.

Complex multi-cellular organisms are made up of both mitotic cells such as epithelial, endothelial (vascular), stromal (fibroblasts), haematopoietic, stem and progenitor cells and post-mitotic differentiated cells including specialized muscle cells and neurons (Campisi and Fagagna., 2007). The proliferative capacity of mitotic cells facilitates the process of tissue repair/regeneration, which is essential for organism survival. However, proliferating cells acquire more genetic/epigenetic alterations than non-dividing cells (Busuttil *et al.,* 2006). Therefore, with increasing age, molecular gain-of-function changes occur that allow abnormal cellular proliferation (hyperplasia), which results in the acquisition of further molecular alterations that can lead to clonal evolution and cancer development. Cancer is an age-related disease, with incidence rates increasing almost exponentially from the mid-point of an organisms lifespan (Campisi., 2013).

Multi-cellular organisms with tissue renewal capabilities have evolved stringent tumour suppressive mechanisms that inhibit the proliferation of potential cancer cells. One such mechanism is cellular senescence, which is characterized by the irreversible growth arrest of mitotic cells in response to potentially oncogenic stimuli.

1.3.1 REPLICATIVE LIFESPAN OF NORMAL AND TUMOUR CELLS

Cellular senescence was first described by Hayflick and colleagues over 50 years ago, when normal human fibroblasts were found to undergo an initial period of active proliferation *in vitro* before gradually decreasing in mitotic activity and entering a period of permanent but viable growth arrest, despite the availability of nutrients, growth factors and space (Hayflick and

Moorhead., 1961, Hayflick., 1965). In complete contrast, human cancer cells that are established in culture proliferate indefinitely. At this time, the immortal phenotype exhibited by cancer cells *in vitro*, was thought to be a cell culture artefact and not a characteristic feature of cancer cells. However, later studies involving normal diploid rodent fibroblasts, demonstrated that malignant transformation, either spontaneously or following carcinogen treatment or transfection with oncogenes (such as *Ha-rasV12*), could only occur within cells that had been previously immortalized (Newbold *et al.,* 1982, Newbold and Overell., 1983). Transfection of normal rodent cells and primary human fibroblasts with the *Ha-rasV12* oncogene, was found to induce premature cellular senescence (Newbold and Overell., 1983, Serrano *et al.,* 1997). Furthermore, spontaneous immortalization of normal human somatic cells (fibroblasts or epithelial cells) has never been found to occur *in vitro* (Stampfer and Yaswen., 2002). Only after treatment with human oncogenes, viral oncoproteins or powerful carcinogens, have rare immortal variants been found to emerge (Newbold *et al.,* 1982, Stampfer and Bartley., 1985). These findings demonstrate the importance of cellular immortality in mammalian carcinogenesis, which is now recognized as one of the hallmarks of human cancer (Hanahan and Weinberg., 2011). Moreover, these findings demonstrate the existence of intrinsic and stringent tumour suppressive mechanisms within mammalian cells that function to prevent cellular immortalization and cancer development upon exposure to oncogenic stimuli.

1.3.2 Cellular Senescence as a Barrier to Immortality

1.3.2.1 Mortality Barriers to Cellular Immortality

In support of the notion that cellular senescence represents a critical tumour suppressive mechanism, *in vitro* studies involving transfection of DNA tumour virus early oncogenes, such as those encoding the simian vacuolating virus 40 (SV40) large T-antigen (SV40-LT) and human papilloma virus (HPV) E6 and E7 proteins, into normal diploid human fibroblasts, provided evidence of a two-step mechanism of cellular immortalization that involved senescence bypass (Wright *et al.,*1989, Wright and Shay., 1992, Ozer *et al.,* 1996, Newbold., 2005). Normal human diploid fibroblasts undergo 50-80 population doublings (PD) *in vitro* before entering replicative senescence, termed Mortality 1 (M1). However, following introduction of SV40-LT or HPV E6 and E7 viral oncoproteins, fibroblasts enter a period of extended vigorous cell growth of around 20-30 PD, before slowing and entering a 'crisis' period known as Mortality 2 (M2), which is characterized by slow cell turnover and extensive cell death. Rarely, a clone of rapidly dividing cells emerges from crisis at a frequency of 10^{-5} to 10^{-8}, which can proliferate indefinitely (Wright and Shay 1992, Newbold., 2005). These findings demonstrate that bypass of replicative senescence pathways, through introduction of viral oncoproteins, leads to an increased propensity for cellular immortalization.

SV40-LT and HPV E6 and E7 proteins are known to bind and sequester the p53 tumour suppressor protein and disrupt the pRb-E2F complex. Taking into account the observations made by viral immortalization studies, these proteins must therefore function as important effectors of cellular senescence pathways (Jha *et al.,* 1998). p53 activates the expression of $p21^{CIP1/WAF1}$, a cyclin-dependent kinase inhibitor (CDKI) that prevents cyclin E/CDK2-mediated phosphorylation (and inactivation) of pRb, which forms a complex with E2F and represses the transcription of target genes involved in cell cycle progression through the G1/S transition and S-phase (reviewed by Campisi and Fagagna., 2007). Two major pathways are known to control activation of p53; DNA-damage response (DDR) and ARF (p14) (reviewed by Ben-Porath and Weinberg., 2005). In response to DNA damage such as DSB, ATM/ATR and Chk1/Chk2 proteins phosphorylate and stabilize p53. The ARF protein is encoded by an alternative splice variant of the *INK4A/CDKN2A* locus, which also encodes the $p16^{INK4A}$ gene. *ARF* and *p16* are under the control of two separate promoters and are translated from different but overlapping open reading frames. *ARF* expression is activated in response to stressful stimuli or following ectopic expression of oncoproteins such as E2F1, c-Myc and Ras (Lowe and Sherr., 2003). Through inhibition of the E3 ubiquitin ligase HDM2, which targets p53 for proteolytic degradation, ARF promotes the stabilization of p53. Therefore, p53 and pRb function as key mediators of cellular senescence in response to potentially oncogenic stimuli such as DNA damage and oncoproteins.

The *p16* gene has also been implicated as an important effector of cellular senescence in mammalian cells (Newbold., 2005). *p16* encodes a CDKI that inhibits cyclin D/CDK4, 6 complexes resulting in hypophosphorylation and activation of pRb. It is thought that p16 is responsible for activating a premature senescence pathway, termed Mortality 0 (M0), in response to accumulated stress *in vitro* (for example, supra-physiological or inadequate growth conditions) or *in vivo* (for example, ageing) (Campisi and Fagagna., 2007). Furthermore, induction of *p16* expression and premature senescence has been observed following exposure to oncogenic RAS, which is known as oncogene-induced senescence (OIS). p16 is solely responsible for mediating stress-associated premature senescence (SIPS) within normal human mammary epithelial cells (HMEC) and keratinocytes (Stampfer and Yaswen., 2002). Inactivation of *p16,* but not *p53*, through epigenetic silencing or deletion of the *p16* locus*,* is associated with bypass of premature senescence *in vitro,* which allows continued proliferation until the p53/p21/pRb-dependent M1 senescence barrier is reached. Interestingly, *p16* is frequently inactivated in many human cancers (Herman *et al.,* 1995). It is believed that, in addition to p53/p21/pRb-mediated replicative senescence pathways, p16/pRb-mediated premature senescence in response to stress or oncoproteins, provides an additional barrier against cellular immortalization, clonal evolution and malignant progression.

1.3.3 TELOMERES, SENESCENCE AND CANCER

1.3.3.1 Telomere Structure and the End Replication Problem

Early genetic studies carried out over 70 years ago, found that chromosomes with damaged termini were unstable and formed ring or dicentric chromosomes through end-to-end fusions, which caused loss of genetic material during subsequent mitoses (Muller., 1938, McClintock., 1941). Chromosome ends were found to possess specialized structures, termed telomeres, that were thought to function to protect chromosome integrity. Telomeres are now known to be highly conserved, specialized nucleoprotein complexes located at the terminal ends of linear eukaryotic chromosomes that consist of short, repetitive DNA sequences and associated proteins including the shelterin complex (Blackburn., 1999, de Lange., 2005). Among different organisms, the number of telomeric sequence repetitions varies from a few kilobases (kb) to greater than 100kb (Gomez *et al.,* 2012). However, the number of telomeric repetitions can also vary among individuals of the same species and also between tissues and chromosomes of the same organism. Human germ-line and adult somatic cells have an average telomere length of around 10kb (Blackburn., 1999, White *et al.,* 2001).

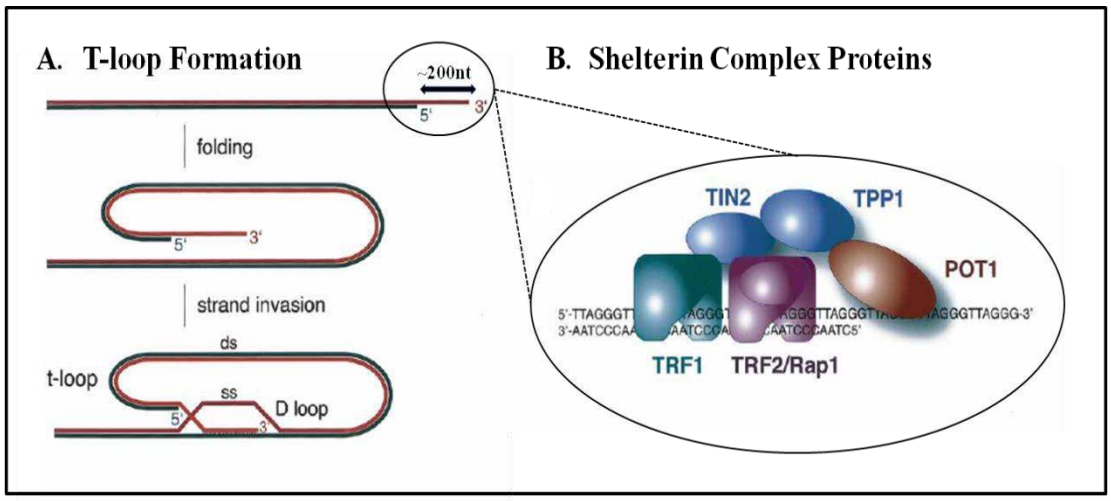


Figure 1.7 - *Schematic representations of* ***(A)*** *T-loop formation by invasion of the 3'-single stranded (ss) overhang into the double-stranded (ds) telomeric sequence to form a D-loop though base-pairing with the complementary C-rich strand.* ***(B)*** *Shelterin complex members and an example of their organization on the repetitive telomeric DNA sequence. Images obtained and adapted from de Lange et al., (2005).*

The human repetitive telomeric DNA sequence (TTAGGG)n, is always oriented 5'-3' towards chromosome termini and consists of a large double-stranded tract and a 3'-single-stranded overhang that protrudes around 200 nucleotides beyond the complementary C-rich strand (Figure 1.7A), which can form complex G-quadruplex structures that disrupt DNA replication or telomere elongation by the telomerase enzyme (Makarov *et al.,* 1997, Blackburn.,

1999). In order to sequester the telomere end, single-stranded 3'-DNA overhangs invade the double-stranded telomeric DNA segments to form telomere loops (T-loop), which are stabilized through displacing G-rich strands and base-pairing with C-rich strands to form triple-stranded displacement loops (D-loop) (Figure 1.7A). T-loop formation and stabilization is mediated by members of the Shelterin complex, which consist of six telomere-specific protein units; TRF1, TRF2, RAP1, TIN2, POT1 and TPP1 (Figure 1.7B, Griffith *et al.,* 1999, de Lange., 2005). Other telomere-associated proteins include DNA helicases, tankyrases and DNA damage sensors such as Rad50. Inhibition of shelterin component members, such as TRF2, results in telomere 'uncapping,' chromosomal end-to-end fusions and activation of the ATM/ATR-mediated canonical DNA damage response pathway, leading to cell cycle arrest, apoptosis or senescence (Griffith *et al.,* 1999, de Lange., 2005). It is therefore thought that t-loop structures function to maintain chromosome integrity and prevent exposure of chromosome ends to DNA damage sensors and subsequent activation of inappropriate DNA damage repair pathways.

Telomeres also provide a solution to the 'end replication problem', which describes the incomplete replication of linear chromosome ends within eukaryotic cells (Watson., 1972, Olovnikov., 1973). During DNA replication, DNA primase enzymes synthesize RNA primer molecules (approximately 10 nucleotides in length) at 200bp-intervals along the lagging strand of the replication fork, which are extended by DNA polymerase to form Okazaki fragments (Okazaki *et al.,*1968). Following removal of RNA primers, DNA ligase and DNA polymerase enzymes seal the Okazaki fragments together to form a continuous DNA strand (Wynford-Thomas and Kipling., 1997). However, removal of the last RNA primer leaves a small region at the chromosome terminal, for which no complementary strand can be synthesized. Therefore, due to incomplete DNA replication and in the absence of compensatory telomere elongation mechanisms, telomeric DNA sequences are lost with each round of cell division. In humans this is equivalent to approximately 150-200bp/chromosome terminal with each cell division (Smorgorzewska and de Lange., 2004).

1.3.3.2 Telomere Maintenance by Telomerase

The de novo synthesis of most eukaryotic telomeres is carried out by the reverse transcriptase ribonucleoprotein enzyme, telomerase, which was first discovered within the *Tetrahymena thermophila* ciliate protozoa by Greider and Blackburn (1989). The human telomerase enzyme is composed of a reverse transcriptase catalytic subunit (hTERT), an RNA template component (hTR) and integral accessory proteins, which together function to catalyze the addition of telomeric repeat sequences (TTAGGG) to the single-stranded 3'-end of telomeric DNA (reviewed by Wyatt., 2010).

The hTERT protein subunit is encoded by the *hTERT* gene located on chromosome 5p15.33, which is around 40kb in length and comprises 16 exons and 15 introns (Daniel *et al.,*

2012). This gene encodes a 1132 amino acid protein, which contains a long N-terminal extension, a central catalytic reverse transcriptase component and and a shorter C-terminal extension (Wyatt., 2010). Within the N-terminal conserved sequence motif TEN (telomerase essential N-terminal domain), is the DAT (dissociates activities of telomerase) region, which has been shown to play an important role in binding the hTR subunit (Figure 1.8A, Friedman and Cech., 1999). Adjacent to the TEN domain is the TRBD (telomerase RNA-binding domain), which contains three conserved RNA binding sequences CP, QFP and T-motif that are involved in positioning the hTR subunit within the hTERT active site (Figure 1,8A, Nicholls *et al.,* 2011). The central reverse transcriptase (RT) domain forms the catalytic core of hTERT and contains conserved sequence motifs, which are involved in positioning the single-stranded 3'-telomeric end within the TERT active site and catalyzing the addition of telomeric sequences. The active site contains a catalytic triad of three conserved aspartic acid residues, which facilitate the sequential addition of nucleotides through a two-metal ion mechanism (Autexier and Lue., 2006, Gillis *et al.,* 2008). The TERT C-terminal extension exhibits the least sequence conservation, but mutations within this region have been shown to influence the subcellular localization of telomerase, enzyme processivity and telomere length maintenence (reviewed by Wyatt *et al.,* 2010).

The hTR component of telomerase is encoded by the *hTR* gene (also known as *hTERC)*, which is located on chromosome 3q26 and is 451bp in length (Nicholls *et al.,* 2011). hTR consists of three conserved domains; (i) the template core domain (or pseudoknot), which contains an 11bp-RNA template strand (5'-CUAACCCUAAC-3') that encodes the telomeric repeat sequence, (ii) conserved regions 4 and 5 (CR4/CR5) and (iii) a box H/ACA domain, which is responsible for tethering telomere-associated proteins, maintaining hTR stability and nuclear localization (Gomez *et al.,* 2012, Wyatt., 2010). Aside from hTERT, hTR has been found to interact with and facilitate the activation of hnRNP A1 (heterogeneous nuclear ribonucleoprotein A1), which functions to direct binding of the telomerase holoenzyme complex to telomere sequences (Nicholls *et al.,* 2011).

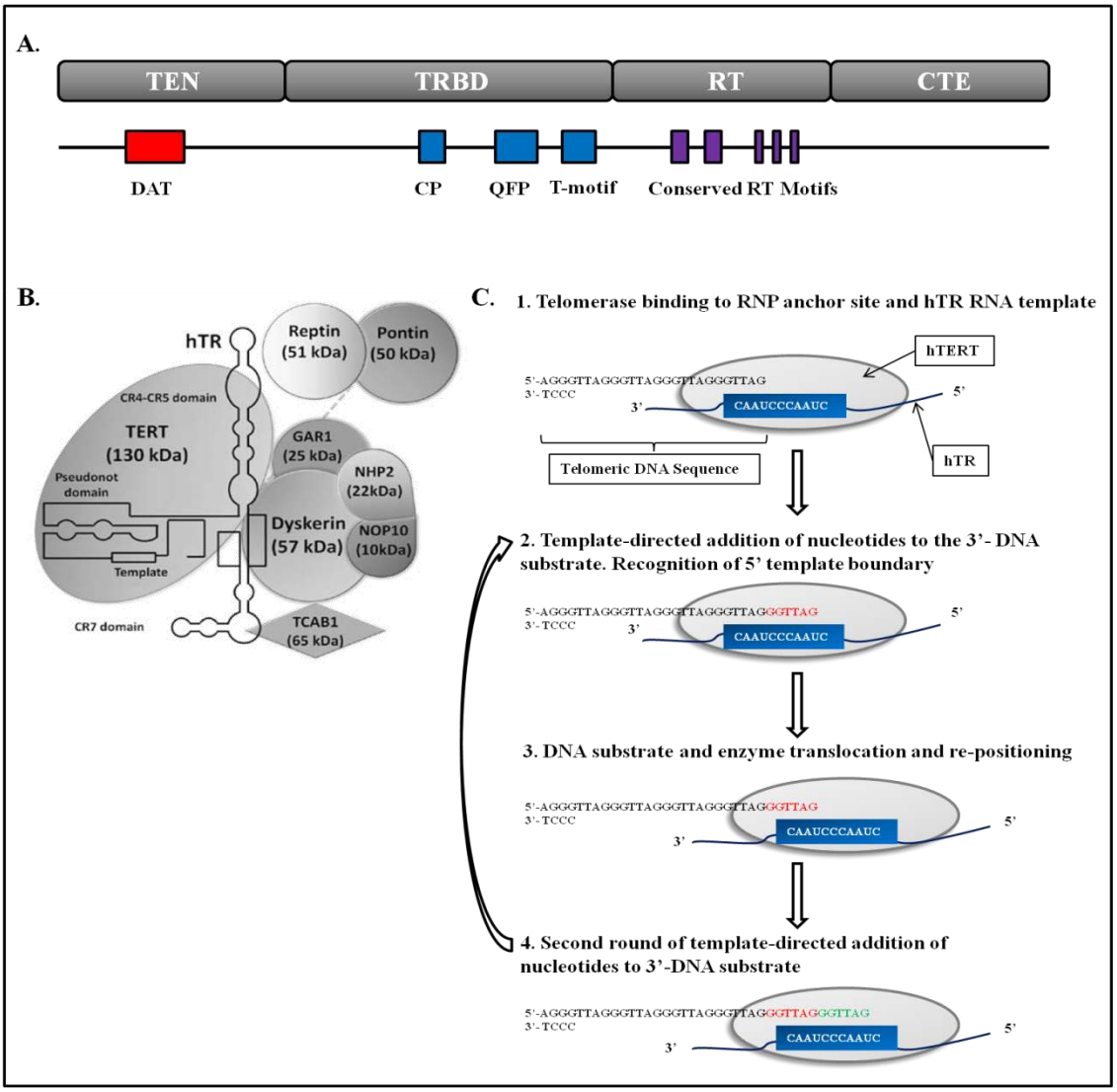


Figure 1.8 - *Schematic representations of* ***(A)*** *the structure of the hTERT protein subunit of telomerase showing the TEN (telomerase essential N-terminal domain), TRBD (telomerase RNA-binding domain), RT (reverse transcriptase), CTE (C-terminal extension) domains and conserved sequence motifs (adapted from Nicholls et al., 2011),* ***(B)*** *the human telomerase holoenzyme complex (image obtained from Gomez et al., 2012) and* ***(C)*** *a simplified model for processive telomere elongation by telomerase (adapted from Autexier and Lue., 2006). Abbreviations: RNP (ribonucleoprotein), hTERT (human telomerase reverse transcriptase), hTR (human telomerase RNA).*

Following *hTERT* mRNA transcription and cytoplasmic translation, hTERT peptides are translocated into the nucleus through interaction with 14-3-3 signaling proteins and NFκB p65, where it is then recruited to Cajal bodies (Wojtyla *et al.,* 2011). Trimethylguanosine (TMG)-capped, pre-cursor hTR mRNA is modified and converted into mature hTR through binding of the RNA-dependent NTPase RHAU and dyskerin to 5'- and 3'-ends of the mRNA respectively (reviewed by Podlevsky and Chen., 2012). TCAB1 (telomerase Cajal body protein 1) then binds to the H/ACA motif and transports mature hTR to Cajal bodies for assembly of the

telomerase ribonucleoprotein complex. Inhibition of TCAB1 has been associated with prevention of telomerase component assembly, disruption of telomerase-telomere interactions and telomere synthesis (Venteicher *et al.,* 2009). Analysis of affinity-purified telomerase from the cervical cancer-derived HeLa cell line by mass spectrometry demonstrated the presence of several other telomere-associated proteins including NOP10, NHP2, GAR1, pontin and reptin, which play essential roles in the biogenesis, stabilization and nuclear localization of telomerase (Figure 1.8B, Venteicher *et al.,* 2008). The highly conserved nucleolar protein dyskerin, together with two closely related ATPase enzymes reptin and pontin, function to assemble the telomerase ribonucleoprotein complex through recruitment and stabilization of the hTR subunit. Dyskerin binds to the hairpin subunit of the H/ACA motif of hTR, along with NOP10, NHP2 and GAR1 proteins, which have been shown to be required for stability and accumulation of hTR *in vivo* (Fu and Collins., 2007). The human telomerase enzyme comprises a 1:1 stoichiometric ratio of hTERT and hTR and has been shown, through single-particle electron microscopy, to display a bi-lobal structure (Sauerwald *et al.,* 2013). It is thought that telomerase is a dimeric complex consisting of two hTERT and two hTR dimers, which has the capacity to extend two telomeric substrates simultaneously. This has been proposed to allow telomerase to bind and extend sister chromatid telomeres in tandem. Following assembly of the dimeric telomerase complex within Cajal bodies, the hnRNA A1 protein binds to the hTR subunit and directs telomerase to telomere ends within the nucleolus (Wojtyla *et al.,* 2011). Confocal microscopy analysis of normal human fibroblasts containing a green fluorescent protein (GFP)-hTERT fusion protein, demonstrated that telomerase is released from the nucleolus into the nucleoplasm to extend telomere lengths during S/G2 phase of the cell cycle (Wong *et al.,* 2002). In contrast, tumour cells were found to display high levels of telomerase within the nucleoplasm during all phases of the cell cycle, which demonstrates that cell cycle-dependent nucleolar association/dissociation of telomerase is dysregulated in cancer cells leading to increased access to telomeric substrates.

The synthesis and elongation of telomeric repeat sequences by telomerase involves the sequential addition of deoxynucleotide triphosphates (dNTPs) to the 3'-hydroxyl group of telomeric ssDNA overhang (Wyatt *et al.,* 2010). The process begins with binding of the telomerase enzyme to anchor sites within the 5'-ssDNA sequence and alignment of the 3'-ssDNA sequence with the template strand located with the hTR component (Figure 1.8C). The hTERT RT domain then catalyzes the addition of dNTPs to the 3'-ssDNA through reverse transcription of the hTR template strand. Once the 5'-template boundary element is reached, telomerase undergoes a translocation reaction whereby the enzyme repositions to the new 5'-ssDNA anchor site and aligns the hTR template strand to initiate another round of dNTP addition. This process of sequential dNTP incorporation is known as nucleotide addition

processivity, which is unique to the telomerase enzyme and can result in the addition of numerous telomere repeats before telomerase disassociates from the telomere.

1.3.3.3 Telomere Dysfunction and Replicative Senescence

In 1990, Harley *et al.,* discovered that telomeric DNA lengths within normal human fibroblasts decrease with increasing passage. However, within immortal human cell lines, such as the HeLa cell line, telomere elongation mechanisms appeared to counteract progressive telomere shortening. Gradual telomere shortening within a yeast strain defective in the *est1* (ever shorter telomeres) gene, was associated with induction of a senescent-like growth arrest, similar to that observed within higher eukaryotic cells (replicative senescence). It was therefore proposed that telomeres act as a 'mitotic clock' to limit the replicative lifespan of normal human somatic cells *in vitro* and *in vivo.* Furthermore, due to the known role of telomeres in stabilizing chromosome termini and protecting against end-to-end fusions, loss of telomeric sequences was thought to explain the higher frequency of dicentric chromosomes observed within senescent human fibroblasts (Harley., 1991).

Using a highly sensitive biochemical assay for measuring the levels of telomerase activity within human cells, Kim *et al.,* (1994) demonstrated that 98% of immortal human cell lines derived from 18 different human tumour types and over 90% of tumour tissue biopsy samples from 12 different tumour types, were positive for telomerase activity, whereas normal human cell strains and noncancerous tissue biopsy samples derived from the same tissues were negative. These findings demonstrate a strong association between cellular immortality and the activation of telomere maintenance mechanisms, which strengthens the hypothesis that progressive telomere shortening within telomerase-deficient, normal human somatic cells, may act as a critical tumour suppressive barrier to cellular immortalization by limiting cellular replicative potential. Subsequent studies using more sensitive methods of telomerase detection have revealed that normal stem/progenitor cells residing within the highly proliferative tissues such as the bone marrow (haematopoietic), skin (hair follicle), and gastrointestinal tract (crypt epithelium) as well as cells within reproductive organs (ovary and testes), germ cells and activated lymphocytes, exhibit detectable levels of telomerase activity (reviewed by Artandi and DePinho., 2010). However, telomerase activity levels within these stem/progenitor cells does not appear to be sufficient to maintain telomere lengths with increasing age (Broccoli *et al.,* 1995).

Definitive proof of a causal relationship between progressive telomere shortening and induction of replicative senescence was provided by Bodnar *et al.,* (1998), who demonstrated that ectopic expression of the gene encoding the telomerase reverse transcriptase subunit (*hTERT*) within telomerase-negative primary human fibroblasts and epithelial cells, was associated with telomere elongation and continued cell division beyond the replicative lifespan

exhibited by negative controls, which displayed progressive telomere shortening and entered replicative senescence. However, *hTERT*-expressing cells were found to display a normal karyotype and did not exhibit any morphological features of transformed cells (for example loss of contact inhibition), indicating that telomerase activity alone was insufficient to confer malignant transformation of normal cells.

Taking these observations into account, together with viral transforming gene studies, it is thought that p53 functions as a key mediator of replicative senescence (M1) induction in response to critically shortened telomeres (Newbold., 2002). In support of this, normal human fibroblasts have been found to exhibit increasing levels of DNA damage response factors such as γ-H2AX, ATM, ATR, 53BP1, BRCA1, Mre11 and ATRIP localized to telomeres, indicating that telomere shortening induces a DNA damage response (Herbig *et al.,* 2004). Co-localization of DNA damage response factors and telomeres, known as telomere-dysfunction induced foci (TIF), have also been identified following inhibition of the telomere-associated TRF2 protein within human fibroblasts (Takai *et al.,* 2003). Disruption of TRF2 binding resulted in telomere uncapping, chromosome-end fusions and rapid activation of ATM/p53-mediated DNA damage response pathways leading to apoptosis. Interestingly, Herbig *et al.,* (2004) found that the telomere-associated TRF2 protein was absent from the majority of TIF in senescent fibroblasts, which demonstrates that destabilization of the protective cap occurs when telomeres become critically shortened. Furthermore, in some senescent cells, single TIF were found to be associated with multiple telomeres, indicating that chromosomal end-associations (a characteristic feature of telomere dysfunction) may also trigger a DNA damage response. There is evidence to suggest that, in any given cell, only one or two critically shortened telomeres are sufficient to produce γ-H2AX foci and initiate a DNA damage checkpoint response (Hemann *et al.,* 2001, Shay and Wright., 2005). Damaged telomeres within senescent fibroblasts were found to activate ATM/Chk1/Chk2/p53/p21 signalling pathway, leading to cell cycle arrest in G1 phase (Herbig *et al.,* 2004). This provides evidence that dysfunctional telomeres are recognized by DNA damage response machinery and are responsible for initiating p53-mediated replicative senescence or pro-apoptotic pathways.

Disruption of p53 function, either by mutation or introduction of SV40 LT antigen prevents p53-mediated growth arrest in response to critical telomere shortening (Newbold., 2005). As a result, DNA damage checkpoint responses are mitigated and cells continue to divide. An increasing number of telomeres become critically shortened, leading to chromosomal end-to-end fusions (dicentric chromosomes), chromosome breakage-fusion-bridge (BFB) cycles and loss of vital genetic material. Cells then enter crisis (M2), where most cells remain and where cell growth is balanced by cell death due high levels of genomic instability. Rare immortal clones that emerge from crisis acquire mechanisms that maintain or extend telomere lengths, allowing them to divide continuously. This is thought to be a critical step in human

carcinogenesis. The majority of these immortal clones maintain telomere lengths through reactivation of telomerase, which is thought to be due to loss of stringent telomerase regulatory mechanisms during crisis (Meyerson *et al.,* 1997, Kim *et al.,*1994, Newbold., 2005). Having acquired a selective growth advantage over neighbouring cells, these immortal variants are predicted to undergo clonal evolution leading to malignant progression.

1.3.3.3.1 Coevolution of Telomerase Activity, Replicative Senescence and Body Mass

As any single cell may undergo malignant transformation, the need for additional lifetime cell divisions within large, long-lived species may confer a greater risk for the development of spontaneous tumours compared with small, short-lived species. However, no correlation between body size and risk of cancer development has been found to exist (Caulin and Maley., 2011). Therefore, large, long-lived species are believed to have evolved tumour suppressive mechanisms that counteract the increased risk for spontaneous tumour development.

Induction of replicative senescence pathways in response to critical telomere shortening is thought to have evolved as a powerful tumour suppressor mechanism that limits the replicative capacity of cells and prevents malignant transformation (Campisi., 2001). Aside from normal human somatic cells, replicative senescence has also been found to occur within fibroblasts from Anthropoids such as New World Primates (squirrel monkey and spider monkey) and Old World Primates (rhesus monkey, orangutan and pigmy chimpanzee), and Ungulates such as sheep, cow and horse (Steinert *et al.,* 2002, Gorbunova and Seluanov., 2009). Fibroblasts derived from these species were also found not to express detectable levels of telomerase. In complete contrast, many somatic tissues of small, short-lived rodents such as the hamster, mouse and rat, have been found to express telomerase activity and enter a telomere length-independent growth arrest termed stress-induced premature senescence (SIPS) (Newbold., 2005). Exposure of mouse somatic cells to supraphysiological levels of oxygen (20%) is associated with slowed growth after around 12-15PD followed by the emergence of immortal clones after a few days. Normal human somatic cells also possess the premature senescence pathway that is activated in response to stress or oncoproteins (see Chapter 1.3.2.1), however spontaneous immortalization of human somatic cells has never been found to occur *in vitro* (Stampfer and Yaswen., 2003). Therefore, replicative senescence is thought to provide an additional mechanism that protects against cancer development within human somatic tissues and those of other large, long-lived species. These observations suggest that significant differences exist between the barriers to immortal transformation and cancer within small, short-lived species and large, long-lived species.

The differences between the telomere biology of humans and mice could be due to differences in lifespan or body mass (Gorbunova and Seluanov., 2009). In order to explore the

evolutionary relationship between telomerase activity, organism lifespan and body mass, Seluanov and colleagues examined telomerase activity within seven somatic tissues of individuals from a set of 15 different rodent species, representing a range of different sizes and lifespans (Seluanov *et al.,* 2007). Interestingly, while the majority of rodent tissues were found to express telomerase activity, tissues from small, but longer-lived rodents such as the grey squirrel and naked mole rat, were also found to express telomerase activity. On the other hand, tissues from the two largest rodent species (beaver and capybara), were not found to express any detectable telomerase activity. Overall, a negative correlation was observed between telomerase activity and body mass, but no correlation was observed between telomerase activity and rodent lifespan. A subsequent study, which examined the proliferation rate and capacity of fibroblasts derived from each of the rodent species, revealed that larger rodent fibroblasts entered replicative senescence in culture, whereas fibroblasts from small, short-lived rodents proliferated continuously (Seluanov *et al.,* 2008). Interestingly, telomerase-positive fibroblasts from small, longer-lived rodents were found to exhibit a slow proliferation rate compared with small, short-lived rodent species. This suggests that small, long-lived species may have evolved alternative mechanisms that protect against tumour development.

Based on these observations, it has been proposed that telomerase repression and replicative senescence are adaptive tumour suppressive mechanisms that have coevolved with an increase in organism body mass, as larger animals contain more cells and therefore have a greater risk for cancer development (Gorbunova and Seluanov., 2009).

1.3.3.4 Telomere Dysfunction and Cancer

It is generally accepted that genetic instability, such as DNA copy number alterations, drives malignant transformation and leads to the development of advanced disease. The initiation of DNA damage pathways in response to one or two uncapped telomeres or chromosome end-associations, is thought to be a powerful barrier to tumour development. However, telomere dysfunction in conjunction with loss of DNA damage signalling checkpoints is believed to be a major mechanism underlying genomic amplifications and deletions in human cancers (Artandi and De Pinho., 2010). The acquisition of telomere maintenance mechanisms during crisis is thought to prevent ongoing telomere dysfunction and genomic instability so that cells remain viable, but retain some level of instability for clonal evolution and malignant progression. This hypothesis is consistent with the observation that telomere lengths within pre-invasive and invasive carcinomas such as breast, pancreatic or prostate cancers, are generally significantly shorter than normal epithelial cell counterparts, which suggests that a period of telomere shortening occurs early in the development of multiple epithelial cell cancers (Meeker *et al.,* 2002, 2004). Concomitant with telomere shortening, pre-invasive colon cancer lesions exhibit high levels of genomic instability, including anaphase bridges (a hallmark of telomere

dysfunction). Up-regulation of telomerase occurs during later stages of colon cancer development, which is thought to promote telomere stability and a reduce the rate of chromosomal alterations during invasive and metastatic stages (Chadeneau *et al.,* 1995, Artandi and De Pinho., 2010). Therefore, telomere dysfunction plays an important tumour suppressive role by limiting the proliferative capacity of human cells, but can also be responsible for initiating chromosomal instability, which is a key driver of malignant progression.

1.3.4 TELOMERASE AND CANCER

The observations made by Kim and colleagues in 1994, demonstrated a specific association between cancer cell immortality and telomerase activity (Kim *et al.,* 1994). Functional proof of this relationship was demonstrated a few years later, after genes encoding the human reverse transcriptase enzyme component (*hTERT*) and the human telomerase RNA subunit (*hTR*) were cloned (Meyerson *et al.,* 1997, Kilian *et al.,* 1997, Feng *et al.,* 1995). Northern analysis of *hTR* expression levels within normal human primary and pre-crisis human cell strains and immortalized cells revealed that *hTR* transcripts could be detected within both telomerase-positive immortal cell lines and telomerase-negative primary and pre-crisis cell strains (Avillon *et al.,* 1996). Despite observing slightly higher *hTR* levels within human tumour samples compared with normal tissue samples, a positive correlation between *hTR* expression and telomerase activity was not observed. These findings demonstrate that an increase in *hTR* expression is unlikely to be responsible for the level of telomerase activity observed within human tumour cells.

On the other hand, studies have shown that telomerase-positive cancer cell lines, primary tumours and normal telomerase-positive human tissues such as the intestine and testes exhibit high levels of *hTERT* expression, whereas telomerase-negative, mortal fibroblast strains or normal human tissues such as the prostate, heart, brain and skeletal muscles do not (Meyerson *et al.,* 1997, Kilian *et al.,* 1997). Using an *in vitro* model of cellular immortalization involving the transformation of normal human lymphocytes and embryonic kidney cells with the Epstein-Barr virus (EBV) and the SV40-T antigen respectively, Meyerson *et al.,* (1997) demonstrated that *hTERT* expression was specifically associated with emergence from crisis and telomerase activation. These findings demonstrate a strong correlation between *hTERT* expression and telomerase activity within normal human tissues, tumours and immortal cell lines, and suggest that de-repression of *hTERT* may be required for telomerase expression within tumour cells. In further support of this, ectopic expression of *hTERT* alone is sufficient to confer immortal transformation of normal human fibroblasts *in vitro* (Counter *et al.,* 1998, Bodnar *et al.,* 1998). In addition, expression of a dominant-negative *hTERT* mutant within telomerase-positive human tumour cells was associated with telomere shortening and cell death

in vitro, and elimination of tumour formation *in vivo* (Hahn *et al.,*1999). These findings not only provide evidence to suggest that the major rate-limiting component of telomerase activity within tumour cells is hTERT, but also demonstrate that inhibition of telomerase is sufficient to restore a limited cellular lifespan and inhibit tumour cell growth.

In further support of the notion that *hTERT* de-repression is a critical step for telomerase reactivation during human carcinogenesis, the high-risk human papilloma virus 16 (HPV16) E6 and E7 oncoproteins have been found to immortalise primary human keratinocytes through induction of *hTERT* transcription and activation of telomerase activity (Klingelhutz *et al.,* 1996, Liu *et al.,* 2008). HPV16 has been linked to cervical, anal and head and neck carcinoma aetiology (zur Hausen., 2002, Gillison *et al.,* 2001). E6 binds directly with *hTERT* regulatory molecules c-Myc, NFX-1 and the E6AP ubiquitin ligase to mediate activation of *hTERT* expression and there is evidence to suggest that E6 also interacts with the hTERT protein to increase telomerase activity through a post-transcriptional mechanism (Liu *et al.,* 2008, 2009).

Using a quantitative real-time polymerase chain reaction (RT-PCR)-based technique, Ducrest *et al.,* (2001) demonstrated that both pre-spliced and post-spliced *hTERT* transcripts could be detected within telomerase-positive tumour-derived cell lines, but not within telomerase-negative human fibroblasts. On average, telomerase-positive cancer cell lines were found to contain 0.2-6 molecules of post-spliced *hTERT* mRNA per cell. A strong correlation between the number of nuclear, pre-spliced *hTERT* transcripts and the number of cytoplasmic, post-spliced *hTERT* transcripts was observed within all telomerase-positive and negative cells analyzed. These findings provide evidence to suggest that telomerase activity within normal and cancer cells is dependent on the transcriptional control of *hTERT.* Using a reporter construct containing sequences upstream of *hTERT,* Cong *et al.,* (1999) demonstrated that the *hTERT* promoter is inactive within normal human cells, but is activated during cellular immortalization. This suggests that telomerase reactivation during cellular immortalization may be due to transcriptional de-repression of *hTERT.* It has therefore been proposed that emergence from crisis and telomerase reactivation may be due to acquisition of a rare genetic or epigenetic change that results in the functional loss of critical *hTERT* transcriptional repressor genes (Newbold., 2005, Stampfer and Yaswen., 2002). The low frequency of spontaneous emergence from crisis ($<10^{-6}$) indicates that stringent mechanisms of *hTERT* transcriptional repression exist within normal human somatic cells. This has prompted researchers to investigate the mechanisms responsible for mediating *hTERT* transcriptional repression within normal human cells and which of these may be disrupted to confer immortal transformation.

1.3.4.1 Extra-Telomeric Functions of Telomerase

Aside from the established functional role of telomerase to extend telomeric repeat sequences, there is new evidence to suggest that extra-telomeric functions of telomerase exist to

promote tumour growth (reviewed by Mocellin *et al.,* 2013). Constitutive activation of the Wnt/β-catenin signalling has been found to promote tumorigenesis of the breast, skin and bone marrow (Fodde and Brabletz, 2013). This is due to the downstream transcriptional activation of a variety of target genes involved in proliferation and differentiation, which is thought to play a major role in both cancer development and metastasis. The telomerase reverse transcriptase subunit (TERT), has been found to interact with the chromatin-remodelling protein BRG1, and form part of the β-catenin transcription complex, that functions to modulate the expression of target genes (Park *et al.,* 2009). In addition, it has been found that under periods of cell stress, TERT is excluded from the nucleus and localizes to the mitochondria, where it protects mitochondrial DNA (mtDNA) and prevents the induction of mitochondrial apoptotic pathways (Ahmed *et al.,* 2008). TERT down-regulation has been shown to cause activation of the pro-apoptotic factor Bax, which leads to induction of apoptosis (Martinez and Blasco., 2011). These observations provide evidence that telomerase plays a critical role, not only in the early events of cancer development, but also in disease progression.

1.3.5 REGULATION OF HUMAN HTERT

Studies have shown that the major rate-limiting component of the human telomerase enzyme, hTERT, is regulated at the transcriptional level by a variety of transcription factors, oncogenes, tumour suppressor genes, hormones and unknown sequences present within the genome (reviewed by Kyo *et al.,* 2008 and Daniel *et al.,* 2012). Epigenetic alterations such as DNA methylation and histone modifications are thought to provide another level of regulation by modulating access of the *hTERT* promoter to activating/repressing factors. Splicing patterns and alternative splice variants of the *hTERT* gene have also been implicated regulating hTERT by maintaining an optimal level of hTERT expression, which is essential for cell survival. Post-translational modifications of hTERT, such as phosphorylation and ubiquitination, are important for modulating hTERT protein stability and nuclear localization, which are critical for telomerase-dependent telomere length maintenance mechanisms within human cancer cells.

1.3.5.1 Characterization of the *hTERT* Promoter

The 5'-promoter region of *hTERT* was cloned by several groups in 1999 (Cong *et al.,* 1999, Takakura *et al.,* 1999, Horikawa *et al.,* 1999). Using a 1.7kb fragment comprising upstream sequences of *hTERT* to control the expression of the firefly luciferase reporter gene, Horikawa *et al.,* (1999) demonstrated that only cancer cell lines (SiHa and RCC23) but not normal human cells (SUSM-1) exhibited high levels of luciferase activity, which provides additional evidence that *hTERT* gene expression is controlled primarily at the transcriptional level. The *hTERT* promoter does not contain detectable TATA or CAAT boxes but is rich is CpG dinucleotides and contains a variety of transcription factor binding sites and hormone

response elements spanning at least -2670bp upstream of the transcription start site (TSS) (Figure 1.9, Kyo *et al.,* 2008).

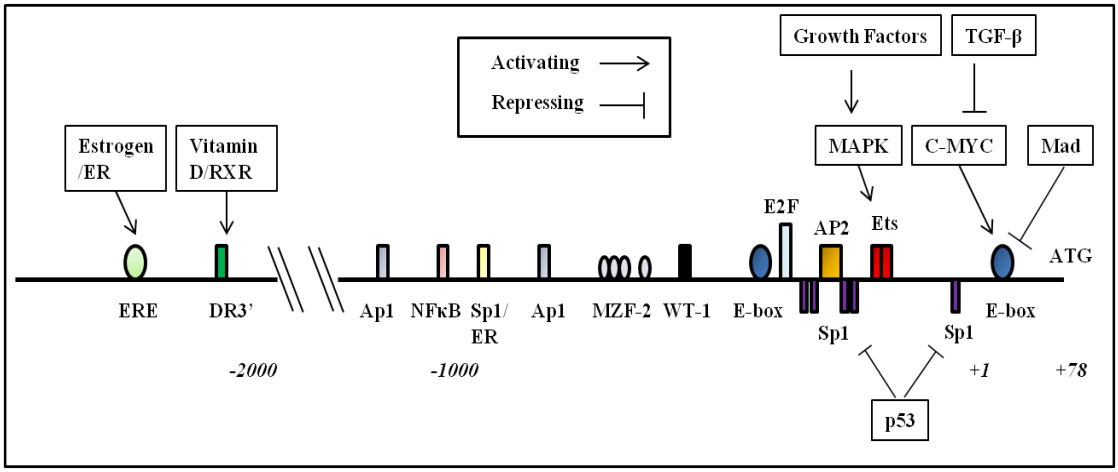


Figure 1.9 - *Schematic representation of the hTERT promoter and the approximate positions of activating and repressing cis-acting elements with known interacting proteins/factors (Adapted from Kyo et al., 2008). Abbreviations: ERE (oestrogen responsive element), ER (oestrogen receptor), RXR (retinoid X receptor), DR3' (degenerated vitamin D3 receptor/retinoid X receptor binding site), WT-1 (Wilms' tumour 1).*

The minimal/core promoter region of *hTERT* is positioned -330bp upstream to +361bp downstream of the TSS and contains two E-box binding elements (-165bp to -160bp and +44bp to +49bp), five zinc finger transcription factor Sp1 binding sites (within 110bp upstream of TSS), two juxtaposed Ets transcription factor binding sites (-22bp to -14bp), an E2F transcription factor binding site (-174bp to -170bp) and a Wilms' tumour suppressor 1 (WT-1) binding site (-283bp to -273bp) (reviewed by Janknecht., 2004). E-box elements (CACGTG) are binding sites for the c-Myc/Max/Mad family of basic helix-loop-helix zipper (bHLHZ) transcription factors. Upstream of the *hTERT* core promoter are several other transcription factor binding sites such as four myeloid-specific zinc finger protein 2 (MZF-2) binding sites (spanning -687bp to -507bp), several activating protein 1 (Ap1/JUN) proto-oncogene binding sites (at -1655bp and -718bp) and a nuclear factor-kappa-B (NFκB) transcription factor complex binding site (Junknecht., 2004, Kyo *et al.,* 2008). Two oestrogen receptor (ER) binding sites are also present upstream of the core *hTERT* promoter sequence (-873bp to -859bp and -2677bp to -2665bp), one of which is a ER half-site coupled with a canonical Sp1 binding site. A vitamin D and retinoic acid nuclear hormone receptor response element DR3' (-2530bp to -2516bp) is positioned adjacent to the most distal ER binding site (Figure 1.9). The presence of multiple binding sites for several transcription factors and hormone receptors that are important effectors for vast array of signalling pathways, suggests that *hTERT* transcription may be controlled and regulated in response to a variety of different stimuli.

1.3.5.2 Transcriptional Regulation

1.3.5.2.1 Transcriptional Activators

1.3.5.2.1.1 Transcription Factors

The bHLHZ transcription factor c-Myc is responsible for regulating a variety of normal cellular functions including proliferation and differentiation (Daniel *et al.,* 2012). Abrogation of c-Myc function, either through mutation or overexpression as a result of gene translocation or amplification, has been implicated in multiple cancer types. Several groups have shown that c-Myc forms a heterodimer complex with Max and binds to E-box elements within the *hTERT* promoter to activate transcription (reviewed by Kyo *et al.,* 2008). However, many of these studies used recombinant c-Myc and exogenous c-Myc overexpression to demonstrate transcriptional activation of *hTERT* through binding of c-Myc to the *hTERT* promoter, which may not reflect the function of endogenous c-Myc *in vivo* (Kyo *et al.,* 2008). Furthermore, studies have reported no correlation between *hTERT* and *c-Myc* expression levels within some cancer cell types, including breast cancer (Kyo *et al.,* 2008, Kirkpatrick *et al.,* 2003). However, Xu *et al.,* (2001) demonstrated that endogenous c-Myc binds to E-box elements within the *hTERT* promoter within exponentially proliferating but not differentiated HL60 human promyelocytic leukaemia cells. Therefore, it is unclear whether deregulation of endogenous c-Myc expression plays a critical role in the transcriptional activation of *hTERT* during carcinogenesis *in vivo*.

In 2003, Goueli and Janknecht demonstrated that bHLHZ upstream transcription factors (USF) 1 and 2 bind more readily to E-box elements within the *hTERT* promoter than c-Myc/Max within both *hTERT*-positive and negative cells. An increase in *hTERT* promoter activity was observed within cancer cells following joint expression of USF1 and USF2, compared with expression of each one alone, indicating that these proteins may function collectively to induce *hTERT* transcription within cancer cells. Exogenous expression of the USF coactivator protein p300, was associated with enhanced *hTERT* promoter activity within *hTERT*-expressing human embryonic 293T cells, whereas inhibition of the p38-MAP kinase (an activator of USF proteins), reduced *hTERT* promoter activity. These findings demonstrate that several upstream signalling pathways modulate USF1 and USF2-mediated activation of *hTERT* transcription. Goueli and Janknecht., (2003) propose that increased proliferative signalling pathways may lead to increased p38-MAP kinase or p300 activity and USF-mediated induction of *hTERT* during human carcinogenesis. However, exogenous overexpression of p38-MAP kinase within *hTERT*-negative normal human fibroblasts was not associated with induction of *hTERT* expression and telomerase activity, which suggests that activation of USF proteins may not be responsible for *hTERT* de-repression during human carcinogenesis.

The zinc finger transcription factor Sp1, has been shown to bind five GC-boxes located on the *hTERT* promoter and activate *hTERT* transcription (Kyo *et al.,* 2000). Substitution mutations within each Sp1 binding site was associated with a slight reduction in *hTERT* transcriptional activity within different cancer cell lines, whereas mutations within all Sp1 binding sites resulted in a greater than 90% loss in *hTERT* promoter activity, even when c-Myc/Max binding elements were intact. Furthermore, examination of Sp1 and c-Myc protein levels within human fibroblasts at various stages during the process of immortal transformation, revealed that Sp1 and c-Myc expression was up-regulated within *hTERT* and telomerase-positive cell clones that had overcome the replicative senescence barrier. These findings indicate that binding of Sp1 to GC-boxes within the *hTERT* promoter maintains transcriptional activation of *hTERT* within cancer cells *in vitro* and also suggest that Sp1 and c-Myc may cooperate to induce *hTERT* transcription during cellular immortalization. In addition, Sp1 has been shown to cooperate with the nuclear factor of activated T cells (NFAT) to induce *hTERT* expression within activated lymphocytes (Chebel *et al.,* 2009). Silencing of *NFAT1* within MCF-7 breast cancer and Jurkat leukaemia cell lines was associated with reduced *hTERT* expression levels. However, Sp1 has been found to be expressed ubiquitously within normal human cells (Kyo *et al.,* 2008). Furthermore, transcriptional repression of *hTERT* by an unknown repressor sequence(s) on human chromosome 3 within RCC23 renal cell carcinoma cells, was not associated with significant changes in Sp1 binding on the *hTERT* promoter (Horikawa *et al.,* 2002). Therefore, Sp1 alone is unlikely to be responsible for mediating transcriptional activation of *hTERT* during immortal transformation.

Studies have shown that members of the Ets family of transcription factors play a functional role in *hTERT* transcriptional regulation (reviewed by Gladych *et al.,* 2011). The Ets family comprises at least thirty members, which share a common erythroblast transformation-specific (ETS) domain. Knockdown of Ets-2, but not Ets-1, within the MCF-7 breast cancer cell line, was associated with a significant reduction in *hTERT* expression and telomerase activity and increased cell death (Dwyer and Liu*.,* 2009). Reconstitution of *hTERT* was found to rescue this effect, indicating that loss of telomerase activity was responsible for induction of cell death. Binding of Ets-2 to two Ets binding sites within the *hTERT* promoter (Figure 1.9) was found to be responsible for Ets-2-mediated induction of *hTERT* expression, as mutation of either site was associated with telomerase repression. Other Ets members including, ER81, PEA2 and FLI1 have also been implicated in telomerase regulation through binding to Ets sites within the *hTERT* promoter within Ewing's sarcoma cell lines (Takahashi *et al.,* 2003). It has therefore been proposed that *hTERT* transcription is regulated by the competitive binding of activating and repressing Ets members. As such, constitutive expression of activating Ets members, such as Ets-2, may be responsible for inducing *hTERT* transcription during carcinogenesis. In addition to gain-of-function mutations, constitutive activation of *hTERT*-inducing Ets

transcription factors may also be due to deregulation of upstream growth factor signalling pathways. Activation of the epidermal growth factor receptor (EGFR) and the epidermal growth factor receptor (EGFR)-related receptor tyrosine kinase HER2, has been associated with induction of *hTERT* transcription through activation of the mitogen activated protein kinase (MAPK) pathway and binding of Ets members to the *hTERT* promoter (Goueli and Janknecht., 2004, Kyo *et al.,* 2008). Therefore increased growth factor signalling may also contribute to Ets-mediated *hTERT* transcription in cancer cells.

1.3.5.2.1.2 Hormones

As shown in Figure 1.9, the *hTERT* promoter contains two oestrogen response elements (ERE) located upstream of the *hTERT* TSS. The oestrogen steroid hormone has been found to up-regulate telomerase activity within breast and prostate cancer cells (Daniel *et al.,* 2012). Studies have shown that ligand-activated oestrogen receptor α (ERα) binds to ERE within the *hTERT* promoter to mediate transcriptional activation of *hTERT* (reviewed by Kyo *et al.,* 2008). There is also evidence to suggest that oestrogen induces telomerase activity within human ovarian cancer cells via Akt-dependent phosphorylation of hTERT (Kimura *et al.,* 2004). Therefore, oestrogen activates telomerase through transcriptional and post-transcriptional regulation of *hTERT*.

The androgen steroid hormone has also been shown to up-regulate *hTERT* transcription within androgen-sensitive LNCap prostate cancer cells (Guo *et al.,* 2003). Using an *in vivo* model of prostate cancer, androgen deprivation was associated with a reduction in *hTERT* mRNA levels and telomerase activity. In 2010, Nourbakhsh and colleagues demonstrated that exposure of ovarian adenocarcinoma cells to androgen hormones testosterone and androstenedione, was associated with a significant increase in *hTERT* expression, hTERT phosphorylation and telomerase activity. Androgen-induced *hTERT* expression was abolished following treatment of cells with phosphotidylinositol 3-kinase (PI3K) inhibitors. Therefore, unlike oestrogen, androgen hormones stimulate *hTERT* expression through an indirect mechanism. Androgen receptors (AR) have been found on the surface of ovarian and prostate cancer cells and can mediate androgen-dependent and androgen-independent growth of prostate cancer cells (Nourbakhsh *et al.,* 2010, Haag *et al.,* 2005). Androgens are also thought to play an important role in the pathogenesis of ovarian cancer, as exposure of normal human ovarian epithelial cell growth to the synthetic androgen mibolerone is associated with increased cell proliferation and inhibition of cell death (Edmonson *et al.,* 2002). Therefore, androgen-dependent up-regulation of *hTERT* expression and telomerase activity may contribute to tumorigenesis within androgen-sensitive tissues.

1.3.5.2.2 Transcriptional Repressors

1.3.5.2.2.1 Transcription Factors

Using a genetic screening approach to identify genes responsible for mediating repression of *hTERT* transcription within normal human somatic cells, Oh *et al.,* (2000) isolated the Mad1 bHLHZ transcription factor. The Mad1/Max heterodimer was shown to bind the *hTERT* promoter at the same E-box elements within the *hTERT* promoter as c-Myc/Max heterodimers. Abrogation of E-box elements by site-directed mutagenesis was associated with a significant increase in *hTERT* promoter activity within normal human diploid fibroblasts, indicating that Mad1 may be responsible for mediating repression of *hTERT* transcription within normal human cells (Oh *et al.,* 2000). Furthermore, exogenous expression of Mad1 was found to suppress *hTERT* transcription within immortalized human kidney cells (293). This effect was reversed upon mutation of E-box binding elements or through increasing amounts of co-transfected c-Myc expression plasmid. These findings suggest that Mad1 and c-Myc compete for the same binding site within the *hTERT* promoter sequence but have opposing effects on *hTERT* transcription. Interestingly, a switch from c-Myc/Max binding to Mad1/Max binding was observed when *hTERT*-expressing human promyelocytic leukaemia cells were induced to differentiate (Xu *et al.,* 2001). Furthermore, exogenous expression of Mad1 within human bladder cancer cells was associated with transcriptional repression of *hTERT* (Daniel *et al.,* 2012). Oh *et al.,* (2012) found that immortalized 293 kidney cells and HeLa cervical carcinoma cells exhibited higher levels of c-Myc protein than normal human kidney cells and fibroblasts, whereas Mad1 protein levels were found to be higher within normal human cells than immortal cell lines. Therefore increased c-Myc expression is thought to contribute to transcriptional activation of *hTERT* during carcinogenesis by causing a shift in Mad1/Max dimerization to c-Myc/Max dimerization. However, studies have shown that some cancer types demonstrate no correlation between *c-Myc* and *hTERT* expression levels (see above), which indicates that induction of *c-Myc* expression cannot account for transcriptional activation of *hTERT* within all human cancer cells.

The CCCTC binding factor (CTCF) is a member of the BORIS (Brother of the regulator of Imprinted Sites) and CTCF gene family that contains eleven highly conserved zinc finger domains and functions as a transcriptional regulator of multiple target genes (Daniel *et al.,* 2012). CTCF has been shown to bind to CCCTC binding sites located within exons 1 and 2 of *hTERT* within telomerase-negative normal human cells but not within telomerase-positive tumour cell lines, which suggests that CTCF may function as a repressor of *hTERT* within normal human cells (Renaud *et al.,* 2007). The ability of CTCF to bind to 5'-exonic regions of *hTERT* was found to be dependent on methylation of CpG sites within the core promoter. Treatment of telomerase-positive tumour cells with the demethylating agent 5'-aza-2'-deoxycytidine (5-azadC) resulted in increased CTCF binding to *hTERT* exonic regions and

transcriptional repression. These findings suggest that changes in DNA methylation patterns around the *hTERT* promoter during tumorigenesis, may play an important role in de-repression of *hTERT* transcription by preventing access of CTCF.

1.3.5.2.2.2 Tumour Suppressor Genes

The Menin tumour suppressor gene, encoded by the gene *MEN-1* (multiple endocrine neoplasia 1), has been found to play a critical role in the transcriptional repression of *hTERT* (Lin and Elledge., 2003). Overexpression of Menin was associated with reduced *hTERT* expression within *hTERT*-positive HeLa cervical cancer cells, whereas depletion of Menin within *hTERT*-negative ALT-positive U2OS osteosarcoma cells was associated with increased *hTERT* expression levels. Importantly, short hairpin-RNA (shRNA)-mediated depletion of Menin within normal human BJ fibroblast cells led to increased telomerase activity and immortal transformation. Menin is thought to mediate *hTERT* transcriptional repression by interacting with AP1 and NFκB binding sites (Figure 1.9), thereby preventing access of transcriptional activators JunD and NFκB to the *hTERT* promoter respectively. These findings suggest that loss of Menin may contribute directly to activation of *hTERT* expression during tumour development. In support of this, individuals carrying germline *MEN-1* mutations have multiple endocrine neoplasia type 1 syndrome, which is characterized by the development of neoplastic lesions within endocrine glands such as the pancreas or pituitary. Somatic mutations in the *MEN-1* gene are prevalent within sporadic endocrine cancer types including parathyroid tumours (54%), gastrinoma (33%), pancreatic neuroendocrine tumours (44%) and insulinoma (17%) (Marx *et al.,* 1999, Jiao *et al.,* 2011). However, *MEN-1* mutations have been identified within less than 1% of breast and ovarian cancer types (catalogue of somatic mutations in cancer, COSMIC, Forbes *et al.,* 2015), therefore, functional loss of Menin cannot account for transcriptional activation of *hTERT* within all cancer types.

The Wilms' tumour 1 (WT-1) transcription factor is an important regulator of cell growth, development, differentiation and apoptosis and has been found to exhibit both tumour suppressive and oncogenic roles in tumorigenesis (Driessche *et al.,* 2012). In 1999, Oh and colleagues demonstrated that WT-1 suppresses *hTERT* transcription and telomerase activity within immortalized human kidney cells (293), but not HeLa cervical carcinoma cells, through binding to a WT-1 binding sequence within the *hTERT* promoter (Oh *et al.,* 1999). Disruption of the WT-1 binding site within the *hTERT* promoter was found to increase *hTERT* promoter activity within 293 cells, but not HeLa cervical carcinoma cells. Therefore, WT-1 may have cell type-specific effects on *hTERT* expression regulation. Sitaram *et al.,* (2010) found that overexpression of WT-1 within clear cell renal cell carcinoma (ccRCC) cells was also associated with downregulation of *hTERT* expression and telomerase activity. Interestingly, WT-1 was found to mediate transcriptional repression of *hTERT* within ccRCC cells, not only through direct binding to the *hTERT* promoter, but also by activating the expression of negative

regulators of *hTERT* such as *SMAD3* and *ETS1,* and repressing the expression of positive *hTERT* regulators including *c-Myc* and *NFX1* (Sitaram *et al.,* 2010). Therefore WT-1 may repress *hTERT* expression through direct and indirect mechanisms. However, the effects of WT-1-mediated *hTERT* transcriptional repression on cell growth were not reported in either study, therefore it is unclear whether reconstitution of WT-1 alone is sufficient to confer *hTERT* repression and restore a limited cellular replicative potential. Furthermore, the lack of WT-1-mediated *hTERT* transcriptional repression within HeLa cells, suggests that loss of WT-1 does not account for activation of *hTERT* expression within all cancer types.

Overexpression of wild type p53 has been shown to down-regulate telomerase activity within multiple cancer cell lines through transcriptional repression of *hTERT* (reviewed by Daniel *et al.,* 2012). The p53 tumour suppressor protein regulates the expression of a variety of target genes to mediate cell cycle arrest or apoptosis in response to stressful stimuli and is the most frequently mutated gene in human cancer (Muller and Vousden., 2014). Abrogation of Sp1 binding sites within the core *hTERT* promoter region, was found to prevent *p53*-mediated transcriptional repression of *hTERT* within human cervical squamous cell carcinoma cells (Kanaya *et al.,* 2000). A similar study demonstrated that endogenous wild type p53 interacts with and sequesters Sp1, thereby preventing Sp1 from binding to the *hTERT* promoter within MCF-7 breast cancer cells (Xu *et al.,* 2000). Interestingly, abrogation of the atypical E2F transcription factor binding site located within the *hTERT* core promoter, was associated with complete elimination of p53-mediated *hTERT* transcriptional repression within the same cell line, indicating that p53 regulates *hTERT* transcription via two independent pathways (Shats *et al.,* 2004). Knockdown of the cyclin-dependent kinase inhibitor (CDKI) p21, which is a transcriptional target of p53, together with Rb family members was also found to reduce p53-mediated transcriptional repression of *hTERT*. It was proposed that *hTERT* repression by endogenous p53 within MCF-7 cells is mediated through activation of the p21/pRb/E2F pathway and recruitment of histone deacetylases (HDAC) to the *hTERT* promoter. However, studies have shown that abrogation of *p53* function, through transduction with the SV40 Large T-Antigen, within normal human fibroblasts and post-stasis (*p16*-silenced) normal human mammary epithelial cells (HMEC), leads to crisis (M2) and the very rare emergence of immortal clones (Gao *et al.,* 1996, Gollahan and Shay., 1996, Van Der Haegen and Shay., 1993, Stampfer and Yaswen., 2003). This suggests that, loss of *p53* alone is insufficient to confer telomerase re-activation within normal human somatic cells, and a second genetic or epigenetic event must occur during crisis to cause *hTERT* de-repression and telomerase re-activation.

Won *et al.,* (2004) demonstrated that pRb-pocket protein (pRb/p107/p130)-E2F complex-mediated recruitment of HDACs is critical for regulating *hTERT* transcription within normal cycling human fibroblasts. Inhibition of HDAC recruitment to pRb-pocket protein-E2F complexes using the CGK1026 small molecule inhibitor or expression of a dominant-negative

E2F mutant, was associated with *hTERT* de-repression and telomerase re-activation. It has been proposed that increased *p16* and *p21* expression, which occurs with progressive cell divisions, results in an accumulation of hypophosphorylated pRb-pocket proteins that bind to E2F to mediate *hTERT* transcriptional repression during senescence. In this way, p16 and p53/p21-mediated activation of the pRb pathway is a powerful tumour suppressive mechanism that prevents *hTERT* de-repression and spontaneous immortalization of normal human cells (Won *et al.,* 2004). Disruption of this pathway may therefore contribute to de-repression of *hTERT* expression during carcinogenesis.

1.3.5.2.2.3 Normal Human Chromosomes

Studies have shown that multiple oncogenes, tumour suppressor genes, transcription factors and hormones, that are de-regulated within different human cancer types, have been found to play a functional role in regulating *hTERT* transcription either directly or indirectly. However, a clear explanation for the tumour-specific expression of telomerase is still lacking. Whole somatic cell fusion of virally transformed or tumour-derived immortal cell lines with normal human cells has been found to produce telomerase-repressed hybrids with a finite replicative capacity, which indicates that telomerase activity and the capacity for indefinite growth behaves as a recessive trait (Pereira-Smith and Smith., 1983). These observations suggest that mechanisms of telomerase repression exist within normal human somatic cells that may be lost/inactivated to confer telomerase re-activation and cellular immortalization during carcinogenesis. In further support of this, monochromosome transfer studies have shown that the introduction of single normal chromosome copies within tumour cell lines, is sufficient to confer *hTERT* and telomerase repression (reviewed by Ducrest *et al.,* 2002).

Table 1.5 - Summary of chromosomes found to repress *hTERT* expression and/or induce senescence pathways within different cancer cell lines

***hTERT*-Repressive Chromosome**	**Cancer Cell Line**	**Cell Type**	**Chromosomes Transferred**	**Reference**
3	21NT	Breast carcinoma	3, 8, 12, 20	Cuthbert *et al.,* (1999)
	RCC23	RCC	3, 7, 11	Horikawa *et al.,* 1998
	KC12	RCC (VHL)	3, 11	Tanaka *et al.,* 1998
	HeLa	Cervical carcinoma	3, 4, 6,11	Backsch *et al.,* 2001
4	HeLa	Cervical carcinoma	3, 4, 6, 11	Backsch *et al.,* 2001
6	FK16A	Immortalized keratinocyte	6, 11	Steenbergen *et al.,* 2001
	SiHa	Cervical carcinoma	6, 11	Steenbergen *et al.,* 2001
7	MeT5A	SV40-transformed mesothelial cell	7	Nakabayashi *et al.,* 1999
10	Li7HM	Hepatocellular carcinoma	2, 4, 5, 10, 16	Nishimoto *et al.,* 2001
17	BP1-E	Immortalized breast epithelial cell	11, 17	Yang *et al.,* 1999
None Tested	TE85	Osteosarcoma	1*	Hensler *et al.,* 1994
	SiHa	Cervical Carcinoma	2*, 3, 6, 7, 9, 11, 12	Tanaka *et al.,* 1999
	CC1	Choriocarcinoma	1, 2, 6, 7*, 9, 11	Tanaka *et al.,* 1999
	JTC-32	Bladder Carcinoma	7, 11*	Tanaka *et al.,* 1999

**Denotes chromosomes that induced senescence within those cell lines in the absence of hTERT/telomerase repression.*

Using the microcell-mediated monochromosome transfer (MMCT) technique and human: mouse monochromosomal hybrid panels, independent studies have shown that chromosomes 3, 4, 6, 7, 10, 17 are able to repress telomerase activity within different cancer types (Table 1.5, Oshimura and Barrett., 1997, Ducrest *et al.,* 2002, Tanaka *et al.,* 2005). For instance, chromosome 3 has been found to repress telomerase activity and restore cellular senescence pathways within breast and head and neck cancer, renal cell carcinoma and cervical carcinoma-derived cell lines (Cuthbert *et al.,* 1999, Tanaka *et al.,* 1998, Horikawa *et al.,* 1999, Backsch *et al.,* 2001). Horikawa *et al.,* (1999) and Tanaka *et al.,* (1999) observed progressive telomere shortening within renal cell carcinoma cell-chromosome 3 hybrids prior to induction of growth arrest. Through deletion mapping of revertant renal cell carcinoma (KC12)-chromosome 3 hybrids that had escaped senescence and microcell transfer of sub-chromosomal chromosome 3 fragments into RCC23 renal cell carcinoma cells, Tanaka *et al.,* (1998) identified a putative repressor sequence within the 3p14.2-p21.1 region. Similarly, deletion mapping of revertant breast cancer (21NT)-chromosome 3 hybrids by Cuthbert *et al.,* (1999) identified two commonly deleted regions localized to 3p12-p21.1 and 3p21.3-p22, one of which overlapped with the commonly deleted region identified by Tanaka *et al.,* (1999). These findings suggest

that a sequence(s) exists on the short arm of chromosome 3, that functions to repress telomerase activity within a variety of cancer types. However, using a genetic complementation approach, Tanaka *et al.,* (2005) demonstrated that whole cell fusion of the 21NT breast cancer cell line with KC12 and RCC23 renal cell carcinoma cell lines (21NTxKC12 and 21NTxRCC23) produced telomerase-repressed hybrids whereas whole cell fusion of both renal cell carcinoma cell lines (KC12xRCC23) produced hybrids exhibiting a similar level of telomerase activity to parental cells (Tanaka *et al.,* 2005). This suggests that at least two sequences may be present on chromosome 3p that function to repress telomerase activity in different cell types. Interestingly, introduction of chromosome 3 into the SiHa cervical carcinoma cell line (expressing HPV 16, E6 and E7 oncoproteins) was found to have no effect on telomerase activity (Tanaka *et al.,* 1999). Furthermore, introduction of chromosome 6 into the HeLa cervical carcinoma cell line, or chromosome 4 into the Li7HM hepatocellular carcinoma cell line, was found to have no effect on telomerase activity or hybrid growth potential, whereas both chromosomes were shown to repress telomerase within SiHa and HeLa cervical carcinoma cell lines respectively (Table 1.5). On the other hand, a 28.9cM region within chromosome 10p15 was found to be responsible for mediating telomerase repression within the Li7HM hepatocellular carcinoma cell line (Nishimoto *et al.,* 2001). Moreover, introduction of chromosome 7 into the MeT5A SV40-immortalized mesothelial cell line was associated with telomerase repression, whereas choriocarcinoma CC1-chromosome 7 hybrids were found to enter cellular senescence without telomerase repression (Nakabayashi *et al.,* 1999, Tanaka *et al.,* 1999). These findings suggest that different sequences exist throughout the genome that function to repress telomerase activity in a tissue-specific manner, which may be lost during carcinogenesis. However, not all chromosomes have been tested within all cancer types. Therefore, it remains possible that common telomerase regulatory sequences exist that function ubiquitously across all tissue types, which have not yet been identified.

Microcell transfer of chromosomes 3, 6, 7 and 10 has been shown to mediate a reduction in telomerase activity through repression of *hTERT* gene expression within a variety of cancer cell lines (Ducrest *et al.,* 2001, Horikawa *et al.,* 1998, Steebergen *et al.,* 2001, Nakabayashi *et al.,* 1999, Nishimoto *et al.,* 2001). This provides further evidence to suggest that the major determinant of telomerase activity is hTERT, which is controlled primarily by the rate of transcription. De-repression of *hTERT* expression through loss of a critical regulatory sequence(s) that functions *in trans* either directly or indirectly via a *cis*-acting element within the *hTERT* gene, is therefore thought to be responsible for telomerase reactivation during carcinogenesis. Results from monochromosome transfer studies show that inhibition of *hTERT* transcription is sufficient to confer telomerase repression and restore the cellular senescence program within multiple cancer types, which demonstrates that targeting cellular immortality is an effective anticancer strategy.

1.3.5.3 Epigenetic Regulation

Epigenetic mechanisms play a major role in regulating the expression of genes within eukaryotic cells by modulating the chromatin structure around gene regulatory regions (reviewed by Jaenisch and Bird., 2003). A major mechanism of epigenetic gene regulation involves methylation of cytosine residues within CpG islands by DNA methyltransferase (DNMT) enzymes. This particular molecular alteration is generally associated with stable repression of target gene expression. Another principle mechanism that influences chromatin structure is the post-translational modification of amino-terminal tails that protrude from histones H3 and H4, which includes acetylation, methylation, phosphorylation, ubiquitylation and sumoylation of particular amino acid residues (Bannister and Kouzarides., 2011). These modifications have important functional consequences for gene expression by recruiting activating/repressive enzymatic protein complexes and chromatin remodelling enzymes that reposition nucleosomes thereby altering the accessibility of factors that regulate gene expression.

Two large CpG islands have been identified within with *hTERT* promoter, which suggests that epigenetic mechanisms may play a role in the transcriptional regulation of *hTERT* (reviewed by Daniel *et al.,* 2012). Consistent with the established role of DNA methylation in gene expression regulation, studies have shown that *hTERT* promoter hypermethylation is associated with *hTERT* gene repression within certain cancer cell types. Bechter *et al.,* (2002), demonstrated that high levels of *hTERT* promoter methylation were associated with low levels of telomerase activity and vice versa, in patients with B-cell chronic lymphocytic leukaemia (B-CLL). In contrast, other studies have reported no significant correlation, or a positive correlation between *hTERT* promoter methylation and *hTERT* expression levels (Daniel *et al.,* 2012). Devereaux *et al.,* (1999) examined *hTERT* promoter methylation through bisulfite genomic sequencing within 37 normal, immortalized and tumour-derived cell lines and found that the promoter was hypomethylated within normal *hTERT*-negative cells, but was either partially or totally methylated within the majority of *hTERT*-expressing cells. An inverse relationship between the degree of *hTERT* promoter methylation and *hTERT* expression has also been reported in gastric cancer and hepatocellular carcinoma (Gladych *et al.,* 2011, Daniel *et al.,* 2012). A comprehensive analysis of *hTERT* promoter methylation patterns within telomerase-positive breast, colon and lung cancer-derived cell lines by Zinn *et al.,* (2007) demonstrated that the *hTERT* promoter was highly methylated within a region positioned 600bp upstream of the TSS, but unmethylated/partially methylated around the TSS. Furthermore, using chromatin immunoprecipitation-methylation-specific polymerase chain reaction (ChIP-MSP) assay, this group also demonstrated that histone modifications associated with active transcription, including histone 3 lysine 9 (H3K9) acetylation and histone 3 lysine 4 (H3K4) dimethylation, were associated with the unmethylated TSS of the *hTERT* promoter, whereas repressive H3K9

and H3K27 trimethyl chromatin marks were associated with methylated regions upstream of the TSS (Zinn *et al.,* 2007). These findings suggest that the unmethylated region around the TSS of *hTERT* may be necessary for *hTERT* expression within cancer cells.

Consistent with these observations, Renaud *et al.,* (2007) found that partial hypomethylation of the core promoter of *hTERT* was necessary for *hTERT* transcription and demonstrated that the CTCF transcription factor could only bind within exon 1 and repress *hTERT* transcription when this region was unmethylated. This was thought to explain why CTCF was found to bind to exon 1 within telomerase-negative normal human fibroblasts, but not within telomerase-positive HeLa and SW480 cervical and adenocarcinoma cell lines respectively. These findings suggest that DNA methylation of specific sites within the *hTERT* promoter may be responsible for the expression *hTERT* within some cancer cells by preventing access of critical *hTERT* repressors. Interestingly, Ge *et al.,* (2010) found that Mad1 mediates transcriptional repression of *hTERT* through recruitment of the histone demethylase RBP2 to the *hTERT* promoter leading to H3K4 demethylation. Depletion of RPB2 was sufficient to confer *hTERT* expression within *hTERT*-negative normal human fibroblasts. Mad1 may therefore play a critical role in maintaining *hTERT* repression within normal human somatic cells through the recruitment of RPB2.

Methylation of specific H3 histone lysine residues has also been found to play an important role in regulating *hTERT* transcription. H3K4 trimethylation is associated with active *hTERT* transcription whereas methylation of H3K9 and H3K20 is associated with *hTERT* repression (Atkinson *et al.,* 2005). The H3K4-specific di- and tri-methyltransferase enzyme SMYD3 has been shown to bind to specific sequences within the *hTERT* promoter and play a critical role in regulating *hTERT* expression (Liu *et al.,* 2007). Ectopic expression of *SMYD3* was found to induce *hTERT* expression within telomerase-negative normal human fibroblasts. Conversely, knockdown of SMYD3 within human colorectal carcinoma and Hodgkin's lymphoma-derived cells was associated with a reduction in H3K4 trimethyl marks and loss of *hTERT* expression. H3K4 trimethylation by SMYD3 was shown to activate *hTERT* transcription through recruitment of histone acetyltransferases (HAT) to the *hTERT* promoter, which promotes binding of activating transcription factors Sp1 and c-Myc (Kyo *et al.,* 2008). These findings are consistent with studies demonstrating that hyperacetylation of core histones within the *hTERT* promoter region is associated with *hTERT* expression (reviewed by Zhu *et al.,* 2010). Treatment of normal human cells and telomerase-negative immortal cell lines with the histone deacetylase (HDAC) inhibitor trichostatin A (TSA), has been found to induce *hTERT* transcription and telomerase activity. Together, these observations suggest that a complex relationship exists between DNA methylation patterns, histone modifications and activating/repressing transcription factors, to control *hTERT* gene expression in human cells.

1.3.5.4 Alternative Splicing

There is strong evidence to suggest that expression of the *hTERT* gene is regulated primarily at the level of transcription. However, RNA processing has also been shown to play an important role in regulating *hTERT* expression (reviewed by Wong *et al.,* 2014). Alternative splicing of gene transcripts is a common mechanism of gene expression regulation that amplifies the coding capacity of genes. The *hTERT* gene is 42kb in length and comprises 16 exons (Figure 1.10). To date, 22 different splice variants of the *hTERT* gene have been identified, which contain exonic deletions and/or intronic insertions that affect the *hTERT* N-terminal, reverse transcription (RT) or C-terminal domains. Only one isoform encodes the full-length transcript while the remaining have lost one or more functional domains and are unable to form a functional telomerase complex for telomere elongation (Hrdlickova *et al.,* 2012).

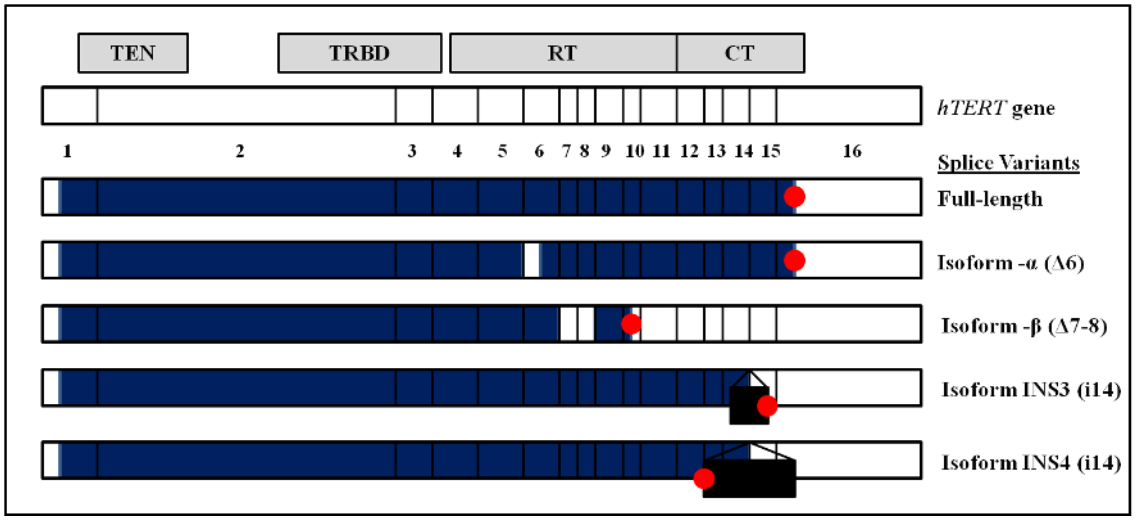


Figure 1.10 - *The structure of the human TERT (hTERT) gene with approximate positions of TEN (telomerase essential N-terminal domain), TRBD (telomerase RNA binding domain), RT (reverse transcriptase) and CT (C-terminal domain) functional domains. The open reading frames (ORF) of the full-length transcript and four splice variants α, β, INS3 and INS4 which have exons 6 and 7-8 deleted (Δ) and intron 14 (i14) inserted respectively, are indicated in the blue shaded regions. Red dots indicate the positions of stop codons and black rectangles represent intronic regions. Diagram adapted from (Hrdlickova et al., 2012).*

The most well characterized alternative splice variants of *hTERT* include the minus alpha (-α) and the minus beta (-β) isoforms (Figure 1.10, Wong *et al.,* 2014). The minus alpha transcript isoform has an in-frame deletion of 36bp from exon 6 due to the use of an alternative 3'-splice acceptor site, which is predicted to remove 12 amino acid residues from the RT domain. This isoform does not possess a functional reverse transcriptase activity, but has been shown to act as a dominant-negative inhibitor of endogenous telomerase activity that induces cellular senescence or apoptosis when overexpressed within telomerase-positive human cell lines (Colgin *et al.,* 2000). Interestingly, two other *hTERT* transcript isoforms known as INS3

and INS4, have also been shown to act as dominant-negative inhibitors of telomerase activity within hepatocarcinoma cells (Figure 1.10, Zhu *et al.*, 2014). Overexpression of both isoforms was associated with telomere shortening and cell growth inhibition. INS3 and INS4 isoforms contain a 159bp and 600bp insertion of sequences from intron 14 respectively, which leads to premature termination and loss of CTD-encoding sequences (Figure 1.10). hTERT is known to form a functional homodimer complex through interactions between the CTD and the N-terminal RNA-interaction domain (RID1). It is thought that INS3 and INS4 proteins mediate telomerase inhibition by forming heterodimer complexes with wild-type hTERT, which disrupts association with hTR components and formation of a functional telomerase complex. Zhu *et al.,* (2014) found that INS3 and INS4 isoforms could only be detected within telomerase-positive tumour-derived cell lines, but not within telomerase-negative primary human cells or ALT-positive cells, which suggests that they may play a functional role in regulating telomerase activity within cancer cells (Zhu *et al.,* 2014).

The minus beta isoform contains a 183bp region of deletion encompassing exons 7 and 8, which leads to a frame-shift and a premature termination codon within exon 10 (Figure 1.10, Wong *et al.,* 2014). As a result, the RT and CT domains are lost and the transcript is predicted to undergo nonsense-mediated mRNA decay (NMD). High expression levels of the minus beta splice variant have been observed within normal human stem cells and cancer cells. Listerman *et al.,* (2013) demonstrated that the minus beta isoform is the most abundant *hTERT* isoform within breast cancer cells and provided evidence to show that it is translated into protein. Overexpression of the minus beta transcript within breast cancer cells caused inhibition of endogenous telomerase activity due to competitive binding for the hTR subunit. Conversely, ectopic overexpression of minus beta was also found to protect breast cancer cells from chemotherapy-induced apoptosis. Similarly, Hrdlickova *et al.,* (2012) found that overexpression of a the human *hTERT* transcript variant termed Δ4-13, which has undergone an in-frame deletion of exons 4 through 13, was associated with increased cellular proliferation through induction of Wnt signalling pathways. This shows that, aside from stabilizing telomere lengths, *hTERT* may play additional roles in cancer pathogenesis through alternative splicing.

Changes in alternative splicing patterns of the *hTERT* gene have been found to occur in a tissue and time-dependent manner during human foetal development (Ulaner *et al.,* 2001). This involves a shift in the expression of full-length *hTERT* transcripts to alternative isoforms that lack a functional RT domain such as minus alpha and minus beta isoforms. Loss of full-length *hTERT* expression correlates with loss of telomerase activity and progressive telomere shortening, which suggests that changes in alternative splicing patterns are responsible for down-regulating telomerase activity during the later stages of foetal tissue development. Interestingly, only a small proportion of total *hTERT* mRNA is spliced into the full-length isoform within telomerase-positive embryonic and adult tissue stem cells and cancer cells

(Wong *et al.,* 2014). Analysis of *hTERT* splice variant expression levels within a panel of cancer cell lines revealed that only 5% of total *hTERT* transcripts were full-length, while the majority (80-90%) were the minus beta isoform (Yi *et al.,* 2001). It is thought that the same program of *hTERT* splicing that occurs during human development is re-activated within cancer cells (Wong *et al.,* 2014). The functional role of all alternative *hTERT* splice variants has not yet been elucidated, however it has been suggested that they may function to maintain the level of telomerase activity required to stabilize telomere lengths over progressive cell divisions. Insufficient levels of telomerase leads to telomere de-stabilization and genomic instability, whereas high levels of telomerase could result in excessive telomere elongation and cell growth inhibition. Therefore, maintaining telomerase activity at an optimal level is critical for cell survival. In support of this notion, overexpression of minus alpha, minus beta, INS3 and INS4 *hTERT* transcript isoforms has been shown to inhibit telomerase activity within immortal human cell lines (Colgin *et al.,* 2000, Zhu *et al.,* 2014). It is also thought that the basal transcription machinery alone is unable to maintain optimal levels of *hTERT* expression and subsequent telomerase activity, therefore alternative splicing provides a mechanism to convert excess transcripts into non-catalytic isoforms (Wong *et al.,* 2014).

1.3.5.5 Post-Translational Regulation

In addition to transcriptional, epigenetic and alternative splicing mechanisms, post-translational modifications of the hTERT peptide have also been implicated in the control of telomerase activity within human cells. Two major covalent modifications of hTERT, phosphorylation and ubiquitination, have been found to be involved in regulating hTERT protein stability, nuclear translocation and the formation of a functional telomerase complex for modulating telomere length homeostasis (Kim *et al.,* 2001, Chung *et al.,* 2012, Kim *et al.,* 2005).

The protein kinase C-alpha (PKC-α) enzyme has been found to interact with and phosphorylate hTERT, which was shown to be important for telomerase activity within human breast and cervical cancer cells (Li *et al.,* 1998, Kim *et al.,* 2001). Exposure of HeLa and CUMC-6 cervical cancer cells to the PKC-inhibitors bisindolylmaleimide I and Gö6978, was associated with elimination of telomerase activity (Kim *et al.,* 2001). Similarly, PKC-α reversed the effects of protein phosphatase 2A (PP2A)-mediated inhibition of telomerase within breast cancer cells, suggesting a dynamic relationship between PP2A (de-phosphorylation) and PKC-α (phosphorylation) in telomerase regulation (Li *et al.,* 1998). Breast cancer cells have been found to exhibit higher levels of nuclear PKC-α expression, therefore it is thought that up-regulation of PKC-α may contribute to telomerase activation during breast tumorigenesis. Interestingly, PKC has also been implicated in modulating telomerase activity during T-cell activation (Bodnar *et al.,* 1996). Induction of telomerase was shown to occur during T-cell activation but was found

to be insufficient for maintaining constant telomere lengths within T-cells in long-term culture. Treatment of T-cells with the PKC-inhibitor bisindolylmaleimide was found to inhibit transient telomerase activation within activated T-cells. Conversely, treatment of peripheral blood mononuclear cells (PBMC), which have been found to express low levels of telomerase activity, with the PKC-activator phorbol myristate acetate (PMA) was associated with increased telomerase activity. Therefore, phosphorylation of hTERT by PKC enzymes is important for regulating telomerase activity within both normal T-cells and PBMCs and cancer cells.

hTERT has also been found to be a phosphorylation target of the Akt serine/threonine protein kinase, which is an important effector of the PI3K-signalling pathway (Kang *et al.,* 1999, Chung *et al.,* 2012). Two putative Akt phosphorylation sites located within amino acid residues 220-229 and 817-826 have been identified within the hTERT peptide sequence (Kang *et al.,* 1999). Purified Akt protein from human melanoma cell lysates was found to phosphorylate hTERT *in vitro*, which provides evidence that hTERT is a direct target of Akt. Furthermore, treatment of human melanoma cells with the specific PI3K-Akt inhibitor wortmannin resulted in a dose-dependent decrease in telomerase activity. On the other hand, treatment of cells with the PP2A inhibitor okadaic acid, resulted in Akt kinase activation and a dose-dependent increase in telomerase activity. These findings provide evidence to show that Akt-dependent phosphorylation of hTERT is important for maintaining telomerase activity within human cancer cells. In 2012, Chung *et al.,* demonstrated that Akt-mediated phosphorylation of hTERT was critical for the immortal transformation of normal human fibroblast cells. Akt kinase was found to phosphorylate serine 227, located within a bipartite nuclear localization signal (NLS) within the hTERT peptide, which was required for mediating nuclear translocation of hTERT. As expected, treatment of telomerase-positive H1299 non-small cell lung cancer (NSCLC) cells with wortmannin resulted in the accumulation of hTERT within the cytoplasm. Interestingly, normal human foreskin fibroblasts (HFF) transfected with mutant serine 227-*hTERT* exhibited similar levels of telomerase activity to those transfected with wild-type *hTERT*. However, unlike wild-type *hTERT*-HFFs, mutant serine 227-*hTERT*-HFFs were found to undergo progressive telomere shortening and induction of senescence pathways, which suggests that Akt-mediated phosphorylation of serine 227 is required for efficient immortalization of human fibroblasts.

Consistent with the observation that phosphorylation is associated with positive regulation of hTERT and telomerase activity, overexpression of the serine/threonine protein phosphatase 2A (PP2A) holoenzyme has been shown to inhibit telomerase activity and disrupt nuclear translocation of hTERT (Xi *et al.,* 2013). The PP2A scaffolding subunit PR65 was found to interact with the T-motif of hTERT and prevent binding of hTERT with 14-3-3θ, which functions to direct nuclear localization of hTERT. In contrast to PKC and Akt kinases, the c-Abl protein tyrosine kinase has been shown inhibit hTERT activity through direct

interaction and phosphorylation of hTERT (Kharbanda *et al.,* 2000). The functional importance of c-Abl-mediated hTERT inhibition was demonstrated by the observed telomere lengthening within c-Abl-deficient cells.

The Makorin RING finger protein 1 (MKRN1) E3 ubiquitin ligase has been found to interact with and ubiquitinate amino acid residues 946-1132 within the C-terminal domain of hTERT and promotes proteasome-mediated degradation of the hTERT peptide (Kim *et al.,* 2005). Overexpression of *MKRN1* within HT1080 fibrosarcoma cells was associated with a significant reduction in hTERT protein levels, telomerase activity and telomere shortening compared with empty vector controls. This indicates that MKRN1 plays an important functional role in modulating telomere lengths within human cancer cells by influencing hTERT protein stability. Interestingly, Oh *et al.,* (2010), also found that the HDM2 E3 ubiquitin ligase enzyme, responsible for targeting the p53 tumour suppressor protein for proteasome-mediated degradation, also interacts with and ubiquitinates five lysine residues within the N-terminal domain of hTERT. Depletion of HDM2 within HCT116 human colon carcinoma cells was associated with increased telomerase activity, whereas ectopic expression of *HDM2* within H1299 human lung cancer cells increased proteasome-mediated degradation of hTERT and caused a reduction in telomerase activity. These findings suggest that HDM2 may function as a negative regulator of hTERT.

1.3.6 TELOMERES, TELOMERASE AND BREAST CANCER

1.3.6.1 Evidence for Telomere Dysfunction and Chromosome Instability in Breast Cancer

Similar to other epithelial cancers, breast cancer development can be characterized by the accumulation of multiple genetic and epigenetic alterations that facilitate progression from normal breast epithelium, to pre-malignant intermediates such as usual ductal hyperplasia (UDH), atypical ductal hyperplasia (ADH) and ductal carcinoma in situ (DCIS) and finally to invasive and metastatic breast carcinoma (Figure 1.2, Heaphy *et al.,* 2009). Chromosome instability (structural and numerical alterations) is a common feature of breast carcinomas, with allelic imbalances (AI) detectable within the earliest breast lesions such as ADH, DCIS and even within usual ductal hyperplasia (UDH) (O'Connell *et al.,* 1997). For example, numerous studies have reported frequent loss of heterozygosity (LOH) on multiple chromosomes including 7q, 16q, 13q, 3p and 19p in breast cancer (reviewed by Miller *et al.,* 2003). Telomere dysfunction is thought to be a major cause of genetic instability during human carcinogenesis (see Chapter 1.3.3.4) and there is evidence to suggest that telomere shortening may be at least partly responsible for the high level of genetic instability observed within human breast carcinomas (reviewed by Meeker and Argani., 2004).

Using southern blot telomere restriction fragment (TRF) analysis, independent studies have shown that breast carcinomas possess shorter telomeres than normal breast tissue (Odagiri *et al.,* 1993, Rogalla *et al.,* 1996, Griffith *et al.,*1999). Odagiri *et al.,*(1993) observed a significant correlation between telomere length and histologic grade within a set of 41 breast carcinoma samples, with the shortest telomere lengths observed within Grade 3 breast carcinomas. These findings contrast those presented by Rogalla *et al.,* (1996), who demonstrated no observable correlation between telomere length and tumour grade. Both studies reported no association between telomere length and tumour volume, hormone receptor (HR) status or lymph node status. Using a titration assay to quantify telomere DNA content within 49 invasive human breast carcinoma samples, Griffith *et al.,* (1999) consistently demonstrated no association between telomere length and tumour volume, grade or stage, but found that tumours with the least telomeric DNA content were more likely to be aneuploid and metastatic. These findings suggest that there is no relationship between telomere lengths and clinicopathological features of breast cancer, however, shorter telomere lengths may be associated with increased genetic instability and metastatic potential.

Employing a telomere fluorescence in situ hybridization technique, which allows telomere length assessment of tissue sections at the single cell level, Meeker and colleagues found that over half of 114 invasive breast carcinoma samples exhibited significant telomere shortening (Meeker and Argani., 2004). 17.5% of samples were found to possess moderately shortened telomeres while around a quarter of samples exhibited normal telomere lengths (21%) or significantly elongated telomeres (5%). Interestingly, 78% of DCIS lesions were found to possess either significantly or moderately shortened telomere lengths, which indicates that telomere shortening occurs within early, pre-invasive breast lesions. In cases of DCIS with adjacent invasive breast lesions, telomere lengths were found to be similar within both lesions, indicating that progression to invasive disease and tumour expansion, was not accompanied by continued telomere shortening. Furthermore, a strong correlation was observed between the presence of short telomeres and chromosome instability, such as anaphase bridges (Meeker and Argani., 2004). Similarly, Heaphy *et al.,* (2009) observed a gradual increase in altered telomeric DNA content and the number of sites of allelic imbalance within a continuum of breast lesions representing each stage of breast tumorigenesis (normal breast tissue, ADH, DCIS and invasive carcinoma). However, no significant changes in telomeric DNA content or allelic imbalance were observed beyond DCIS, indicating that levels of telomere dysfunction and genetic instability plateau at DCIS.

Interestingly, Meeker and Argani (2004) also observed moderate telomere shortening within normal luminal epithelial cells of the terminal duct lobular unit (TDLU). This was thought to be linked to the observations made by Holst *et al.,*(2003), which demonstrated that ductal and luminal cells within histologically normal breast epithelium, exhibit methylation (and

inactivation) of the *p16* promoter. Promoter hypermethylation of *p16* has been associated with spontaneous bypass of the stress-associated senescence barrier (termed stasis) within normal human mammary epithelial cells (HMECs) *in vitro*, leading to extended cell growth and continued telomere shortening until cells reach the telomere-dependent, replicative senescence barrier (Romanov *et al.,* 2001). Together, these findings suggest that telomere shortening may play an important role in the early stages of breast cancer development.

1.3.6.2 Telomerase Activity and *hTERT* Expression in Breast Cancer

By means of a highly sensitive polymerase chain reaction (PCR)-based telomerase detection assay, known as the telomere-repeat amplification protocol (TRAP) assay, independent studies have shown that over 90% of human breast carcinomas exhibit detectable levels of telomerase activity, whereas normal breast tissues do not (Hiyama *et al.,* 1996, Clark and Osborne., 1997, Hoos *et al.,* 1998, Poremba *et al.,* 1998). These studies also demonstrate a correlation between telomerase activity and tumour stage, nodal metastases and decreased disease-free survival, which suggests that higher levels of telomerase activity is associated with a more aggressive disease. Poremba *et al.,* (1998) found that, unlike invasive breast carcinomas, normal breast tissue and ADH lesions did not exhibit telomerase activity. Interestingly, 59% of DCIS lesions were found to exhibit detectable levels of telomerase activity, indicating that telomerase re-activation may occur at this stage of breast carcinogenesis. Others have reported that around 75% of *in situ* breast lesions and 88% of lobular and ductal carcinomas express detectable levels of telomerase activity, compared with only 5% of adjacent tissues (Shay and Bacchetti., 1887). DCIS lesions also exhibit telomere shortening and chromosomal instability (see above). It is thought that telomerase reactivation occurs during the DCIS stage of breast cancer development, which results in telomere stabilization and reduced chromosomal instability (Artandi., 2003). These findings support the notion that telomerase re-activation is a critical step during human carcinogenesis that provides a mechanism of overcoming the growth-inhibiting effects of critical telomere shortening.

Using a real-time quantitative reverse transcription-PCR (qRT-PCR) assay to detect and quantify *hTERT* transcript levels, studies have reported increased but heterogeneous expression of *hTR* within pre-invasive ADH and DCIS lesions and invasive breast carcinomas relative to normal breast epithelium (Yashima *et al.,* 1998, Poremba *et al.,* 2002). Bieche *et al.,* (2000) found that around 75% of invasive breast carcinomas expressed detectable levels of *hTERT*, whereas normal breast tissue samples showed complete absence of *hTERT* expression. Furthermore, increased levels of *hTERT* expression were associated with shorter relapse-free survival, higher histopathological tumour grade and absence of hormone receptors ER and PgR, indicating that *hTERT* expression also correlates with more aggressive disease. In contrast, studies have reported heterogeneous *hTERT* expression patterns within both normal breast

epithelium and malignant breast tumours, with a subset of normal epithelial cells displaying higher *hTERT* expression levels than tumour cells (Hines *et al.,* 2005, Liu *et al.,* 2004). These findings contradict the hypothesis that *hTERT* de-repression and telomerase re-activation are critical events during breast carcinogenesis and suggest that breast tumours may arise from *hTERT*-positive epithelial cells. Analysis of four different *hTERT* splice variants (full-length, α, β, α+β) within normal breast tissue specimens, benign breast lesions and breast cancers by Zaffaroni *et al.,* (2002), demonstrated that expression of the full-length *hTERT* transcript was associated with the presence of active telomerase. While breast carcinoma samples were found to express the full-length variant as well as different combinations of other alternative splice variants, five out of six (83%) peri-tumoral normal breast tissue samples taken at a distance of 3cm away from the malignant tumour, expressed the truncated *β-hTERT* isoform and three out of six (50%) normal breast tissue samples were found to express the full-length *hTERT* variant. Out of these, only one normal breast tissue sample was found to exhibit telomerase activity, which could suggest that full-length *hTERT* expression levels may have been too low to confer telomerase activity within the remaining samples. Furthermore, the majority (80%) of normal breast samples taken from breast tissue surrounding benign breast lesions (fibroadenomas, fibrocystic lesions and duct papillomatosis) were not found to express any detectable *hTERT* splice variants. These findings indicate that normal breast epithelial cells may express *hTERT* splice variants that encode catalytically inactive protein products, which could account for the high level of *hTERT* expression observed by Hines *et al.,* (2005) and Liu *et al.,* (2004) and provide an explanation for the lack of telomerase activity observed within normal breast tissues observed by others (see above). Furthermore, these findings suggest that alternative splicing patterns of *hTERT* may play an important role in regulating *hTERT* expression and telomerase activity in breast cancer cells.

Consistent with other human carcinomas, such as skin tumours, gastric, renal cell and ovarian carcinomas, a significant correlation between *hTERT* mRNA levels and telomerase activity has been observed within human breast carcinomas, which is consistent with the hypothesis that *hTERT* is the critical rate-limiting component of telomerase enzyme activity (Kirkpatrick *et al.,* 2003). Furthermore, ectopic expression of *hTERT* within normal human mammary epithelial cells is sufficient to confer bypass of replicative senescence, immortal transformation and resistance to transforming growth factor β (TGF-β), which is a common feature of malignancy in human carcinomas (Stampfer *et al.,* 2000).

1.3.6.3 *hTERT* regulation in Breast Cancer

Epidemiological and *in vitro* studies have demonstrated that oestrogen plays an important role in breast cancer aetiology, with certain established breast cancer risk factors, such as post-menopausal obesity, exogenous hormones (contraceptive pill, HRT) and early age

at menarche, associated with increased systemic exposure to oestrogen (reviewed by Althuis *et al.,* 2004). In 1999, Kyo and colleagues were the first to show that oestrogen is a direct and indirect transcriptional activator of *hTERT* within breast cancer cells. Following treatment with 17β-estrodiol (E2), oestrogen receptor (ER)-positive MCF-7 breast cancer cells were found to exhibit increased levels of *hTERT* expression and telomerase activity. This was found to be due to direct, ligand-activated binding of the ER to the imperfect oestrogen responsive element (ERE) located approximately 2.7kb upstream from the *hTERT* core promoter region. In addition, oestrogen-induced transactivation of *hTERT* was found to be mediated by induction of *c-Myc* expression and binding of c-Myc/Max heterodimers to E-box elements within the *hTERT* core promoter (Kyo *et al.,* 1999).

The importance of ligand-activated ER-signalling on the transcriptional regulation of *hTERT* in breast cancer cells was demonstrated by Marconett *et al.,* (2011), who found that inhibition of E2-stimulated *hTERT* expression by indole-3-carbinol (I3L) was associated with cell cycle arrest in G1 and induction of cellular senescence pathways. I3L is a glycolysis product of glycobrassicin, which is a naturally occurring compound found within cruciferous vegetables such as broccoli and cabbage. I3L was found to inhibit E2-induced *hTERT* expression through stimulating both the degradation of ER-α and the production of threonine 579 (Thr579)-phosphorylated Sp1, thereby disrupting the binding of both transcriptional regulators to an ERE-Sp1 composite element within the *hTERT* promoter. Treatment of MCF-7 cells with the selective oestrogen receptor modulator raloxifene, was also found to inhibit E2-induced transcriptional activation of *hTERT* by inhibiting ERE-induced *hTERT* expression, phosphorylation (and activation) of Akt, the PI3K/Akt/NFκB signalling cascade, hTERT phosphorylation and interaction with NFκB (Kawagoe *et al.,* 2003). These findings demonstrate that oestrogen not only functions as a direct and indirect transcriptional activator of *hTERT*, but also influences post-translational modifications and interactions with factors that control the intracellular localization of hTERT. Treatment of MCF-7 cells and MCF-7 mouse xenograft models with melatonin was associated with a dose-dependent reduction in *hTERT* mRNA levels *in vitro* and a significant reduction in telomerase activity, tumour weight and metastatic potential *in vivo* (Leon-Blanco *et al.,* 2003). A later study found that melatonin inhibits *hTERT* expression within MCF-7 cells by blocking E2 or xenoestrogen (cadmium)-ER induced *hTERT* transcription (Martinez-Campa *et al.,* 2008). Therefore, the oncostatic effects of melatonin on tumour growth can be attributed, in part, to a significant reduction in *hTERT* expression and telomerase activity.

In addition to oestrogen, the progesterone sex steroid hormone has also been found to regulate *hTERT* expression within breast cancer cells (Wang *et al.,* 2000). Transient exposure of breast cancer cells to progesterone was associated with rapid induction of *hTERT* expression, which peaked at around 12 hours after exposure before declining. Treatment of cells with a

MEK (serine/threonine kinase) inhibitor prevented progesterone-stimulated *hTERT* transcription, indicating that activation of the mitogen activated protein kinase (MAPK) pathway may be involved in mediating progesterone-induced *hTERT* transcriptional activity. In contrast, long-term exposure to progesterone (over 48 hours) was associated with inhibition of oestrogen-induced *hTERT* transcriptional activation, which was thought to be due to up-regulation of *p21* expression. These findings demonstrate that *hTERT* is a target of both oestrogen and progesterone steroid hormones, which display different effects on *hTERT* transcription within breast cancer cells.

Introduction of c-Myc, but not viral oncoproteins such as HPV16-E7 or cellular oncoproteins such as Ras, cyclin D1 or cdc25C/A, has been found to induce telomerase activity and extend the proliferative capacity of normal HMECs *in vitro* (Wang *et al.,* 1998). Induction of *hTERT* expression following *c-Myc* transduction was rapid, with elevated *hTERT* mRNA levels observed within one passage. Telomere lengths within *c-Myc*-transduced HMECs were found to be stabilized, compared with negative control HMECs, which underwent progressive telomere shortening with each round of cell division. Telomere lengths within human tumour cells are generally short but stable. Therefore, transduction of *c-Myc* within normal HMECs, was associated with alterations in telomere dynamics similar to that observed within tumour cells (Wang *et al.,* 1998). Studies have shown that *c-Myc* is amplified and overexpressed within 16% and 22% of breast cancers respectively (Deming *et al.,* 2000, Bieche *et al.,* 1999). Therefore, the results presented by Wang *et al.,* (1998) suggest that de-regulation of *c-Myc* may contribute to breast tumorigenesis, in part, through induction of *hTERT* expression. c-Myc activates *hTERT* transcription through binding to two canonical E-box (CACGTG) elements located both upstream and downstream of the transcription start site (TSS) (see Chapter 1.3.5.2.1.1).

c-Myc has also been found play a critical role in mediating Aurora-A kinase-induced transcriptional activation of *hTERT* within breast and ovarian epithelial cell lines (Yang *et al.,* 2004). Aurora-A is a serine/threonine kinase involved in regulating G2/M cell cycle control (reviewed by Anand *et al.,* 2003). The *Aurora-A* gene is located within 20q13.2, which has been found to undergo frequent amplification in multiple human carcinomas such as colorectal, gastric, breast and ovarian. Specifically, *Aurora-A* has been shown to be overexpressed within up to 62% of human breast cancers, which is thought to be a major cause of spindle checkpoint dysfunction and aneuploidy during carcinogenesis. Ectopic expression of Aurora-A within MCF-10A breast epithelial cells, was found to induce telomerase activity through transcriptional activation of *c-Myc*, which led to binding of c-Myc to the upstream E-box element and activation of *hTERT* transcription (Yang *et al.,* 2004). Therefore, aside from chromosome missegregation, Aurora-A may also contribute to breast carcinogenesis through induction of *hTERT* expression.

Overexpression of the c-Myc-cooperating oncogene Bmi-1, originally identified as a driver of B-cell lymphomas in mice, has been found to induce telomerase activity and immortalize normal HMECs *in vitro* (Dimri *et al.,* 2002). Bmi-1 is a member of the polycomb-group (PcG) family of transcriptional repressors and controls cell proliferation through regulation of the *INK4A* locus, which encodes two tumour suppressor genes *p16* and *ARF* (Jacobs *et al.,* 1999). It is thought that Bmi-1 cooperates with c-Myc to drive tumorigenesis by blocking c-Myc-mediated induction of *ARF*, which allows HDM2-mediated degradation of p53 and inhibition of apoptosis. Dimri *et al.,* (2002) found that overexpression of *Bmi-1* within two different post-stasis (bypassed M0 through *p16* silencing) HMEC strains, resulted in extended replicative capacity, telomerase activation and immortal transformation through transcriptional activation of *hTERT.* In contrast, overexpression of *Bmi-1* within normal human fibroblasts was insufficient to confer telomerase activation or immortalization, indicating cell-type specific differences in *hTERT* regulation between human epithelial cells and fibroblasts. Bmi-1-induced *hTERT* transcription did not appear to involve c-Myc, as mutation of c-Myc-binding sites within the *hTERT* promoter was associated with increased promoter activity. This suggests that binding of the c-Myc/Max competitor Mad1/Max, may be important for mediating transcriptional repression of *hTERT* within normal HMECs. *Bmi-1* expression was also found to be higher within breast cancer cell lines and immortalized HMECs relative to a non-tumorigenic breast cell line (Dimri *et al.,* 2002). Silva *et al.,* (2007) demonstrated a strong correlation between plasma *Bmi-1* expression and poor disease-free and overall survival within breast cancer patients. Therefore, deregulation of *Bmi-1* may contribute to breast tumorigenesis through induction of *hTERT* expression and immortal transformation.

The epidermal growth factor receptor (EGFR)-related receptor tyrosine kinase HER2, which is overexpressed within 20-30% of human breast cancers, has also been shown to induce *hTERT* expression and telomerase activity within breast cancer cells (Goueli and Janknecht., 2004). Breast tumour tissue specimens and cell lines overexpressing HER2 were found to display higher levels of *hTERT* expression and telomerase activity than HER2-negative specimens or cell lines. Upon exposure to the HER2-inhibitor AG825, HER2-overexpressing SKBR3 breast cancer cells were found to exhibit a significant reduction in telomerase activity. HER2 was shown to induce telomerase activity through activation of Ras and Raf downstream effector proteins and collaboration with the ERK transcription factor ER81, which binds to two Ets sites within the *hTERT* promoter leading to transcriptional activation. Therefore, HER2 induces *hTERT* transcription by activating the MAPK signalling cascade. Co-transfection of the V664E oncogenic *HER2* mutant and *ER81* within telomerase-negative normal human fibroblasts was sufficient to confer induction of *hTERT* expression and telomerase activity. These findings suggest that induction of *hTERT* expression by oncogenic HER2 may be a contributing factor in the re-activation of telomerase during the development of many breast

tumours, as early pre-invasive DCIS lesions have been found to exhibit abnormal HER2 expression (Burstein *et al.,* 2004).

In addition to initiating the MAPK signalling cascade, HER2 has also been found to mediate transcriptional activation of *hTERT* through the PI3K/Akt/NFκB/c-Myc pathway (Papanikolaou *et al.,* 2011). Irradiation of tumour cells derived from *HER2*-overexpressing breast cancer biopsies, resulted in increased radio-resistance and elevated expression of *hTERT* and telomerase activity due to increased binding of c-Myc/Max complexes to E-box elements within the *hTERT* promoter. In contrast, irradiation of tumour cells derived from *HER2*-negative breast tumours was associated with increased Mad1/Max binding to the *hTERT* promoter. HER2 was found to induce *c-Myc* transcription through initiating the PI3K/Akt/NFκB pathway, thereby leading to indirect activation of *hTERT* expression. These findings demonstrate another mechanism by which HER2 activates *hTERT* transcription in breast cancer cells, which may be activated in response to radiotherapy and contribute to radio-resistance. Therefore HER2-induced *hTERT* expression may not only contribute to breast cancer development but also therapeutic resistance.

Aside from growth factor and hormone receptors, transcription factors and oncogenes, epigenetic mechanisms have also been implicated in regulating *hTERT* gene expression within breast cancer cells. By examining the micrococcal nuclease (MNase) digestion pattern of a 26.4kb region of the *hTERT* gene, encompassing around 10kb of 5'-flanking sequences and 16kb of sequences downstream of the TSS, Szutorisz *et al.,* (2003) investigated the chromatin structure around the *hTERT* gene within *hTERT*-repressed and *hTERT*-positive cells to identify *cis*-acting regulatory elements that may be responsible for tumour cell-specific expression of *hTERT*. Two discrete sites located within the first 1kb of intron 2 of the *hTERT* gene*,* were found to be susceptible to MNase-digestion within *hTERT*-positive tumour cells and an SV40-immortalized human fibroblasts cell line, but not within *hTERT*-negative normal human fibroblasts or an SV40-immortalized human fibroblast cell line that uses an alternative lengthening of telomeres (ALT) pathway of telomere maintenance. Interestingly, no differences in the pattern of MNase sensitive sites were observed in the 5'-flanking region of *hTERT*-positive cells and *hTERT*-negative cells. These findings suggest that an 'open' chromatin conformation around intron 2 may be responsible for tumour-specific *hTERT* expression. In support of this, introduction of a normal copy of chromosome 3 into the *hTERT*-positive 21NT breast cancer cell line produced *hTERT*-repressed hybrids that displayed a change in the MNase-sensitivity pattern around intron 2 of *hTERT,* which resembled normal human fibroblasts. The MNase-sensitivity pattern associated with active *hTERT* transcription was restored upon loss of the exogenous chromosome 3 copy, however not all segregants were found to re-express *hTERT*. These observations indicate that a sequence(s) present on chromosome 3 functions to repress *hTERT* transcription within breast cancer cells by altering

the chromatin conformation around intron 2 of the *hTERT* gene. It was proposed that functional loss of this repressor sequence during breast tumorigenesis may lead to an 'open' chromatin state that is required, but not sufficient, to confer *hTERT* expression and telomerase re-activation. These findings indicate that epigenetic events may play a critical role in *hTERT* de-repression and cellular immortalization during breast tumorigenesis.

1.3.7 Conflicting Views on the Origins of Telomerase in Cancer

In 1994, Kim and colleagues demonstrated that the majority of human tumours possess active telomerase activity, whereas the majority of human somatic tissues, aside from the ovaries and testes, do not (Kim *et al.,* 1994). It is now known that telomerase is active within developing embryonic tissues until around 21 weeks of gestation, at which point it is either down-regulated and maintained at low levels within stem/progenitor cells of self-renewing adult tissues including gastrointestinal crypt, hair follicle bulbs, lymphoid tissue and glandular prostate tissue, or repressed completely within certain adult tissue compartments, such as mesenchymal tissues (Forsyth *et al.,* 2002). Unlike embryonic stem cells, which display high levels of telomerase activity and an immortal phenotype, tissue stem cells and their progeny (progenitor and transit amplifying stem cells), which reside within tissue micro-environmental niches, are not immortal and exhibit reduced self-renewal capacity (reviewed by Kong *et al.,* 2014). However, intestinal stem cells (ISC), which reside within intestinal crypts and function to replace epithelial cells shed from the epithelium every day, are in a constitutive state of activation and can undergo several thousand cellular divisions throughout a human's lifespan (Pech and Artandi., 2011). Similar to immortal human cell lines, human ISCs exhibit telomerase activity and do not appear to show a limited proliferative capacity. However, despite the presence of telomerase activity within many human tissue stem cell compartments, adult stem cells have been found to undergo progressive telomere shortening with increasing age (Shay and Wright., 2010). Therefore, despite the lack of sufficient telomerase to maintain stable telomere lengths, it is thought that low levels of telomerase activity within adult stem/progenitor cells delays the rate of telomere attrition to support rapid cell division and differentiation for the replacement of lost/damaged cells during tissue renewal or repair.

Studies involving the immortal transformation of normal human fibroblasts and epithelial cells using viral oncoproteins have demonstrated that the functional loss of genes *p53*, *pRb* and *p16*, which are abrogated in a significant proportion of human cancers, is associated with an increased propensity for immortal transformation (Newbold., 2005). The rare emergence (around one in ten million cells) of an immortal variant from crisis is almost always accompanied by de-repression of *hTERT* and telomerase re-activation (Newbold., 2005, Shay and Wright., 2010). Based on these observations, it is thought that stringent mechanisms of

hTERT transcriptional repression exist within the majority of normal human somatic cells that provide a powerful barrier to cellular immortalization, clonal evolution and malignant progression. Therefore, the acquisition of an immortal phenotype during crisis is thought to be due to a rare mutational or epigenetic event that leads to functional loss of a critical *hTERT* repressor sequence(s). In support of this, chromosome transfer studies have shown that repression of *hTERT* transcription and telomerase activity can be achieved within different human cancer types, through introduction of single copies of normal human chromosomes (see Chapter 1.3.5.2.2.3). The observed effect on *hTERT* transcription is thought to be due to reconstitution of a *hTERT* repressor sequence(s) present on the introduced chromosome copy, that was lost during immortal transformation.

Despite these observations, a second hypothesis for the origin of telomerase activity within human cancers has also been proposed, which is that cancer cells arise from telomerase-positive stem cell populations residing within self-renewing tissues (reviewed by Sell., 2004). Human tumours are known to display intra-tumoral heterogeneity, which is thought to be due to the acquisition of mutations or epigenetic alterations as tumour cells undergo clonal evolution. However, tumours also display non-genetic, heterogeneous patterns of differentiation, which reflect aspects of normal differentiation patterns that occur within the original tissue (reviewed by Reya *et al.,* 2001). For example, breast tumour cells display variable expression patterns of normal differentiation markers such as the oestrogen receptor or milk proteins. Consequently, the degree of tumour 'de-differentiation' is an important prognostic marker that is used by pathologists to grade tumours and predict clinical outcomes. During the normal process of organogenesis, adult stem cells divide by asymmetric cell division to produce one daughter cell that retains the same stem cell properties, while the other becomes a more differentiated progenitor or transit amplifying cell that gives rise to a variety of functional cell types specific to that organ (Shay and Wright., 2010). Analogous to this process, it is thought that the variable degree of differentiation observed within human tumours originates from a deregulated process of organogenesis, initiated by a cancer stem cell (CSC) or tumour initiating cell. The degree of differentiation within the tumour is thought to reflect the stage of maturation at which the normal stem cell acquired a tumorigenic alteration and became a CSC (Sell., 2004). In support of the CSC hypothesis, Tomasetti and Vogelstein (2015) recently found a significant correlation between the total number of tissue stem cell divisions and the lifetime risk of developing cancer within that particular tissue in 25 different cancer subtypes. Due to inherent flaws in the process of DNA replication, around three mutations are predicted to occur each time a normal cell divides. Therefore, the accumulation of 'replicative mutations' within normal stem cells is thought to explain why certain tissue types give rise to human cancers more frequently than other tissue types.

Unlike the clonal evolution theory of tumour formation, which suggests that the majority of the tumour cells possess self-renewing capacity, the CSC hypothesis proposes that tumours possess a hierarchical organization with only a small subpopulation of tumour cells capable of self-renewing and generating tumour cells (Visvader and Lindman., 2012). In support of the CSC theory, *in vitro* and *in vivo* studies have shown that the majority of tumour cells are incapable of initiating tumour formation (Shay and Wright., 2010, Reya *et al.,* 2001). This was first reported by Park *et al.,* (1971), who found that only 1 in 10^2-10^4 mouse myeloma cells could form colonies in an *in vitro* colony-forming assay. Furthermore, when transplanting human cancer cells into immuno-deficient mice, hundreds of thousands to millions of cells are required to form a tumour (Shay and Wright., 2010). These observations suggest that the majority of human tumour cells are unable to proliferate extensively, while only a small subpopulation of cells are clonogenic. Furthermore, CSCs or tumour initiating cells, which express stem/progenitor cell surface markers, have been identified within multiple cancer types including primary glioblastoma multiform (GBM), breast, prostate and multiple myeloma. Studies have shown that CSCs derived from breast, pancreatic glioblastoma, neuroblastoma and prostate cancers express *hTERT* mRNA and telomerase activity (Kong *et al.,* 2014). A comparison of telomerase activity within CSC and non-CSC tumour populations derived from resected glioma specimens, revealed that only the CSC population possessed detectable levels of telomerase activity (Castelo-Branco *et al.,* 2011). Interestingly, inhibition of telomerase using the telomerase inhibitor Imetelstat, was associated with DNA damage, growth arrest and cell maturation of neural CSCs and rapid inhibition of the clonogenic survival of $CD138^-$ multiple myeloma CSCs (Castelo-Branco *et al.,* 2011, Shay and Wright 2010). These findings not only indicate that CSCs rely on telomerase activity to maintain stable telomere lengths for self-renewal, but also demonstrate that CSCs are sensitive to anti-telomerase therapies. This is consistent with the observation that CSCs derived from GBM and prostate cancers have been found to possess short telomere lengths (Shay and Wright., 2010). In many cases, cells within pre-neoplastic lesions have been shown to possess shortened telomere lengths (Meeker *et al.,* 2002). It has therefore been proposed that tumour initiating cells or CSCs originate from a subpopulation of transit amplifying cells located within tissue stem cell compartments, that have undergone telomere shortening, possibly as a result of chronic inflammation (Shay and Wright., 2010). Analogous to the mortality barriers encountered by normal human fibroblasts and epithelial cells, these cells are predicted to bypass replicative senescence and enter crisis in the absence of *p53*. Immortal variants that emerge have indefinite self-renewing capacity due to up-regulation or re-activation of telomerase activity and stabilization of telomere lengths.

These observations have important implications for the development of anti-telomerase therapies in the treatment of human cancers and pose important questions that remain to be answered. Namely, what genetic/epigenetic alterations are responsible for up-regulating

telomerase activity and maintaining stable telomere lengths within CSCs and are they different to those responsible for telomerase re-activation within normal human fibroblasts and epithelial cells? Do all human tumours originate from telomerase-positive tissue stem/progenitor cells, or do tumours arise from the immortalization and clonal expansion of telomerase-negative, functional cell types such as fibroblasts and epithelial cells?

1.3.8 TELOMERASE-INDEPENDENT PATHWAYS OF TELOMERE MAINTENANCE IN CANCER

In order to examine the role of telomerase in cancer, Blasco and colleagues constructed a telomerase-knockout mouse strain by deleting the telomerase RNA component (mTR) from the mouse germline (Blasco *et al.,* 1997). Rodents are known to possess active telomerase in adult tissues, which is active throughout the organisms lifespan (Greenberg *et al.,*1998). As a consequence, primary rodent cell cultures have long telomeres, which do not shorten over time with increasing cell divisions *in vitro*. $mTR^{-/-}$ mice were found to lack detectable telomerase activity but survived for six generations, likely due to the presence of long telomeres in the original mouse strain (Blasco *et al.,* 1997). Telomere lengths shortened at a rate of around 4.8kp/generation and cells derived from generation 4 (G4) mice lacked detectable telomeric repeat sequences and were found to display multiple chromosome abnormalities such as chromosome end-to-end fusions. Telomere shortening and induction of replicative senescence pathways in normal human somatic cells is considered to be a powerful protective barrier to immortal transformation. Therefore, it was surprising to find that telomerase-deficient cells derived from all $mTR^{-/-}$ mice generations could be transformed by viral oncogenes and produce tumours in athymic mice (Blasco *et al.,* 1997). These findings indicate that telomerase-independent telomere-maintenance mechanisms exist within mammalian cells.

The existence of alternative mechanisms of telomere maintenance in eukaryotic cells was first observed following inactivation of telomerase activity (through deletion of est-1) within the *Saccharomyces cerevisisae* yeast strain (Lundblad and Szostak., 1989). The majority of est-1 mutants were found to undergo telomere shortening and cell death, however a small population of immortal cells arose that maintained their telomere lengths through amplification and acquisition of sub-telomeric elements via a RAD52-mediated homologous recombination pathway (Lundblad and Blackburn., 1993). Evidence for the existence of telomerase-independent telomere maintenance mechanisms in human cancer cells, stems from the observation that most, but not all, cancer cells possess active telomerase (Kim *et al.,* 1994). In addition, Bryan *et al.,* (1995) found that around 43% of DNA tumour virus (SV40 and HPV)-transformed human fibroblasts were dependent on a telomerase-independent mechanism of

telomere maintenance. These cells were found to possess long but heterogeneous telomeres, ranging from very short (undetectable) to up to 50kb in length.

It is now known that around 15% of all human cancers maintain telomere lengths through a telomerase-independent mechanism involving homologous recombination (HR), known as alternative lengthening of telomeres (ALT) (reviewed by Cesare and Reddel., 2010). ALT-positive tumour cells can be distinguished from telomerase-positive and normal human cells by the presence of ALT-associated promyelocytic leukaemia (PML) bodies (APB), which are large round nuclear structures consisting of telomeric DNA, PML protein, telomere-binding proteins such as TRF1 and TRF2 and proteins involved in DNA recombination and synthesis including RAD51, RAD52 and replication factor A (Yeager *et al.,* 1999). It is thought that APBs represent the site of ALT activity. In addition, ALT-positive cells exhibit very heterogeneous telomere lengths (which may change rapidly) and increased levels of recombination at telomeres. Furthermore, the ALT phenotype is associated with significantly higher levels of extrachromosomal telomeric DNA compared to telomerase-positive and normal human cells, which can take the form of linear double-stranded DNA (dsDNA), double or partially single-stranded telomeric circles (t-circles), G-rich strand circles (G-circles) or C-rich strand circles (C-circles) (Cesare and Reddel., 2010). Despite these unique characteristics, telomeres within ALT-positive cells still retain many typical features, such as the ability to form t-loops and the presence of shelterin complex protein components.

The ALT phenotype has been observed within a 5-15% of carcinomas such as breast, renal, bladder, endometrium and lung (Henson and Reddel., 2010). However, this phenotype is more common among sarcomas, such as liposarcomas and osteosarcomas, and central nervous system tumours such as glioblastoma multiform (an adult malignant brain tumour) and astrocytomas, compared with epithelial cell malignancies (Cesare and Reddel., 2010). A comprehensive survey of the ALT phenotype within over 6000 primary tumour specimens encompassing a wide range of human tumour types, demonstrated that a subset of adenocarcinomas from the prostate, small intestine, colon and pancreas do not exhibit any features of ALT, indicating that certain tumour types rely solely on telomerase activity for telomere length maintenance (Heaphy *et al.,* 2011).

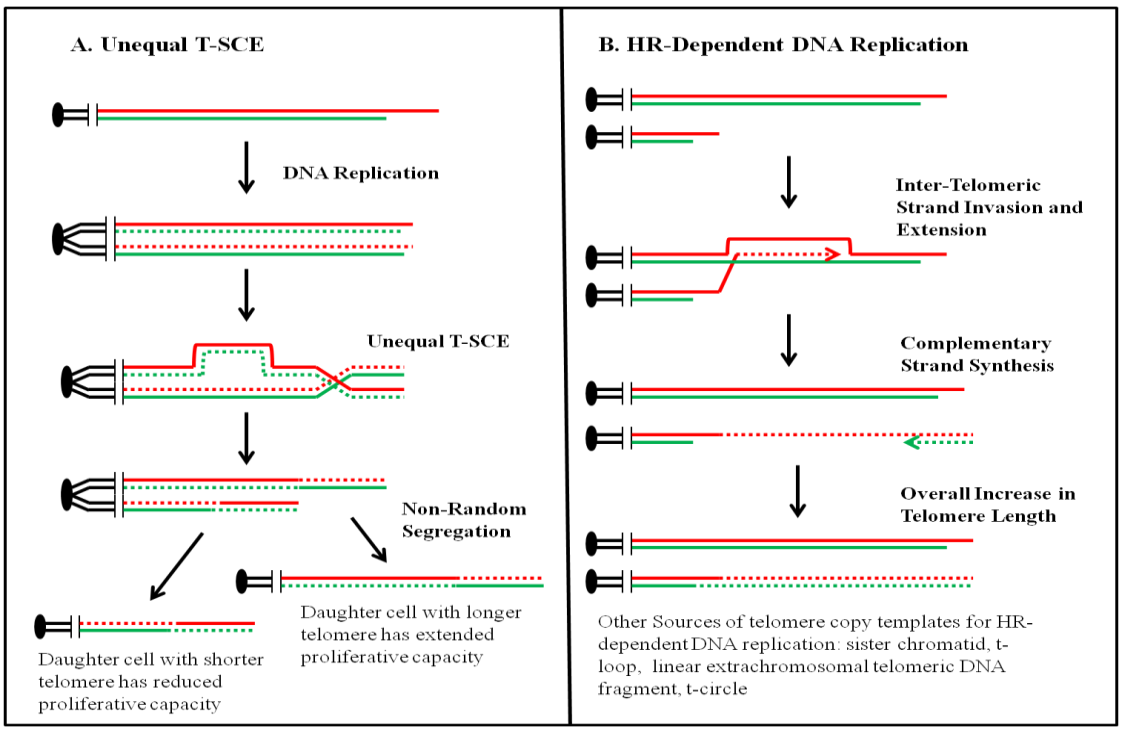


Figure 1.11 - *Schematic representations of two models of alternative lengthening of telomeres (ALT):* ***(A)*** *unequal telomere sister chromatid exchange (T-SCE) and* ***(B)*** *homologous recombination (HR)-dependent DNA replication. Image adapted from Cesare and Reddel., (2010).*

Physical evidence of homologous recombination-mediated telomere maintenance mechanisms within ALT-positive cells was provided by Dunham and colleagues in 2000. Plasmid tags inserted within telomeres were found to be copied to other chromosome ends within ALT-positive but not telomerase-positive cell lines, indicating that inter-telomeric templating and recombination may occur within ALT cells. Furthermore, telomere sister chromatid exchanges (T-SCEs) have been found to occur at a higher frequency within ALT-positive cells compared with telomerase-positive or normal human cells (Cesare and Reddel., 2010). Two hypothetical recombination-dependent models for telomere maintenance and elongation within ALT-positive cells have been proposed; unequal T-SCE exchange and HR-dependent DNA replication (Figure 1.11A). It is thought that nicks within telomeric DNA sequences of ALT-positive cells, disrupt DNA replication and causes unequal T-SCE. In this model, exchange of telomeric DNA between sister chromatids results in the extension of one telomere at the expense of another. This would not result in any net gain in telomere length unless elongated and shortened telomeres were each segregated into two separate daughter cells (Figure 1.11A, Durant., 2012). Therefore, through non-random segregation, one generation would possess longer telomeres and an extended proliferative capacity. It has also been proposed that ALT may result from HR-dependent DNA replication, which involves the synthesis of a new telomeric DNA sequence by inter-telomeric strand invasion of an adjacent

chromosomal telomere (Figure 1.11B). This method of telomere-templated DNA synthesis can involve other copy templates such as sister chromatid telomeres, where the 3'-ssDNA overhang from one chromatid invades the homologous sequence of the sister chromatid telomere to prime DNA replication. Alternatively, linear or circular extrachromosomal telomeric DNA sequences such as double-stranded DNA fragments, t-circles or C-circles, are thought to provide templates for HR-mediated telomeric DNA synthesis through strand-invasion or rolling-circle replication mechanisms (Cesare and Reddel., 2010).

Through genetic analyses, the MRE11-RAD50-NSB1 (MRN) complex has been found to play an important role in mediating telomere elongation within ALT-positive cells. In normal human cells MRN protein complex members have been found to interact with the telomere-associated protein TRF2 during different cell cycle phases, and are thought to facilitate t-loop formation (Zhu *et al.,*2000). Furthermore, the MRN complex functions as a DNA damage sensor that recruits ATM to DSB sites and facilitates resection of the DNA ends for repair by homologous recombination (Lee and Paull., 2007). RNA-interference (RNAi)-mediated disruption of RAD50, has been found to result in telomere erosion and induction of senescence pathways (Potts and Yu., 2007). It is thought that the MRN complex may promote HR-dependent telomeric DNA replication within ALT cells by recruiting ATM and initiating a recombination process of DNA repair, whereby the 3'-ssDNA overhang invades a neighbouring telomeric DNA strand (adjacent/sister chromosome or extra-chromosomal sequence) and DNA polymerase catalyzes extension of the 3' sequence (Cesare and Reddel., 2010).

The mechanisms by which ALT pathways are suppressed within normal human cells and the events that occur during carcinogenesis that re-activate ALT pathways is unknown. Whole somatic cell fusion of ALT-positive and normal human cells was found to produce hybrids that displayed rapid telomere erosion and induction of a senescent-like phenotype, which indicates that normal human cells possess at least one mechanism that functions to repress ALT pathways (Perrem *et al.,* 1999). Due to the high frequency of ALT-positive cells produced by SV40-LT immortalization, it is possible that functional loss of p53 may play a role in activation of ALT pathways during human carcinogenesis (Bryan *et al.,*1995, Newbold., 2002). The existence of a telomerase-independent pathway of telomere maintenance and elongation in cancer, poses a potential problem to the application of anti-telomerase therapies. ALT-positive tumours are resistant to anti-telomerase therapies, which indicates that a proportion of carcinomas, sarcomas or central nervous system tumours may not respond to this treatment type (Heaphy *et al.,* 2011). In addition, it is not known whether activation of ALT pathways presents a potential resistance mechanism to anti-telomerase therapies (Newbold., 2002). Therefore, by understanding the mechanisms that repress ALT-pathways and *hTERT* transcription within normal human somatic cells, and monitoring the consequences of anti-

telomerase therapies on tumour cell growth, we can develop more effective therapies that target both mechanisms of telomere maintenance that underlie cancer cell immortality.

1.3.9 THERAPEUTIC STRATEGIES FOR TARGETING TELOMERASE

The telomerase ribonucleoprotein holoenzyme is responsible for maintaining stable telomere lengths within the majority of human cancers (Kim *et al.,* 1994). By counteracting the effects of progressive telomere shortening, telomerase prevents recognition of eroded chromosome ends as double-stranded DNA breaks (DSB) by DNA repair machinery, subsequent activation of DNA damage response pathways and induction of senescence or apoptosis. In this way, telomerase is responsible for sustaining the indefinite growth capacity of cancer cells. Telomerase therefore represents an attractive anti-cancer target and has been the focus of the scientific community to develop strategies that inhibit its activity (Pal *et al.,* 2015). A proof of principle of this approach was provided by Hahn *et al.,* (1999), who demonstrated that inhibition of telomerase activity within human cancer cell lines by introducing a dominant-negative *hTERT* mutant, was associated with telomere shortening and cell growth arrest *in vitro* and elimination of tumorigenicity *in vivo*.

Unlike cancer cells, the majority of normal human somatic cells do not express detectable levels of telomerase. However, as discussed earlier, some actively dividing normal somatic cells, such as stem/progenitor cells located within self-renewing tissues, require low levels of telomerase activity to facilitate tissue repair/regeneration (see Chapter 1.3.7). The importance of telomerase within adult stem cells has been demonstrated through the study of individuals with defective telomerase activity (Kong *et al.,* 2014). Dyskeratosis congenita (DC) is a rare genetic disease either caused by inheritance of X-linked *DKC1* mutations or autosomal dominant/recessive *hTERT* and *hTR* mutations (Vulliamy and Dokal., 2006). These genes encode important subunits and associated proteins that are essential for telomerase holoenzyme complex biogenesis, stability and function. In the absence of telomerase activity, the stem cells of DC individuals have defective telomere maintenance and undergo critical telomere shortening, which manifests pathologically as bone marrow failure, oral leukoplakia, nail dystrophy and increased risk of cancer. Therefore, telomerase plays an integral role in human tissue renewal. There is also some evidence to show that telomerase is active during S-phase of the cell cycle within primary human fibroblasts (Masutomi *et al.,* 2003). Therefore, the development of therapeutic strategies that aim to inhibit telomerase activity within cancer cells, may have an adverse effect on the regulation of telomere structure within normal human cells, and the self-renewing capacity and function of normal adult stem cells. However, despite the presence of higher levels of telomerase activity, cancer cells have been found to possess shorter telomere lengths than normal human somatic cells (Shay and Wright., 2006). Therefore, normal

stem/progenitor cells would theoretically remain unaffected by telomerase inhibitors due to their lower-mitotic activity and longer telomere lengths.

An important limitation of targeting telomerase as a therapeutic anti-cancer strategy is the period of time required for short telomeres to become critically shortened and induce cell cycle arrest or apoptotic pathways (Shay and Wright., 2006). During this lag-phase, cancer cells would continue to divide and the tumour mass would increase before telomeres become critically shortened. On the other hand, treatment of cancers with chemotherapy and/or radiotherapy is associated with a rapid reduction in tumour burden. However, resistance to chemotherapeutic agents and disease relapse is an inevitable consequence of this treatment approach. Small subpopulations of cancer stem cells (CSC)/tumour initiating cells, which depend on telomerase activity for their indefinite self-renewal capacity, are thought to be responsible for mediating resistance to these anti-cancer therapies (Kong *et al.,* 2014). Therefore, using telomerase inhibitors in combination with chemotherapeutic agents and/or radiotherapy has been proposed as a potential strategy to reduce the tumour burden and prevent therapeutic resistance simultaneously (Shay and Wright., 2006).

A variety of approaches have been developed to inhibit telomerase activity, the majority of which target the reverse transcriptase (hTERT) or telomerase RNA (hTR) components (Pal *et al.,* 2015, Philippi *et al.,* 2010). These include the use of: (1) nucleic acid-based biologicals such as small interfering RNA (siRNA) or anti-sense oligonucleotides (AS-ODN) that interfere with *hTERT* and *hTR* translation (2) ribozymes and hammerhead ribozymes that cleave hTERT and hTR components respectively, (3) Gene-directed pro-drug therapy (GDEPT) that exploits the high promoter activities of *hTERT* and *hTR* within cancer cells, to induce the expression of pro-apoptotic 'suicide' genes, (4) nucleoside analogues such as azidothymidine triphosphate (AZT) (5) dominant-negative *hTERT* mutants, (6) G-quadruplex targeting agents that stabilize complex G-quadruplex secondary structures and prevent access of telomerase to the telomeric 3'-ssDNA overhang, (7) induction of antigen-presenting cells (T-lymphocytes or dendritic cells) against peptides derived from hTERT to stimulate an immune response against hTERT-positive cells.

Studies involving the inhibition of telomerase through the use of AS-ODNs that target template and non-template regions of the hTR subunit, have demonstrated a reduction in telomerase activity within cancer cells *in vitro* (reviewed by White *et al.,* 2001). However, the majority of these studies did not report progressive telomere shortening as a result of continued treatment. Furthermore, some studies found that cells exhibited slowed growth or apoptosis in a shorter time period than would be expected if the AS-ODN had targeted telomerase specifically. Independent studies involving the use of hammerhead ribozymes to target the template region of hTR also showed a reduction of telomerase activity within endometrial and melanoma cells *in vitro*, but no change in cellular proliferative capacity (Yokoyama *et al.,* 1998, Folini *et al.,*

2000). In addition, studies have shown that treatment of cancer cell lines with the reverse transcriptase inhibitor 3'-azido-3'-deoxythymidine (AZT), was associated with telomerase inhibition but not telomere shortening or induction of cell growth arrest following prolonged exposure (White *et al.,* 2001). These findings suggest that some telomerase inhibitors are non-specific and have off-target effects that may lead to side effects *in vivo*.

The hTR template antagonist GRN163L, also known as imetelstat, is a promising agent that has been found to inhibit telomerase activity within cancer cells *in vitro* and *in vivo* (Herbert *et al.,* 2005). GRN163L has been found to induce cell growth arrest, apoptosis and reduce colony formation and tumorigenicity within a variety of cancer cell lines including lung, breast, prostate, ovarian, cervical, glioblastoma, hepatoma and melanoma (reviewed by Ruden and Puri., 2013). Furthermore, GRN163L was found to restore sensitivity to trastuzumab within HER2-positive breast cancer cell lines (Goldblatt *et al.,* 2009). Unlike typical AS-ODNs, which bind to and prevent translation of target mRNA sequences, GRN163L consists of a 5'-palmitoyl-TAGGGTTAGACAA-3' oligonucleotide sequence that is complementary, and binds with high specificity, to the hTR template strand thereby preventing elongation of telomeric sequences by telomerase. The 5'-palmitoyl moiety makes the oligonucleotide sequence lipid-soluble, which facilitates transport across cell membranes. GRN163L has undergone (and is currently undergoing) several stage I/II clinical trials where it has been tested alone or in combination with other monoclonal antibodies and/or chemotherapeutic agents to assess dose limiting toxicities, pharmacokinetics and objective response rates for a variety of solid tumour malignancies, lymphoma and multiple myeloma (Ruden and Puri., 2013). A phase I clinical trial investigating GRN163L in patients with metastatic or recurrent breast cancer, in combination with paclitaxel and bevacizumab demonstrated an overall response rate of 53.8% (Kozloff *et al.,* 2010). A phase I clinical trial investigating the effect of GRN163L treatment on reversing trastuzumab resistance in *HER2*-positive breast cancer patients is currently ongoing (Clinical Trials Phase Identifier: NCT0126592).

Other promising strategies for telomerase inhibition include the use of telomerase-based immunotherapies, which are designed to stimulate an immune response against telomerase-positive cancer cells through induction of CD4+ and CD8+ cytotoxic lymphocytes (CTL) (reviewed by Ruden and Puri., 2013). Telomerase-positive cancer cells express telomerase-associated antigens (TAAs), such as hTERT peptides, on their surface through major histocompatibility complexes (MHC). A variety of telomerase targeting vaccines have been developed that aim to stimulate CTL-mediated elimination of these cells. These include GV1001 and GRNVAC1, which have been tested within phase I/II clinical trials and have been shown to stimulate CD4+ and CD8+ immune responses with minimal effects on normal cells (Ruden and Puri., 2013). GV1001 is an MHC class II-restricted vaccine containing sixteen

amino acids of the hTERT active site, while GRNVAC1 contains autologous dendritic cells that have been transfected *ex vivo* with the *hTERT* mRNA sequence.

The existence of a telomerase-independent telomere maintenance mechanism (ALT), presents a potential problem for the use of telomerase-targeting agents to inhibit cancer cell growth. Following telomerase inhibition, it is thought that re-initiation of telomere erosion and cell crisis during the lag-phase may facilitate the emergence of possible resistance mechanisms, such as activation of ALT (see Chapter 1.3.8). Using $Atm^{-/-}$ knockout mouse engineered with an inducible *mtert* allele to model the effects of telomerase re-activation and inhibition in T-cell lymphoma, Hu *et al.,* (2012) found that following telomerase extinction, the rate of tumour growth was reduced but soon increased due to the acquisition of ALT-mediated mechanisms of telomere maintenance. This was accompanied by the overexpression of *PGC-1β* (peroxisome proliferative activated receptor, gamma, coactivator 1 beta), which encodes an important regulator of mitochondrial function. Knockdown of *PGC-1β* or *SOD2* (superoxide dismutase 2) was associated with a significant inhibition in ALT-positive tumour cell growth. Hu *et al.,* (2012) proposed that, increased DNA damage signalling caused by ALT-mediated telomere maintenance mechanisms could lead to increased mitochondrial damage and a subsequent increase in intracellular reactive oxygen species (ROS). To counteract these potentially cytotoxic effects, ALT-positive cells up-regulate the expression of genes involved in maintaining mitochondrial function and reducing oxidative damage. These observations provide an insight into possible anti-telomerase resistance mechanisms in cancer cells and demonstrate how these may be counteracted by combination regimens that target the PGC pathway. However, *in vitro* studies suggest that activation of ALT pathways in response to anti-telomerase inhibitors is rare in human cancers (Shay *et al.,* 2012). Although, it is argued that *in vitro* experiments do not fully recapitulate clinically-relevant tumours *in vivo* due to limited cell numbers. Therefore, a greater understanding of whether and at what frequency ALT-positive human cancer cells arise in response to anti-telomerase therapies, and what pathways may be activated that constitute potential therapeutic targets for ALT inhibition, is required.

AIMS AND OBJECTIVES

The aim of my project is to investigate the role of chromosome 17, chromosome 3 and single genes/sequences localised to chromosome 3p, in the regulation of *hTERT* transcription and telomerase activity within breast cancer cells.

In order to achieve this aim, my objectives are as follows:

I. To determine the existence of telomerase repressor sequences localized to chromosomes 3 and 17 within a single breast cancer cell line using the microcell-mediated monochromosome transfer (MMCT) technique.

II. To investigate the functional role of chromosome 3p-encoded genes *SETD2, FLJ, BAP1, RASSF1, PARP-3* and *PBRM1* as candidate repressors of *hTERT* transcription and telomerase activity in breast cancer cells.

III. To investigate chromosome 3p structure during the multi-step process of normal human mammary epithelial cell (HMEC) immortalization.

CHAPTER 2

2 GENERAL MATERIALS AND METHODS

2.1 CELL CULTURE MANIPULATION

2.1.1 GENERAL EQUIPMENT FOR CELL CULTURE

Cell culture manipulation was carried out in a LaminAir HB2448 laminar flow safety cabinet (Heraeus Instruments, DE). All cell lines and strains were maintained in fully humidified (>95%) HeraCell incubators (Heraeus Instruments, DE) at 37ºC with either 5% or 10% carbon dioxide (CO_2). Visualization and monitoring of cell cultures was performed using an Olympus CK40 inverted phase contrast microscope and photomicrographs of cells were obtained using an Olympus 1X71 inverted microscope attached to a coolSNAP cf camera (Photometrics, US). Centrifugation of cell suspensions was carried out using a Sorvall Legend T swinging-bucket rotor bench-top centrifuge (ThermoScientific, UK).

Generally, all cell lines were maintained in sterile disposable plastic culture dishes with the following surface areas; 9.5cm^2 (6-well plate), 9cm^2 (P35), 21cm^2 (P60) or 55cm^2 (P100) (Sarstedt, DE). Pre-sterilized, individually wrapped serological pipettes (Sarstedt, DE) were used to transfer liquid volumes (2ml-35ml). Smaller volumes (<1ml) were transferred using Gilson pipettes with RNAse and DNAse-free sterile filter-tips (Sarstedt, DE or Starlabs, UK). Glass Pasteur pipettes were heat-sterilized (130ºC) before being used to aspirate waste liquid volumes.

2.1.2 CELL LINES AND CULTURE CONDITIONS

Both human and rodent-derived cell lines and strains were cultivated for the purposes of this project (Table 2.1 and Chapter 2.1.2.2). Growth medium was purchased as filter-sterilized 500ml liquid stock solutions (Gibco, US or HyClone, GE Healthcare, UK) to which appropriate supplements were directly added, depending on the specific requirements of each cell line (Table 2.2). To ensure that complete media were free from contaminants, 5ml aliquots were incubated at 37ºC for at least 5 days and monitored for microbial or fungal growth. Complete media were stored at 4ºC until required. 500ml stock solutions of foetal bovine serum (FBS, Gibco, US) supplement, were aliquoted into 50ml Falcon tubes and stored at -20ºC. Depending on manufacturer's guidelines, supplements were either stored at 4ºC or -20ºC.

2.1.2.1 Human Cell Lines/Strains

Table 2.1 - Origin and features of each human cell line/strain utilized.

Cell Line	Patient Details	Cancer Type	Tumour stage	Cell Line Tissue Origin	Tumorigenic	Reference
BT20	C, F, 74 years,	Breast, IDC	-	Mammary gland	Yes	Lasfargues and Ozello, 1978
BT474	C, F, 60 years	Breast IDC	-	Breast Duct	Yes	Lasfargues *et al.* 1978
GI101	F, 57 years	Breast IDC	III	Breast Duct	Yes	Hurst *et al.* 1993
HCC1143	C, F, 52 years	Breast IDC	IIA	Breast Duct	-	Gazdar *et al.* 1998
H5S78T	C, F, 74 years	Breast CS	-	Mammary Gland	No	Hackett *et al.,* 1977
HMEC LONZA*	F	Normal HMEC cell strain	N/A	Breast - Reduction Mammoplasty Tissue	-	Lonza Group Ltd
HMEC 184*	F, 21 years					Stampfer and Bartley 1984
HMEC 240L*	F, 19 years					Garbe *et al.,* 2009
MCF7	C, F, 69 years	Breast, AC	IV	Pleural Effusion	No	Soule *et al.,* 1973, Clarke *et al.,* 1990
MCF-10A	C, F, 36 years	Breast FBD	-	Breast	No, spontaneously immortalized	Soule *et al.,* 1990
MTSV	-	Normal Breast epithelium	N/A	Breast Lumen	No, SV40-immortalized	Bartek *et al.,* 1991, D'Souza *et al.,* 1993
PC3-hTERT	M, 62 years	Prostate AC	-	Prostate	Yes, Telomerized by Newbold group (Brunel University)	Kaighn *et al.,* 1979
21NT	F, 36	Breast, IDC and DCIS	III	Breast	Yes	Band *et al.*, 1990
21MT				Pleural Effusion	Yes	

(*) Denotes HMEC cell strains that were cultivated by Dr. Hemad Yasaei. Abbreviations: C (Caucasian), F (Female), M (Male), IDC (Infiltrating/Invasive Ductal Carcinoma), CS (Carcinosarcoma), AC (Adenocarcinoma) HMEC (Human Mammary Epithelial Cell), FBD (Fibrocystic Disease), DCIS (Ductal Carcinoma In Situ).

Table 2.2 - Individual human cell line growth media and supplements

Cell Line	Medium	Supplements
21NT, 21MT	MEM-α (1X)	10% FBS, 10mM HEPES, 0.1mM NEAA, 1µg/ml Insulin, 2.8µM Hydrocortisone, 12.5ng/ml EGF
BT474, H5S78T, BT20	DMEM/F12 (1:1, 1X)	10% FBS, 1% Glutamax
MTSV	DMEM/F12 (1:1, 1X)	10% FBS, 1% Glutamax, 0.5µg/ml Hydrocortisone
MCF-10A	DMEM/F12 (1:1, 1X)	10% FBS, 1% Glutamax, 5µg/ml Insulin, 1µg/ml Hydrocortisone, 10ng/ml EGF
HCC1143	RPMI 1640 (1X)	10% FBS, 1mM Sodium Pyruvate, 10mM HEPES
GI101	RPMI 1640 (1X)	10% FBS, 2mM Glutamine, 10µg/ml Insulin
MCF-7	EMEM (1X)	10% FBS, 0.1mM NEAA, 1mM Sodium Pyruvate
PC3-hTERT	F12 (1X)	7% FBS, 1% Glutamax

Abbreviations: FBS (foetal bovine serum), MEM (modified Eagle's medium), DMEM (Dulbecco's MEM), EMEM (Eagle's minimal essential medium), F12 (Ham's F12), HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), NEAA (non-essential amino acids), EGF (Epidermal Growth Factor).

The HMEC cell strains (LONZA, 184 and 240L) were cultured by Dr. Hemad Yasaei and were provided to me upon request, either as a semi-confluent monolayer or as a cell pellet.

2.1.2.2 Rodent A92 and Human: Rodent Monochromosomal Hybrid Cells

The A92 cell line is a sub-clone of the A9 mouse fibroblast cell line, which in turn was derived from mouse L-cells (Littlefield, 1964). This cell line was cultured in DMEM (1X, Gibco, US) supplemented with 10% FBS and maintained at 37ºC with 5% CO_2.

The human: rodent somatic cell hybrid A9-Hytk17 is a rodent fibroblast cell line (A9), carrying a single, intact copy of human chromosome 17. This hybrid was generated as part of a panel of 23 human: rodent hybrids, representing all 22 human autosomes and the X chromosome (Cuthbert et al., 1995). The normal human donor chromosomes are derived from a human male skin fibroblast cell strain (1BR.2); each human chromosome was tagged with a selectable hygromycin phosphotransferase-thymidine kinase (Hytk) fusion gene marker (Lupton *et al.* 1991). The Hytk marker comprises the *E.coli* hygromycin phosphatase gene (*hph)* and the *Herpes Simplex* virus thymidine kinase gene (*HSVtk)* (Lupton *et al.* 1991). Stable integration of the Hytk fusion gene within each human chromosome of the human: mouse hybrid panel allows the stable transfer of human monochromosomes into target mammalian cells using microcell-mediated monochromosome (MMCT) techniques. In the presence of the hygromycin B (hygB) antibiotic, only hybrids containing the Hytk-tagged chromosome are resistant and able to survive. Selection for the loss of the tagged human chromosome from hybrids can be achieved through exposure of cells to ganciclovir (GCV), which is converted by the Hytk-encoded thymidine kinase enzyme into a cytotoxic nucleotide analogue. The A9-Hytk17 hybrid was used to study the effects of functional gene transfer during this project. This cell line was cultured in

DMEM (1X, Gibco, US), supplemented with 10% FBS and 400U/ml hygromycin B (Calbiochem, DE) and were maintained at 37ºC at 10% CO_2.

The human: mouse monochromosome hybrid A9-Neo3 was also used as a donor of normal human chromosome 3 for the purposes of gene transfer during this project (Coriell Cell Repositories, Camden, NJ). The normal copy of human chromosome 3, within this hybrid, is derived from a female lung fibroblast cell line, and has been tagged with a neomycin (neo) antibiotic resistance gene. A9-Neo3 cells were cultured in DMEM (1X, Gibco, US), supplemented with 10% FBS and 400µg/ml geneticin (G418, Invitrogen, US).

2.1.3 SERIAL SUB-CULTIVATION OF CELL LINES (PASSAGING)

All cell lines used within this study were cultured as anchorage-dependent cell monolayers and were subject to a standard protocol of serial sub-cultivation. This protocol was used to passage 70-90% confluent dishes containing cells growing in log-phase. First, all required reagents were warmed to 37ºC in a water-bath for at least 15 minutes prior to cell manipulation. Equipment and reagent bottles were cleaned and sterilized using 70% Industrial Methylated Spirits (IMS) diluted in de-ionized water. Following aspiration of cell culture media, 1-2ml of sterile 1X phosphate buffered saline solution (PBS, Severn Biotech, UK) was used to wash residual medium from the cell monolayer. Immediately after aspiration of PBS, 1-2ml of sterile 1X TryPLE Express Enzyme solution (Gibco, US) was added. Cells were then incubated at 37^{o}C for 5-10 minutes and regularly observed under the microscope to monitor detachment from the dish surface. In order to avoid prolonged exposure to dissociation enzymes within the TryPLE Express solution, the side of the dish was firmly tapped to detach any cells remaining after this incubation period. Rarely, a sterile 16cm cell scraper (Sarstedt, DE) was used to detach cells that remained adherent even after incubation or agitation. The resulting cell suspension was transferred to a new culture vessel containing fresh complete medium. Cells were sub-cultured at a ratio of 1:2 to 1:10 depending on cell growth rate and downstream requirements.

To maintain a constant pH balance and supply of nutrients, cell monolayers were re-fed with fresh complete culture medium every 2-3 days.

2.1.4 CRYOPRESERVATION AND RECOVERY OF CELLS

Stocks of each cell line were created for long-term storage in liquid nitrogen (-196ºC) Firstly, cells at 70-90% confluence, growing in log-phase, were detached from the dish surface using the aforementioned method and the resulting cell suspension was transferred to a 15ml Falcon tube. To obtain any residual adherent cells, the dish was washed once with complete medium and the solution added to the cell suspension. The mixture was then centrifuged at 1500rpm for 5 minutes. After careful aspiration of the supernatant, the Falcon tube was tapped

firmly to loosen the cell pellet. Depending on the original dish size (and therefore cell yield), 0.5-1ml of freezing mixture consisting of 90% FBS and 10% Dimethyl Sulfoxide (DMSO) was used to re-suspend the cell pellet. The cell solution was then transferred to a sterile plastic screw-top ampoule (Sarstedt, DE), placed in a metal rack and slowly cooled in the gaseous phase of a liquid nitrogen container (at a rate of ~1ºC per minute). The following day, the ampoule was transferred to the liquid nitrogen phase for long-term storage.

To re-establish cell lines in culture following storage in liquid nitrogen, ampoules were first rapidly thawed at 37ºC in a water bath. Cell suspensions were then transferred into 1 or 2 culture vessels containing complete growth media, and incubated under normal growth conditions. The following day, the cell culture medium was refreshed to remove any traces of DMSO.

2.1.5 Isolation and Propagation of Individual Cell Colonies/Clones

A standard protocol was developed for the isolation and propagation of cell colonies arising from treated 21NT cells undergoing antibiotic selection for: (i) stable cell hybrids containing an intact copy of a single MMCT-transferred human chromosome and (ii) cells containing transfected human gene cDNA clones that had been virally integrated into the host genome. Selection and growth of antibiotic-resistant cell colonies was carried out using P100 cell culture dishes, in order to maximize the distance between colonies that may arise on the same dish. Following microscopic identification of a cell colony, a permanent black marker pen was used to draw a circle around the colony on the underside of the culture dish, in order to indicate colony size and position. After aspiration of cell culture media, the dish was washed once with pre-warmed 1X PBS. Sterile forceps were used to press a sterile plastic cloning cylinder (Cole-Parmer, Scienceware) into a layer (approximately 5mm thick) of pre-sterilized Vaseline® to coat the underside rim of the cylinder. Using the same forceps, the cylinder was then transferred to the P100 culture dish and firmly pressed down upon the marked colony. The Vaseline® coating was used to seal the cloning cylinder to the dish surface. Depending on the size of the cloning cylinder, 100-200µl of pre-warmed 1X TryPLE Express was pipetted into the cylinder, before placing the dish in a 37ºC incubator for 5-10 minutes to enable cell detachment. The resulting cell suspension was retro-pipetted several times and transferred to a P35 dish containing 2ml of complete medium and the appropriate selection antibiotic. Fresh medium was applied to the dish after 48 hours.

2.1.6 Cell Number and Viability Estimation

Cell number and viability was assessed using a Countess® Automated Cell Counter (Invitrogen, US), according to manufacturer's guidelines. Cells growing in log-phase were harvested and pelleted using the aforementioned procedure. Depending on the size of the culture dish and the observed level of confluence prior to cell detachment, cell pellets were resuspended

in 1-2ml of culture medium. 10µl of cell suspension were transferred to a sterile Eppendorf tube, followed by 10µl of 0.4% Trypan blue stain (Invitrogen, US). After thorough mixing, 10µl of the cell solution were transferred into a single chamber of a Countess® Cell Counting Chamber Slide. The slide was then immediately placed into the automated cell counter and both the cell number (number of cells/ml) and viability (viable cells/ml) were ascertained and recorded.

2.2 GENOMIC DNA ISOLATION AND QUANTIFICATION

Cell monolayers growing in log-phase (70-90% confluent) were harvested and centrifuged to obtain cell pellets as mentioned previously. After aspiration of the supernatant, 1ml of pre-warmed 1X PBS was used to wash pellets and remove residual growth media. Following aspiration of the supernatant cell pellets were immediately stored at -80ºC until required.

DNA extraction was carried out using the Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's guidelines. Cell pellets were first removed from storage and thawed on ice. 600µl of Nuclei Lysis solution was then immediately added to the pellet and the solution retro-pipetted several times to facilitate complete cell lysis. Cell lysates were transferred to a sterile 1.5ml Eppendorf tube, followed by the addition of 3µl of RNAse A solution. The mixture was then incubated at 37ºC for 15-30 minutes in a pre-heated water bath. The cell lysate mixtures were then cooled to room temperature for 5 minutes before 200µl of Protein Precipitation solution was added. After vigorous vortexing for 20 seconds, samples were incubated on ice for 5 minutes to enable protein precipitation. Samples were then centrifuged at 13,000 x g for 4 minutes at 4ºC. Sample supernatants, containing genomic DNA, were carefully transferred to fresh 1.5ml Eppendorf tubes containing 600µl of 2-propanol (Sigma-Aldrich, US). Sample tubes were inverted several times before pelleting the DNA by centrifugation at 13,000 x g for 1 minute at room temperature. After decanting the supernatant, DNA pellets were washed once by adding 600µl of 70% ethanol solution (diluted in nuclease-free water, Ambion, Thermo Fisher Scientific, US), and centrifuging at 13,000 x g for 1 minute at room temperature. Sample supernatants were then decanted and DNA pellets air-dried for 15 minutes by inverting the tubes on clean tissue paper. DNA pellets were dissolved in 50µl of DNA Rehydration solution (10mM Tris-HCl, 1mM EDTA) by incubating at 4ºC overnight. The following day, samples were transferred to a -20ºC freezer for long-term storage.

The concentration of sample DNA was ascertained using the NanoDrop 2000 UV/Vis Spectrophotometer (Thermo Scientific). The spectrophotometer was first calibrated using DNA Rehydration Solution, before reading the absorbance of sample solutions at 260nm and 280nm

wavelengths to determine the DNA concentration (ng/µl). As a measure of sample purity, only samples with an $A_{260/280}$ absorbance ratio of around 1.8 were kept for downstream analysis.

2.3 QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (QRT-PCR)

2.3.1 RNA EXTRACTION AND QUANTIFICATION

Extraction of RNA from cell lines or strains was carried out using peqGOLD TriFast reagent (Peqlab) according to manufacturer's guidelines. Cell culture media was first aspirated from dishes containing 70-90% confluent cell monolayers growing in log-phase. Cell monolayers were washed once with pre-warmed 1X PBS to remove any traces of culture media, before 1ml of TriFast reagent was added to lyse the cells. After retro-pipetting several times, cell lysates were transferred into a sterile 1.5ml Eppendorf tube and incubated at room temperature for 5 minutes. Following the addition of 200µl of molecular biology-grade chloroform (Sigma, US), cell lysates were vortexed vigorously for 20 seconds. To enable phenol-chloroform phase separation, sample mixtures were incubated at room temperature for 5 minutes and then centrifuged at 12,000 x g for 5 minutes at 4ºC. The aqueous-phase, containing the RNA, was carefully pipetted into a fresh 1.5ml Eppendorf tube containing 500µl of 2-propanol. RNA was then precipitated by incubating samples on ice for 10-15 minutes. Samples were centrifuged at 12,000 x g for 10 minutes at 4ºC to pellet precipitated RNA. After decanting the supernatant, RNA pellets were washed twice with 75% ethanol (diluted in nuclease-free water, Ambion, Thermo Fisher Scientific, US) by gentle vortexing and centrifugation at 12,000 x g for 10 minutes at 4ºC. After the final wash step, the ethanol solution was decanted and RNA pellets were air-dried by leaving tubes inverted for 5 minutes at room temperature. RNA pellets were then dissolved in 30-50µl of nuclease-free water (Ambion, Thermo Fisher Scientific, US) by incubating samples on ice for 30 minutes. For long-term storage, samples were transferred to a -80ºC freezer.

Sample RNA concentrations were determined using the NanoDrop 2000 UV/Vis Spectrophotometer (Thermo Scientific) as described for the quantification of DNA samples, except that the instrument was first calibrated using nuclease-free water. Only RNA samples with an $A_{260/280}$ absorbance ratio of between 1.6-2.0, were kept for downstream gene expression analysis.

2.3.2 ELIMINATION OF DNA FROM RNA EXTRACTS

Removal of contaminating DNA from RNA sample extracts was achieved using the Deoxyribonuclease I (DNAse I), Amplification Grade enzyme (Invitrogen, US). To each

reaction mixture containing 1µg of RNA, 1µl of 10X DNAse I Reaction Buffer and 1µl of (1U/µl) DNAse I enzyme was added, followed by nuclease-free water (Ambion, Thermo Fisher Scientific, US) to increase the final volume to 10µl. Solutions were then incubated at room temperature for 30 minutes. To de-activate the DNAse I enzyme, 1µl of 25mM Ethylenediaminetetraacetic acid (EDTA) was added before reaction mixtures were incubated at 65ºC for 10 minutes.

2.3.3 Reverse Transcription of RNA to Complementary DNA (cDNA)

The High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, US) was used to reverse transcribe 1µg of DNAse I-treated RNA into cDNA, according to manufacturer's guidelines. Reaction mixtures consisted of: 2µl 10X RT Buffer, 0.8µl 100mM deoxynucleotides (dNTPs), 2µl 10X RT Random Primers, 1µl MultiScribe® MuLV Reverse Transcriptase enzyme (50U/µl), 1µl RNAse OUT™ Recombinant Ribonuclease Inhibitor (40U/µl, Invitrogen, US) and 2.2µl of nuclease-free water (Ambion, Thermo Fisher Scientific, US). For larger amounts of starting RNA (2µg), reaction component volumes were scaled up accordingly. cDNA synthesis was carried out by incubating reaction mixtures at 25ºC for 10 minutes, followed by 37ºC for 120 minutes and then 85ºC for 5 minutes using a thermal cycler (DNA Engine Tetrad 2, MJ Research). Samples were then immediately stored at -20ºC, or an equal volume (20µl) of nuclease-free water was added to dilute the cDNA (1:1) prior to storage.

2.3.4 Gene Expression Analysis by qRT-PCR

Gene expression levels were determined using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, US). Details of gene primer and probe sequences and the mRNA transcripts detected by each primer pair are detailed in Table 2.3. Reaction mixtures for all genes consisted of: 5µl 10X iTaq™ Universal SYBR® Green Supermix or Universal Probes Supermix (BioRad, US), 2µl undiluted or 1:1 diluted cDNA sample, gene primers with/without probes and nuclease-free water up to 10µl (Table 2.4). Reaction mixture components, aside from sample cDNA, were added to a sterile 1.5ml Eppendorf tube. After gentle vortexing, the reaction mixture was pipetted into wells of a 96-well microplate (MicroAmp® Optical 96-Well Reaction Plate, ABI, US). 2µl of cDNA sample was then added to appropriate wells before sealing microplates with optically-clear adhesive film (MicroAmp® Optical Adhesive Film, ABI, US). After centrifugation at 1000xg for 30 seconds at room temperature, microplates were loaded into the qRT-PCR thermal cycler. Exposure of the reaction mixture to light was minimized throughout the procedure. SDS 2.3 (ABI, US) software was used to set up thermal cycling parameters (Table 2.4), assign appropriate detectors (SYBR® or probe-labelled) and label wells with sample names. All samples for one gene were analysed on a single plate and every sample was analysed in triplicate. Non-Targeting Controls (NTC) containing nuclease-free water in place of cDNA were included for every gene analysed.

Where possible, positive and negative control samples for each target gene were included. To facilitate downstream quantification of target gene expression levels, the mRNA levels of one or more endogenous control genes (*GAPDH, TOP1* and *YWHAZ)* were also determined using the same cDNA stock for each sample.

Table 2.3 - Primer and probe sequences and transcript accession numbers

Gene	Primer Name	Sequence (5'-3')	Transcript (s) Accession No.*
hTERT[1]	hTERT-F	GAGCTGACGTGGAAGATGAGC	-
	hTERT-R	GGTGAACCTCGTAAGTTTATGCAA	
	hTERT-Pr	6-FAM-CACGGTGATCTCTGCCTCTGCTCTCC-TAMRA	
GAPDH[1]	GAPDH-F	GAAGGTGAAGGTCGGAGT	NM_001289746.1 NM_002046.5
	GAPDH-R	GAAGATGGTGATGGGATTTC	
SETD2	SETD2-F	ATTGAGTTTTTCTTCCTCTTGTGAGAT	NM_014159.6
	SETD2-R	CCCAACCTAAGTTTCTGAGCTCTT	
	SETD2-Pr	6-FAM-CACATGTGGATGGCTTGCACTCATCA-MGB	
FLJ/KIF9	FLJ-F	TGACAGTGCTTGGTGCCTTTC	NR_033373.1
	FLJ-R	CCATACTCGTTGCAAGGAGGA	
P21/CDKN2A	P21-F	AGCATGGAGCCTTCG	NM_001195132.1 NM_000077.4 NM_058197.4
	P21-R	ATCATGACCTGGATCGG	
BAP1	BAP1-F	AGAAATACTCACCCAAGGAG	NM_004656.3
	BAP1-R	TCCTTCTCTGGTCATCAATC	
PBRM1	PBRM1-F	ACGGAAAATCAACATGAGTG	NM_018313.4
	PBRM1-R	TGCCTTCATATTCTGCTTTC	
RASSF1	RASSF1-F	CTTGAACAAGGACGGTTC	NM_001206957.1 NM_170713.2 NM_170714.1 NM_007182.4 NM_170712.2
	RASSF1-R	TGGGCAGGTAAAAGGAAG	
PARP3	PARP3-F	CTGGAAAGTAAACCAAGAAGG	NM_005485.4 NM_001003931.2
	PARP3-R	TCTCTGAGGCAAAGTAGATG	
P53	P53-F	N/A	N/A
	P53-R	N/A	
P16	P16-F	N/A	NM_000077.4 NM_001195132.1
	P16-R	N/A	
TOP1	TOP1-F	N/A	N/A
	TOP1-R	N/A	
YWHAZ	YWHAZ-F	N/A	N/A
	YWHAZ-R	N/A	

**Accession numbers obtained from the National Centre for Biotechnology Information (NCBI).*
[1]hTERT and GAPDH primer sequences obtained from Ducrest et al., (2001). Abbreviations: F (Forward), R (Reverse), Pr (Probe), 6-FAM (Carboxyfluorescein), MGB (major groove binder), TAMRA (Carboxytetramethylrhodamine).

Table 2.4 - Primer-specific thermal cycling parameters and final reaction primer and probe concentrations

Primer Source	Sigma	ABI	Primer Design	Other
Gene Name	*PARP-3, PBRM1, RASSF1, BAP1, p21*	*P16*	*TOP1, YWHAZ*	*hTERT*, GAPDH, SETD2,FLJ, p53*
Primer (µM)	0.45	0.9	0.3	0.3
Probe (µM)	-	0.25	-	0.15
Thermal Cycling Parameters	Step 1: 95ºC 30sec Step 2: (40cycles) 95ºC 15min, 58ºC 30sec, 72ºC 15sec	Step1: 95ºC 10min Step 2: (40cycles) 95ºC 15sec, 60ºC 1min		

**hTERT transcripts were amplified for 50 cycles.*

2.3.5 GENE EXPRESSION QUANTIFICATION

After completion of the thermal cycling program, sample amplification curves, and assigned baseline fluorescence and threshold values were inspected to confirm (i) consistency of cDNA quality across all samples, (ii) no detectable amplification of the NTC control, (iii) that appropriate threshold and baseline fluorescence values for each gene were assigned (iv) where possible, expected amplification curves of both positive and negative control samples. Cycle threshold values (Ct) were then imported into qbase^{PLUS} data analysis software program (Biogazelle). This software is designed to calculate the normalized relative quantities (NRQ) for each sample based on the Ct values obtained for target and endogenous control genes.

NRQ values were calculated by qbase^{PLUS} software using the following formulae (adapted from Hellemans *et al*. 2007):

1. Target gene normalization using a single endogenous control gene:

$$\mathbf{NRQ = E^{-\Delta\Delta Ct}} = \frac{E^{\Delta Ct_{end}}}{E^{\Delta Ct_{toi}}}$$

2. Target gene normalization using multiple endogenous control genes:

$$\mathbf{NRQ} = \frac{E_{toi}^{\Delta Cq,toi}}{\sqrt[f]{\prod_{i=1}^{f} E_{end_i}^{\Delta Cq,end_i}}} = \frac{RQ_{toi}}{geometric\ mean(RQ_{end})}$$

E = amplification efficiency

end = endogenous control gene

toi = target gene of interest

All target and endogenous control genes included in this project were assumed to have a 100% amplification efficiency (E = 2).

2.4 Determination of Telomerase Activity

2.4.1 Protein Extraction and Quantification

Cells growing in log-phase were harvested and counted using aforementioned methods. Following centrifugation, cell pellets were washed once with 1X PBS and immediately stored at -80ºC. After thawing on ice, cells were lysed by adding 200µl/10^5-10^6 cells of TRAPeze® 1X CHAPS Lysis Buffer (Chemicon®, Merck Millipore, DE) and thoroughly mixing several times before incubating on ice for 30 minutes. Cell lysates were then centrifuged at 12,000xg, for 20 minutes at 4ºC. 20µl of each sample supernatant was set aside for protein quantification and the remainder aliquoted into multiple tubes and immediately stored at -80ºC.

The protein concentration of each sample was determined using the CB-X Protein Assay (G-Biosciences) according to manufacturer's guidelines. This colorimetric assay relies on the absorbance of the CB-X protein dye, which changes according to the amount of protein within each sample. Firstly, bovine serum albumin (BSA, 2mg/ml) was diluted in CHAPS buffer to produce a set of eight protein standards ranging from 0.05-2µg/µl. These were used to construct a calibration curve of absorbance values at 595nm for each protein standard (Fig 2.1). The linear trend line equation was used to determine the protein concentration of sample extracts.

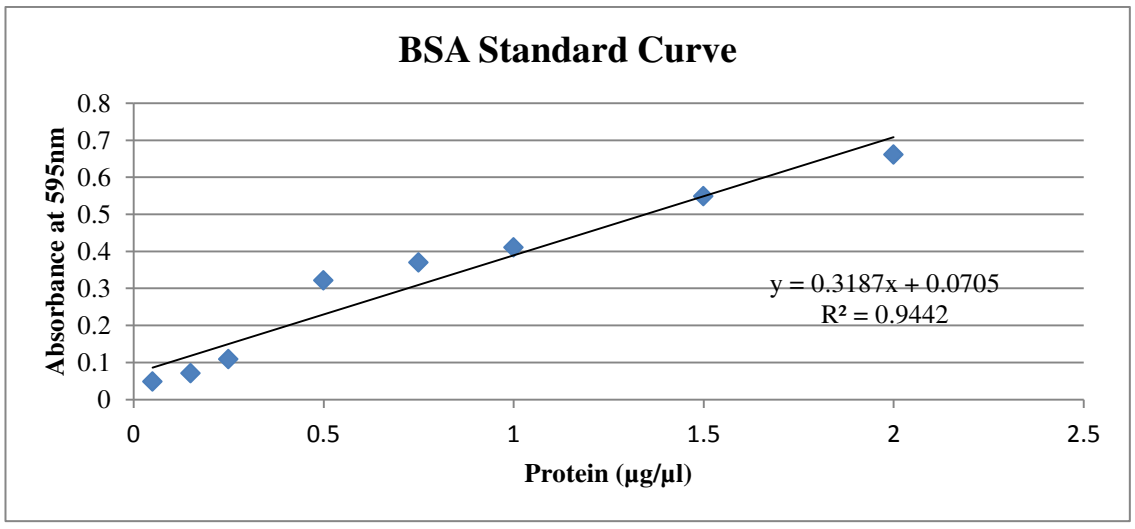


Figure 2.1 - *Standard calibration curve of BSA protein (2mg/ml) diluted in CHAPS lysis buffer.*

Samples were prepared for quantification, by first transferring 10µl of BSA standards or sample extracts to a 96-well microplate (Corning, US), followed by 200µl of CB-X Assay Dye.

Solutions were then incubated at room temperature for 5 minutes before loading the microplate into a spectrophotometer (xMark Microplate Absorbance Spectrophotometer, BioRad, US) and reading the absorbance at 595nm. Sample extracts and BSA standards were read in duplicate wells and each microplate was read three times in succession. The mean of six absorbance readings for each sample was then determined before subtracting the average background absorbance (CHAPS buffer only). These final values were used to construct a BSA standard curve and estimate protein concentrations of sample extracts (Fig. 2.1).

2.4.2 TELOMERE REPEAT AMPLIFICATION PROTOCOL (TRAP) ASSAY

Telomerase activity was determined using an qRT-PCR-based TRAP assay protocol adapted from Wege *et al*. (2003). Reaction mixtures for each sample consisted of 12.5µl 10X iTaq™ Universal SYBR® Green Supermix, 0.05µg/µl ACX primer, 0.1µg/µl TS primer (Table 2.5), 250ng sample protein extract, and nuclease-free water to bring the total volume to 25µl. Protein extracts were thawed on ice and diluted to 62.5ng/µl using CHAPS lysis buffer. A stock solution containing all reaction mixture components except the protein sample, were loaded into 96-well microplates (MicroAmp® Optical 96-Well Reaction Plate, ABI, US) followed by diluted protein extracts. A maximum of nine samples, twelve control samples and five standards were assayed on a single microplate at any one time. A serial dilution of telomerase-positive PC-3-hTERT protein extracts was carried out to generate a standard curve and enable quantification of telomerase activity within unknown samples. These consisted of five standards ranging from 400ng to 25ng protein, diluted in CHAPS lysis buffer. The 21NT breast cancer cell line was used as a telomerase-positive control and as an untreated wild-type control sample (Cuthbert *et al.* 1999, Ducrest *et al.* 2001, Szutorisz *et al.* 2003) and either HMEC 184 or HMEC 240L cell strains were used as normal human controls. Negative controls for each sample were generated by heat-inactivating telomerase within aliquots of sample protein extracts at 85ºC for 10 minutes. An additional non-template control (NTC) consisting of CHAPS lysis buffer in place of protein sample, was also included. All samples, controls and standards were analysed in triplicate. After sealing with optical adhesive film (MicroAmp® Optical Adhesive Film, ABI, US), microplates were loaded into the ABI Prism 7900HT thermal cycler. The SDS 2.3 software program was used to set up a thermal cycling program consisting of an initial incubation step at 25ºC for 20 minutes to enable endogenous telomerase to elongate the TS primer substrate molecule, followed by PCR amplification of extended fragments at 95ºC for 10 minutes and 35 cycles of 95ºC for 30 seconds and 60ºC for 90 seconds.

Table 2.5 - Primer sequences used in the qRT-PCR TRAP assay

Primer Name	Sequence (5'-3')
TS (Telomerase Substrate)	AATCCGTCGAGCAGAGTT
ACX (Anchored Return Primer)	GCGCGG(CTTACC)$_3$CTAACC

2.4.3 *Quantification of Telomerase Activity*

Once the thermal cycling program had completed, amplification plots of positive and negative control samples were first inspected before telomerase activity was quantified within sample extracts. Figure 2.2 shows typical amplification plots of control samples obtained from a single assay run, that demonstrate: (i) the absence of contaminating telomerase inhibitors within the reaction mixture (Figure 2.2A), (ii) that sample cross-contamination is unlikely to have occurred during assay set-up (Figure 2.2B) and (iii) a minimal degree of technical variation introduced by pipetting errors (Figure 2.2 A and C). From average Ct values of PC-3-hTERT protein standards, SDS 2.3 software was used to generate a standard curve plot (Fig 2.2C and D) and calculate the telomerase activity of unknown samples based on their Ct values. These values were exported and presented as a bar-graph with error bars showing the standard deviation of sample replicates.

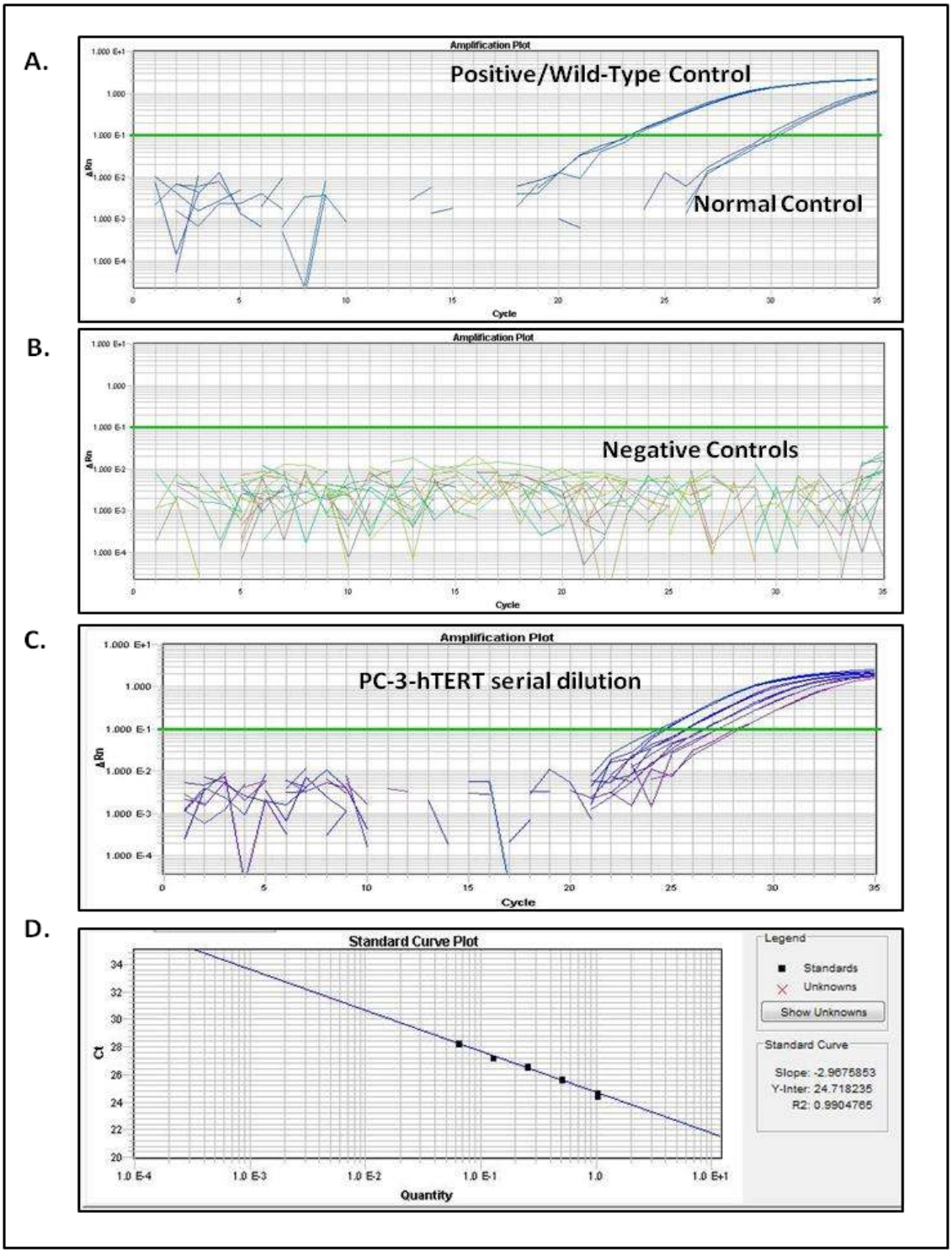


Figure 2.2 - *Representative amplification and standard curve plots of control samples and standards included in each qRT-PCR-based telomere repeat amplification protocol (TRAP) assay (SDS2.3). Baseline-corrected normalized fluorescence signals (ΔRn) of* ***(A)*** *Telomerase positive wild-type (21NT) and normal human (HMEC) control samples,* ***(B)*** *heat-treated samples and CHAPS-only NTC control* ***(C)*** *serial dilution standards of PC-3-hTERT protein extract. The green line shows the amplification threshold value. Image* ***(D)*** *A typical standard curve plot of Ct values obtained from PC-3-hTERT serial dilution standards.*

2.5 GENE COPY NUMBER VARIATION (CNV) ANALYSIS

CNV analysis of target genes was carried out using pre-designed Taqman® Copy Number Assay primers developed by ABI (Applied Biosystems, US), that detect and amplify specific 86-105bp intronic or exonic regions of *SETD2* and *FLJ* gDNA sequences (Table 2.6). In order to normalize target gene copy number, assays were run in a duplex reaction with a ribonuclease P RNA component H1 (*RNaseP*) Taqman® Reference Assay. *RNaseP* is assumed to be present as two copies within each sample. To distinguish between target and reference sequences within a duplex qRT-PCR reaction, target gene copy number probe sequences are labelled with 5'FAM - 3'MGB and the reference gene probe sequences are labelled with 5'VIC - 3'TAMRA.

Table 2.6 - Details of the size and genomic region targeted by pre-designed CNV primers (ABI, US)

Gene Name	NCBI Location (Build 37)	Assay Reference Number	Assay Gene Location	Amplicon Length (bp)
SUMF1	3p26.1c: 4,443,756	Hs06663284_cn	Intron 7	81
RAR-β	3p24.2a: 25,542,710	Hs01803834_cn	Intron 2-Exon 3	106
SETD2	3p21.31: 47,057,898-47,205,467	Hs02774936_cn	Exon 21	105
		Hs00431122_cn	Exon 15	98
		Hs01027663_cn	Intron 6-Exon 7	86
		Hs02852048_cn	Exon 3	91
		Hs04751634_cn	Intron 1	105
FLJ	3p21.31: 47,243,557	Hs05895570_cn	Intron 2	100
PARP-3	3p21.2a: 51,979,562	Hs01428519_cn	Exon7	85
BAP1	3p21.1e: 52,439,941	Hs02357352_cn	Intron9-Exon 10	108
PBRM1	3p21.1e: 52,657,538	Hs06624309_cn	Intron 14	108
NA*	3p14.2b: 61,252,882	Hs06677725_cn	-	111
CADM2	3p12.1a: 85,911,377	Hs04769003_cn	Intron 5	110
RNaseP (Reference)	14q11.2: 20,343,071	4403326	Exon 1	87

*Annotations: * NA (Not Applicable)*

Reaction mixtures for each target gene consisted of 10µl of 2X Taqman® Universal PCR Mastermix, 1µl of 20X Taqman® Copy Number Assay, 1µl 20X Taqman® Reference Assay, 4µl of 5ng/µl gDNA extracts and 4µl nuclease-free water up to a total volume of 20µl. Reaction mixtures without gDNA were first pipetted into wells of a 96-well microplate (MicroAmp®, ABI, US). Samples were analysed in triplicate and an NTC was included for each target gene analysed. The HMEC 184 cell strain was assumed to have two copies of every target gene analysed, and was used to calibrate gene copy number within all other samples. Following the addition of gDNA into appropriate wells, microplates were sealed with optical adhesive film (MicroAmp®, ABI, US) and centrifuged at 1000xg for 30 seconds at room temperature. After loading the microplate into the ABI Prism 7900HT Sequence Detection System, the following thermal cycling parameters were set up: 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Raw amplification data was imported into the Copy Caller™ Software data analysis program, which calculates target gene copy number of each sample using the ΔΔCt method of relative quantification. For each sample, the following calculations were applied:

1. Normalization of target gene (t) with *RNase P* reference gene (r) within each well (w):
   $(\Delta Ct)_w = (Ct)_{t,w} - (Ct)_{r,w}$

2. The arithmetic mean of sample (s) ΔCt replicates (n):

   $\mu(\Delta Ct)_s = \frac{\Sigma^n (\Delta Ct)w}{n}$

3. Calibration of sample ΔCt values relative to HMEC 184 ΔCt values (ΔΔCt):
   $\Delta\Delta Ct = \mu(\Delta Ct)_s - \mu(\Delta Ct)_{HMEC\ 184}$

4. Fold change in amplification for each sample relative to HMEC 184 (RQ):
   $RQ = 2^{(-\Delta\Delta Ct)}$

5. Calculated target gene copy number (CN):
   CN = 2 x RQ

6. Calculated CN values obtained for each sample were then used to estimate target gene copy number:

Calculated CN value	**Predicted Gene CN**
$0 \leq CN < 0.5$	0
$0.5 \leq CN < 1.5$	1
$1.5 \leq CN < 2.5$	2
$2.5 \leq CN < 3.5$	3

2.6 CLONING

2.6.1 TRANSFORMATION OF CHEMICALLY-COMPETENT E.COLI WITH PLASMID VECTORS

Plasmid constructs were transformed into OneShot® TOP10 Chemically Competent E.coli (Invitrogen, US) according to manufacturer's guidelines. For each transformation reaction, one vial of OneShot® TOP10 E.coli was removed from -80°C storage and thawed on wet-ice for around 30 seconds. 1-5µl of 10pg-100ng plasmid DNA was then added to the E.coli cell suspension and gently mixed before incubating on ice for 30 minutes. To heat-shock the E.coli cells, vials were placed inside a pre-warmed water bath and incubated at 42°C for 30 seconds. Vials were immediately transferred to wet-ice for two minutes before adding 250µl of room-temperature S.O.C medium. Transformed E.coli were then incubated at 37°C for 1 hour on an orbital shaker at 200rpm. Using aseptic techniques, 20-200µl of bacterial suspension were spread onto 3-5 dishes containing pre-sterilized 4% high-salt Luria Bertani (LB) Agar (Sigma-Aldrich, US) with 100µg/ml ampicillin. Dishes were then inverted and incubated overnight at 37°C. The following day around 5-10 individual colonies were picked by gently scraping the surface of the plate with a sterile pipette tip and transferring into a Falcon tube containing 5ml of pre-sterilized room-temperature LB-Broth and 100µg/ml ampicillin. Bacterial suspensions were then returned to the orbital shaker and incubated at 37°C overnight at 200rpm. The following day, cultures were either stored at 4ºC for a maximum of five days, or were prepared for long-term storage as glycerol stocks.

2.6.2 PREPARATION OF BACTERIAL GLYCEROL STOCKS

To create 25% glycerol stock solutions, 750µl of bacterial suspensions growing in log phase were transferred to fresh pre-sterilized Eppendorf tubes together with 250µl of pre-sterilized 100% glycerol solution before gently mixing and storing immediately at -80°C.

2.6.3 PROPAGATION OF TRANSFORMED E.COLI

Bacterial glycerol stock solutions were removed from -80°C and thawed on wet ice. Using aseptic techniques, 50µl of glycerol solution was used to inoculate Falcon tubes containing 5ml of pre-sterilized 2.5% LB-broth (high salt, Sigma, US) and 100µg/ml ampicillin. Bacterial cultures were then incubated at 37°C overnight in an orbital shaker at 200rpm. The following day, 0.5ml of bacterial culture was used to inoculate conical flasks containing 200ml of pre-sterilized 2.5% LB-broth and 100µg/ml ampicillin. Bacterial cultures were returned to the orbital shaker and incubated at 37°C overnight at 200rpm.

2.6.4 *EXTRACTION AND PURIFICATION OF PLASMID DNA CONSTRUCTS FROM E.COLI*

Plasmid constructs were extracted from E.coli using the QIAfilter Midi Plasmid Purification Kit (Qiagen, NL). First, bacterial cells were harvested by centrifugation at 5000xg for 15 minutes at 4°C. After decanting the supernatant, pellets were completely resuspended in 10ml of Buffer P1 (50mM Tris-Cl pH 8, 10mM EDTA, 100µg/ml RNase A). Bacterial cells were then lysed by adding 10ml of Buffer P2 (200mM NaOH, 1% SDS), vigorously inverting tubes 4-6 times and incubating for 5 minutes at room temperature. To precipitate genomic DNA, 10ml of chilled neutralization buffer P3 (3M potassium acetate, pH 5.5) was added to lysate solutions and mixed by inverting tubes 4-6 times. Lysates were then transferred to QIAfilter Midi Cartridges and incubated at room temperature for 10 minutes. During this incubation period, QIAGEN-tips were equilibrated with 4ml of Buffer QBT (750mM NaCl, 50mM MOPS, 15% isopropanol, 0.15% Triton® X-100). Plungers were then inserted into QIAfilter Midi tubes and the lysate filtered into equilibrated QIAGEN tips. Plasmid DNA binds to an anion-exchange resin as bacterial lysates pass through QIAGEN tips by gravity flow. To remove contaminating RNA from the resin, Buffer QC (1mM NaCl, 50mM MOPS, 15% isopropanol) was added to QIAGEN tips and allowed to clear by gravity flow. Elution of plasmid DNA from the resin was achieved by adding 5ml of Buffer QF (1.25M NaCl, 50mM Tris-Cl pH 8.5, 15% isopropanol) to QIAGEN tips and collecting eluates in 50ml Falcon tubes. Plasmid DNA was precipitated by adding 3.5ml of room-temperature isopropanol and by centrifuging the solution at 5000xg for 1 hour at 4°C. Sample supernatants were carefully decanted and DNA pellets were washed once with 2ml of 70% ethanol by centrifugation at 5000xg for 1 hour at 4°C. After decanting supernatants, DNA pellets were air-dried for 5-10 minutes and re-dissolved in nuclease-free water. DNA quality and final concentrations were ascertained using the NanoDrop 2000 UV/Vis Spectrophotometer (Thermoscientific, UK), as described in Chapter 2.2, before storing samples at -20ºC.

2.7 TRANSFECTION OF TARGET GENES WITHIN THE 21NT CELL LINE

2.7.1 *TRANSFECTION PROCEDURE*

Overexpression of target genes within the 21NT breast cancer cell line was achieved by transfection of plasmid DNA constructs consisting of target gene cDNA, using the *Trans*IT®-BrCa Transfection Reagent (Mirus) according to manufacturer's guidelines. This chemical-based method of gene delivery into mammalian cells, relies on the formation of cationic lipopolyplexes consisting of polymers, lipids (present within transfection reagent mixture) and plasmid DNA, that are then taken up into cells by endocytosis. As a negative control, plasmid

constructs without target gene cDNA (empty vectors) were transfected into 21NT cells at the same time, using the identical procedure.

24 hours prior to transfection, 21NT cells were plated at $2.5x10^5$ cells/P60 dish and cultured under normal growth conditions (Chapter 2.1.2, Table 2.2). The following day, cells were visualized under a light microscope to ensure that they were 60-80% confluent. To generate *Trans*IT®-BrCa Transfection Reagent-plasmid DNA complexes, 500µl of Opti-MEM I Reduced Serum Medium (Invitrogen, US), 5µg of linearized plasmid DNA and 7.5µl of transfection reagent (1:1.5 ratio of plasmid DNA to transfection reagent) were mixed together in a sterile 1.5ml Eppendorf tube and incubated at room temperature for 25 minutes. The transfection reagent-DNA plasmid complexes were then added drop-wise to P60 dishes containing 21NT cells, which were gently agitated back-and-forth and side-to-side to ensure even distribution. Dishes were immediately returned to the incubator.

2.7.2 TRANSFECTION EFFICIENCY

In order to determine the efficiency of target gene delivery using the above transfection reagent protocol, a GFP-labelled lentiviral vector construct (LNV-GFP) developed by my colleague Mr. Hassan Khonsari (PhD student supervised by Dr. Michael Themis, Brunel University London) was transfected into three individual P60 dishes of 21NT cells using the aforementioned procedure. 48 hours post-transfection, a Floid® Cell Imaging Station (Life Technologies, US) fluorescence microscope, was used to visualize cells and obtain at least three images of different areas of each dish at x20 magnification (Figure 2.3). The total number of cells and the number of green fluorescent cells, within three images/dish of LNV-GFP-transfected 21NT cells was determined. The efficiency of gene delivery (transfection efficiency) was estimated by calculating the average proportion of cells displaying green fluorescence for all three images/dish. As shown in Figure 2.3, the overall transfection efficiency for this procedure was found to be around 20%.

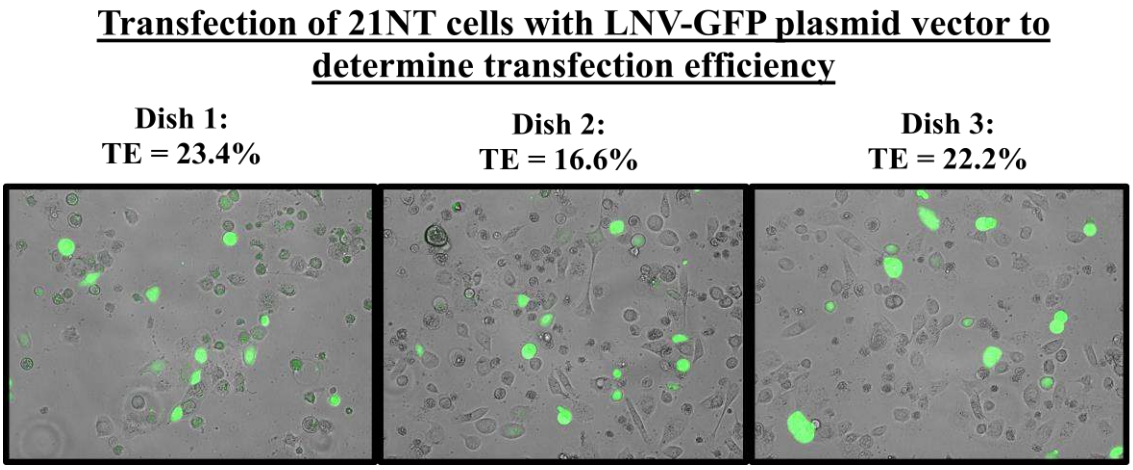


Figure 2.3 - *Determination of transfection efficiency. Representative fluorescence microscopy images (x20 magnification) of three individual P60 dishes of 21NT cells (seeded at 2.5 x 10^5 cells/dish) 48 hours after they were transfected with a lentiviral vector-green fluorescent protein (LNV-GFP) plasmid vector construct using the Mirus Transfection Reagent protocol. The proportion of green fluorescent cells was used to estimate the efficiency of gene delivery into 21NT cells using this method. Abbreviations: TE (transfection efficiency).*

2.7.3 Colony Forming Unit (CFU) Assay

The aforementioned transfection procedure was used to transfect two P60 dishes of 21NT cells; the first with control empty vector plasmids and the second with target gene plasmid constructs. 48 hours post-transfection, both dishes were passaged at a ratio of 1:8 into four P60 dishes. To select for 21NT cells that had undergone stable integration of plasmid DNA, dishes were maintained in complete culture medium supplemented with the appropriate selection antibiotics for two weeks. At this point, culture medium was aspirated and cells were washed once with pre-warmed sterile 1X PBS. In order to visualize and count the number of stable colonies present on each dish, cells were fixed with 5ml of 100% industrial methylated spirits (IMS) and stained with 3ml of Methyl Blue stain (Sigma Aldrich, US). The number of stable empty vector control and target gene colonies that arose following two weeks of antibiotic selection, was expressed as the mean ± SD of four dishes for each plasmid construct. A student's t-test was used to compare the mean number of stable empty vector control and target gene colonies that emerged.

2.8 STATISTICAL FORMULAE

2.8.1 POPULATION DOUBLINGS (PD)

To monitor cell population growth, the following formula was used to calculate the number of times the cell population had doubled in size over a certain time period (Kumar *et al.,* 2014):

$$\mathbf{PD = [(\log_{10}C_1)/(\log_{10}2)] - [(\log_{10}C_0)/(\log_{10}2)]}$$

C_1 = Final cell number

C_0 = Original cell number

CHAPTER 3

3 THE USE OF MICROCELL-MEDIATED MONOCHROMOSOME TRANSFER (MMCT) TECHNIQUES TO DETERMINE THE EXISTENCE OF TELOMERASE REPRESSOR SEQUENCES LOCATED ON CHROMOSOMES 3 AND 17 WITHIN THE 21NT BREAST CANCER CELL LINE

3.1 INTRODUCTION

The human telomerase enzyme has been found to be active within over 85% of human cancers, but is undetectable within the majority of normal human somatic tissues, (Kim *et al.,* 1994). It has been shown that the major rate-limiting factor for telomerase activity is the expression of the *hTERT* gene, which encodes the reverse transcriptase component of telomerase (Meyerson *et al.,* 1997, Counter *et al.,* 1998). This is supported by the finding that *hTERT* transcript molecules can only be detected within human cancer cell lines and not normal somatic cells (Ducrest *et al.,* 2001). Therefore, changes in the regulation of *hTERT* gene transcription could be responsible for the observed telomerase activity within cancer cells.

Studies have shown that normal human somatic cells can undergo telomerase activation and immortalization *in vitro* (Counter *et al.,* 1998, Ozer., 2000). Transfection of normal human fibroblasts with viral oncogenes has been found to extend the proliferative potential of cells beyond the replicative senescence barrier, due to the inhibition of important cell cycle regulatory proteins, p53 and pRb (Wright and Shay., 1992). Cells continue to divide for a further 20-30 population doublings (PD) until entering growth crisis. This is thought to be due to the continued erosion of telomeric sequences, which provokes substantial chromosomal instability and cell death (Chang *et al.,* 2003). Rarely, a colony of immortal cells emerges from crisis, the majority of which display active telomerase. It has been hypothesized that telomerase reactivation occurs due to loss of an *hTERT* transcriptional repressor sequence(s) during cell crisis. There is evidence to show that cellular immortalization is an essential prerequisite for clonal evolution and malignant transformation of cells (Newbold *et al.,* 1982, Counter *et al.,* 1998). As a result, cellular senescence pathways are thought to have evolved as a protective mechanism against immortalization and tumorigenesis.

The functional role of telomerase in cancer development, together with the observation that telomerase is active within the majority of human tumours and not within most normal human tissues, has led to the search for an effective strategy to inhibit telomerase activity in

cancer. One such approach involves the identification of important sequences responsible for regulating telomerase within normal human somatic cells that are functionally inactivated during cancer development. In this way, a greater understanding of the critical events that occur during carcinogenesis can be developed, and effective mechanisms of telomerase repression can be elucidated. Whole cell fusion of immortal human cell lines with normal human fibroblasts has been found to repress telomerase activity and restore limited growth potential, which shows that telomerase activity behaves like a recessive trait. (Pereira-Smith and Smith., 1983). In addition, cell fusion of two different parental immortal human cell lines (somatic genetic complementation) can also result in telomerase repression and growth arrest, which indicates that more than one event can occur during cancer development, that leads to *hTERT* deregulation and telomerase reactivation (Pereira-Smith and Smith, 1988).

In order to develop an effective strategy to study human gene function and narrow the search for genome sequences involved in normal cell growth regulation, Cuthbert *et al.,* (1995) developed a library of human: rodent monochromosomal 'donor' hybrids, representing all 22 normal human autosomes and the X-chromosome, maintained within a rodent fibroblast background. This hybrid panel provides a means of stable, functional gene transfer by facilitating the introduction of single, intact copies of normal human chromosomes into any mammalian recipient cell line. Each human chromosome copy originated from a normal human adult fibroblast cell strain, and was tagged with a selectable hygromycin-phosphotransferase (*Hytk*) fusion gene marker, to allow both positive and negative selection of hybrids following transfer into recipient cells. Cytogenetic characterization of each human: rodent hybrid, confirmed that human monochromosomes could be stably maintained within a rodent background, except chromosome 9, which underwent deletions on the short-arm. This was likely due to the presence of anti-proliferative genes, such as those encoded by the *CDKN2* locus (Cuthbert *et al.,* 1995).

During an initial screen for telomerase regulatory sequences, introduction of a normal copy of human chromosome 3 was found to repress telomerase activity within a head and neck carcinoma cell line (Newbold., 1997). The same effect was observed following introduction of chromosome 3 into a primary breast carcinoma cell line (21NT). Up to 78% of chromosome 3 hybrids were found to be telomerase repressed, compared with around 10% of hybrids containing chromosomes 8, 12 or 20 (Cuthbert *et al.,* 1999). The majority of telomerase-repressed chromosome 3 hybrids were found to enter delayed, permanent growth arrest, and display morphological features of senescence. Deletion mapping of chromosome 3 within telomerase-positive segregant hybrids, revealed two discreet regions of deletion on the short arm of chromosome 3 (3p21.3-p22 and 3p12-p21.1). These findings provided strong evidence that chromosome 3 harbours sequences that repress telomerase activity and inhibit the growth of breast cancer cells.

Introduction of a normal copy of human chromosome 17 into a benzo(a)pyrene-transformed MCF-10F human breast epithelial cell line, resulted in a significant reduction in telomerase activity and a 90% reduction in cell growth (Yang *et al.,* 1999). Chromosome 17 is known to harbour the *p53* tumour suppressor gene, which is ranked as the second most commonly mutated gene in breast cancer (Bamford *et al.,* 2004). Several independent studies have shown that *p53* can mediate *hTERT* transcriptional repression within breast, pancreatic and prostate cancer cells (Kusomoto *et al.,* 1999, Xu *et al.,* 2000, Shats *et al.,* 2004). A reduction in *hTERT* transcription and telomerase activity was observed following activation of endogenous *p53* within a breast carcinoma cell line, through sequestration of Sp1, a known transcriptional activator of *hTERT* (Xu *et al.,* 2000). However, *p53*-mediated transcriptional repression of *hTERT* was found to be dependent on the presence of wild-type *p53* within breast cancer cells. In support of this, transduction of wild-type *p53* into pancreatic cells harbouring *p53* mutations, resulted in *hTERT* transcriptional repression (Kusomoto *et al.,* 1999). Shats *et al.,* (2004), provided evidence of an alternative mechanism of *hTERT* repression in breast cancer cells, whereby p53 activates p21 and E2F proteins, which repress *hTERT* transcription through histone deacetylation. These observations provide evidence that multiple loss/mutation events can occur during breast carcinogenesis, that lead to *hTERT* de-repression and telomerase reactivation. The high frequency of *p53* mutations in breast cancer (23%, Catalogue of Somatic Mutations in Cancer, COSMIC; Forbes *et al.,* 2015), together with evidence demonstrating an important role of *p53* in *hTERT* regulation, could suggest that loss of *p53* function may be a common event in breast carcinogenesis that contributes to telomerase reactivation.

The 21NT cell line used within the study by Cuthbert *et al.,* (1999), has been shown to possess a single insertion mutation within the *p53* coding region, that results in a premature stop codon and non-functional p53 protein (Liu *et al.,* 1994). Introduction of a normal copy of human chromosome 3 into this cell line has been shown to repress telomerase activity and restore a limited growth potential (see above) suggesting that at least two loss/mutation events could be responsible for *hTERT* deregulation. Therefore, in order to further understand whether multiple sequences could function to regulate telomerase activity within a single breast cancer cell line, the effect of introducing a single copy of human chromosomes 3 and 17 into the 21NT cell line, on the level of telomerase activity, was investigated. The same human: rodent monochromosome hybrid cell lines employed by Cuthbert *et al.,* (1999) were used as donors of chromosomes 3 and 17 within this study, and telomerase activity was determined using a quantitative telomere-repeat amplification protocol (TRAP). In order to explore a relationship between telomerase activity and cell proliferation, the growth rate and replicative potential of hybrids was also examined.

3.2 MATERIALS AND METHODS

3.2.1 MICROCELL-MEDIATED MONOCHROMOSOME TRANSFER (MMCT) PROCEDURE

The MMCT procedure was used to transfer human chromosomes 3 and 17 from human: rodent monochromosome donor hybrids A9-Neo3 and A9-Hytk17 donor hybrids, to the primary human breast cancer cell line, 21NT. First, donor hybrids A9-Neo3 and A9-Hytk17 were seeded in twelve 24cm^2 flasks (Nunclon Surface, Nunc), at cell densities described in Table 3.1. Hybrid donor cells were maintained in DMEM (1X) and 20% FBS (Gibco, US) in the absence of selection antibiotics (Chapter 2.1.2.2). On the same day, 21NT cells were seeded at 1.5x10^6 cells/dish in two P100 cell culture dishes and maintained under normal growth conditions for three days (Chapter 2.1.2.1). 24 hours after seeding, colcemid was added to flasks containing A9-Neo3 and A9-Hytk17 donor hybrids at final concentrations detailed in Table 3.1. Flasks were then incubated at 37^{o}C for 48 hours, to induce microcell formation. Exposure of cells to colcemid, inhibits the formation of cell spindles, leading to cell cycle arrest during metaphase. After prolonged exposure of cells to colcemid, cells attempt to re-enter the cell cycle and proceed to G1 phase. In the absence of complete mitosis, nuclear membranes form around single or small groups of chromosomes positioned randomly in the cell, leading to the formation of micronuclei (McNeill and Brown 1980).

Table 3.1 Conditions used for micronucleation of human: rodent donor hybrid cells

Human: Rodent Donor Hybrid	Seeding Density/24cm^2 Flask	Colcemid Concentration (µg/ml)
A9-Hytk17	1.5x10^6	0.075
A9Neo3	1.25x10^6	0.1

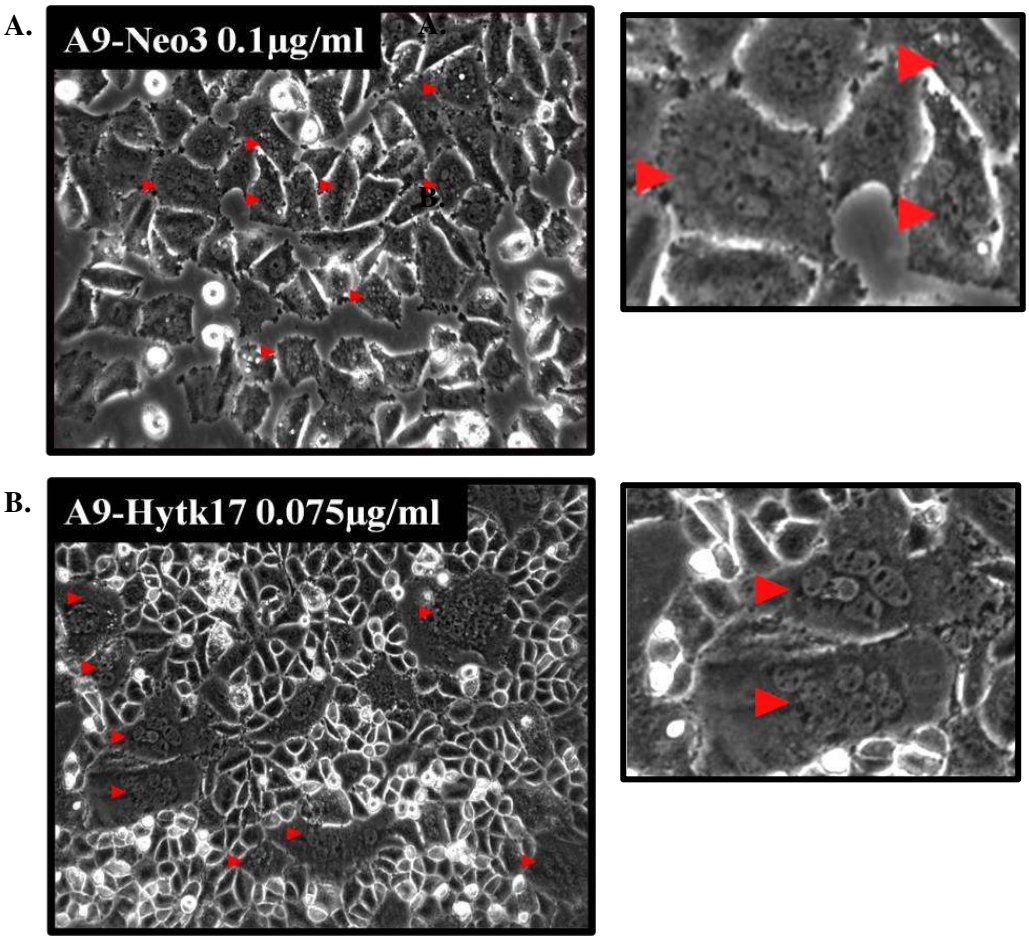


Figure 3.1 - *Representative photomicrographs of human: rodent monochromosome hybrid donor cells 48 hours after incubation with colcemid (x100 magnification).* ***(A)*** *A9-Neo3 hybrids incubated with 0.1µg/ml colcemid.* ***(B)*** *A9-Hytk17 hybrids incubated with 0.075µg/ml colcemid. Red arrows indicate micronucleated cells and adjacent images show an enlarged section of the original image, demonstrating the presence of micronucleated cells.*

After 48 hours, donor cells were observed under a microscope to confirm microcell formation (Figure 3.1A and B). Culture media was then aspirated and 30ml of pre-warmed cytochalasin B solution (10µg/ml, Sigma, US) was added to each flask. The cytochalasin B mycotoxin permeates micronucleated donor cells and inhibits the formation of cytoplasmic microfilaments, leading to 'out-pocketing' of micronuclei from the cell surface (Veomett *et al.,* 1974). Flasks were weighed and paired according to similar weights (±0.03g). Each well of a 6-well rotor (6x250ml, Sigma, US), was filled with 75ml of pre-warmed (37ºC) water, before placing paired flasks in opposite wells of the rotor. Flasks were centrifuged at 9642xg for 1 hour and 10 minutes at 37ºC in Sigma laboratory centrifuges (6K15, Philip Harris). High-speed centrifugation facilitates complete extrusion of micronuclei from the cell cytoplasm, resulting in the production of individual microcells. These consist of micronuclei surrounded by a thin cytoplasmic layer. Following centrifugation, flasks were inspected for evidence of breakage and weighed to determine whether liquid had entered or left the flasks. If a difference in weight of

±0.02g was observed, flasks were immediately discarded. The majority of cytochalasin B solution was then decanted from each flask, leaving approximately 2ml behind. By tapping the sides of flasks, microcell pellets present in the bottom corners were resuspended into the residual solution. A sterile plugged Pasteur pipette was used to pool microcell suspensions from each flask into a single 50ml Falcon tube. Microcells were then pelleted by centrifugation at 4500xg for 5 minutes at room temperature. Following aspiration of the supernatant, microcells were resuspended in 10ml of pre-warmed, serum-free DMEM (1X), for every six flasks originally harvested. Microcell suspensions were divided into 10ml aliquots, before being filtered through 8µm and 5µm polycarbonate filters (Nucleopore Track-Etch Membranes, Whatman®) to isolate microcells containing single chromosomes. Filtered 10ml aliquots were centrifuged at 4500xg for 5 minutes at room temperature. Following aspiration of the supernatant, microcell pellets were resuspended in 3ml of 100µg/ml phytohaemagglutinin (PHA-P), and pooled into one Falcon tube. A P100 culture dish containing 70-90% confluent 21NT cells was then washed with pre-warmed, serum-free MEM-α (1X) three times. Microcell suspensions were then pipetted onto recipient 21NT cell monolayers and dishes were gently swirled before incubating at 37ºC for 25 minutes. As a negative control, another subconfluent P100 dish of 21NT cells, was subject to the same procedure except for the addition of microcells.

Following incubation, PHA-P solutions were aspirated before 1ml of polyethylene glycol solution (PEG) consisting of 42% PEG and 8% DMSO in serum-free DMEM (1X), was slowly added to the corner of the dish. The PEG solution was aspirated after exactly 1 minute and dishes were washed three times with serum-free MEM-α (1X). Each wash step consisted of vigorous agitation for 1 minute, in order to remove as much of the PEG solution as possible. Complete culture medium was then added to the 21NT cell monolayer, before returning the dish to the incubator. The same procedure was also carried out for the negative control dish of 21NT cells. 24 hours later, dishes were passaged at a ratio of 1:10, and cultured in complete growth medium. Positive selection of 21NT-Neo3 and 21NT-Hytk17 hybrids, was carried out by supplementing culture media with 400µg/ml neomycin (G418) and 400U/ml hygromycin (hyg B) respectively. The presence of neomycin and hygromycin antibiotic resistance gene markers within donated human chromosomes 3 and 17 respectively, confer a selective growth advantage for 21NT cell hybrids containing introduced chromosome copies. These concentrations of antibiotic are cytotoxic to 21NT cells that do not gain a single copy of either exogenous chromosome following microcell-fusion. As expected, extensive cell death was observed 5-7 days after the addition of appropriate selection antibiotics following fusion of 21NT cells with microcells containing tagged chromosomes 3 or 17.

P100 dishes containing 21NT-HyTK17 and 21NT-Neo3 hybrids were incubated at 37ºC and 5% CO_2 and re-fed with fresh complete culture medium containing selection antibiotics, every week for 3-4 weeks. Dishes were monitored regularly for the growth of hybrid cell colonies, which were either isolated and propagated on individual P35 cell culture dishes as described previously (Chapter 2.1.5), or harvested *in situ* for protein extraction. Hybrid colonies consisting of cells with vacuoles or an enlarged cytoplasm with very few mitotic cells, were harvested *in situ*.

Rarely, colonies of donor A9 donor hybrid cells were observed after approximately two weeks of selection, indicating that these cells had escaped the filtration step of the MMCT procedure. A9 donor cells were distinguishable from 21NT hybrids by their cellular morphology and growth pattern. On a single dish, they typically manifested as multiple, rapidly growing, dense colonies with several surrounding satellite colonies. As soon as contaminating A9 donor hybrids were identified, dishes were treated with hypoxanthine aminopterin thymidine (HAT, 1X, Sigma, US). Aminopterin is an inhibitor of dihydrofolate reductase; a key enzyme in the *de novo* pathway of purine and pyrimidine nucleotide synthesis (Chung *et al.,* 2001). Therefore in the presence of HAT, cells activate the salvage pathway of DNA synthesis in order to survive and continue replicating. The A9 fibroblast cell line used in the construction of human: rodent monochromosomal hybrids by Cuthbert *et al*., (1995), is deficient in the *hprt* gene, that encodes a critical enzyme involved in the salvage pathway; hypoxanthine-guanine phosporibosyltransferase (Cos *et al.,* 1974). A9 donor cell growth arrest was observed approximately three days after HAT was added to the culture medium of affected dishes. This enabled further discrimination of 21NT hybrid colonies, that were able to survive and grow larger in size.

Transfer of human chromosome 17 from A9-Hytk17 donor hybrids to 21NT cells by MMCT, was carried out by our laboratory cell culture technician, Mrs. Alison Marriott. After 3-4 weeks in selection, P100 dishes containing 21NT-Hytk17 colonies were provided to me. As described above, colonies were either picked and cultured as individual cell lines, or harvested *in situ*.

3.2.2 TELOMERASE ACTIVITY OF 21NT-HYBRID AND 21NT CELL COLONIES

P35 dishes containing 70-90% confluent individual 21NT-Neo3 and 21NT-Hytk17 hybrid cell clones, were harvested and either (i) counted as described previously (Chapter 2.1.6) or (ii) whole cell populations were pelleted without counting and immediately stored at -80ºC. For cell colonies that were counted, aliquots of $3x10^5$ cells were pelleted and stored at -80ºC. The remaining cell suspensions of a sub-set of hybrid colonies were re-established in P60 dishes

and maintained in complete medium with appropriate selection antibiotics, in order to determine cell growth rate and replicative potential.

Protein extraction and quantification of hybrid colony cell pellets was carried out as described previously (Chapter 2.4.1). A semi-confluent P35 dish reportedly yields an average of $9x10^5$ cells (Corning, Life Sciences, US). Therefore, cell pellets harvested from P35 dishes with an unknown cell number, were lysed in 200µl of CHAPS buffer. The same volume was used to lyse cell pellets containing $3x10^5$ cells.

Lysis of hybrid colonies *in situ*, was carried out by first marking the position and size of the colony on the underside of the P100 dish with a permanent marker. After aspiration of culture media, hybrid colonies were washed once with pre-warmed 1X PBS. Cloning cylinders were then placed over colonies and fixed in place using pre-sterilized Vaseline (Chapter 2.1.5). 50µl of CHAPs lysis buffer was then pipetted into each cloning cylinder and mixed thoroughly by retro-pipetting to cause complete cell lysis. Cell lysates were transferred into a sterile Eppendorf tube and subject to the same procedure for protein extraction and quantification as cell pellets. The qRT-PCR TRAP assay was used to quantify telomerase activity of all 21NT-Neo3 and 21NT-Hytk17 hybrids generated (Chapter 2.4).

The clonal variation in telomerase activity of wild-type 21NT cells was determined by first seeding 21NT cells at 10^4 cells/P100 dish. Nine cell colonies were picked using cloning cylinders (Chapter 2.1.5), and maintained as individual cell lines on P35 dishes. As soon as they were subconfluent, cells were harvested and pelleted. Following protein extraction and quantification, the telomerase activity of individual colonies was measured using the qRT-PCR TRAP assay, and quantified relative to the telomerized prostate cancer cell line (PC-3-*hTERT,* Chapter 2.1.2.1).

3.2.3 CELL GROWTH AND REPLICATIVE POTENTIAL

As described above, a proportion of 21NT-Neo3 and 21NT-HyTK17 hybrid colonies were re-established in P60 culture dishes after $3.5x10^5$ cells were taken from cell suspensions for protein extraction and determination of telomerase activity. Remaining cells were re-established in P60 dishes and maintained under normal growth conditions in appropriate selection antibiotics. At 70-90% confluence, the cell number was determined and $3.5x10^5$ cells were re-established on P100 dishes containing complete culture media. This procedure of serial sub-cultivation and cell counting was repeated a maximum number of five times, before colonies were cryo-preserved (Chapter 2.1.4). The same procedure was carried out using wild type 21NT cells.

Hybrid colony and 21NT cell growth was assessed by calculating the total number of population doublings (PD) that had occurred between seeding $3.5x10^5$ cells and harvesting at 70-90% confluence (Chapter 2.8.1). The cumulative PD(s) of each hybrid colony and 21NT cells was determined over five serial passages or until cell growth arrest was observed.

The PD/day for 21NT cells and hybrid colonies, was calculated by dividing the number of PD(s), by the number of days between passages. These values were then used to calculate the mean population doubling time (PDT), defined as the time taken (hours) for a cell population to double in size, for all hybrid colonies and the 21NT cell line.

3.3 RESULTS AND DISCUSSION

In order to investigate whether normal human chromosomes 3 and 17 harbour sequences that repress telomerase within the primary breast cancer cell line 21NT, copies of both chromosomes present within human: mouse monochromosome hybrid donors A9-Neo3 and A9-Hytk17 respectively, were transferred by MMCT into 21NT cells to generate 21NT-Neo3 and 21NT-Hytk17 hybrid clones. Four independent MMCT experiments were carried out to generate two sets of 21NT-Neo3 and two sets of 21NT-Hytk17 hybrid colonies. A total of twenty-seven 21NT-Neo3 and sixteen 21NT-Hytk17 hybrid colonies were recovered (Table 3.2). One 21NT-Neo3 and two 21NT-Hytk17 hybrid colonies entered growth arrest *in situ* following 3-4 weeks in selection. These hybrids demonstrated morphological features of senescence: large, granular cells, with an enlarged cytoplasm (Figure 3.2). However, the small proportion of *in situ* senescent hybrids observed following introduction of both chromosome 3 and 17 (4% and 13% respectively), could indicate that it is a relatively uncommon occurrence, which is unrelated to the introduced chromosome copy. Due to the absence of actively dividing cells (Figure 3.2), these hybrid colonies were not picked, but were harvested *in situ*. Remaining 21NT-Neo3 and 21NT-Hytk17 hybrids were picked and could be successfully re-established on individual P35 dishes.

Table 3.2 - Summary of hybrid colonies generated and the number of colonies that senesced and entered growth arrest *in situ*.

Hybrid Colony	Number of colonies generated (per 10^6 cells)		Total	Number of colonies that entered growth arrest *in situ* (IS)
21NT-Neo3	MMCT 1	11 (3.7)	27	1
	MMCT 2	16 (5.3)		
21NT-Hytk17	MMCT 1	6 (2)	15	2
	MMCT 2	9 (3)		

Abbreviations: IS (In situ)

The frequency of 21NT-Hytk17 hybrid colonies recovered/10^6 cells following MMCT-mediated transfer of human chromosome 17, was considerably lower compared with the frequency of 21NT-Neo3 hybrid colonies recovered/10^6 cells (Table 3.1). This could indicate the presence of a tumour suppressor sequence(s) on normal human chromosome 17 that inhibits the growth of 21NT cells. However, it is possible that there were differences in the number of micronucleated A9-Neo3 and A9-Hytk17 hybrids produced during the initial stages of the MMCT procedure, which could have led to downstream differences in the number of microcells obtained and the total number of hybrids generated.

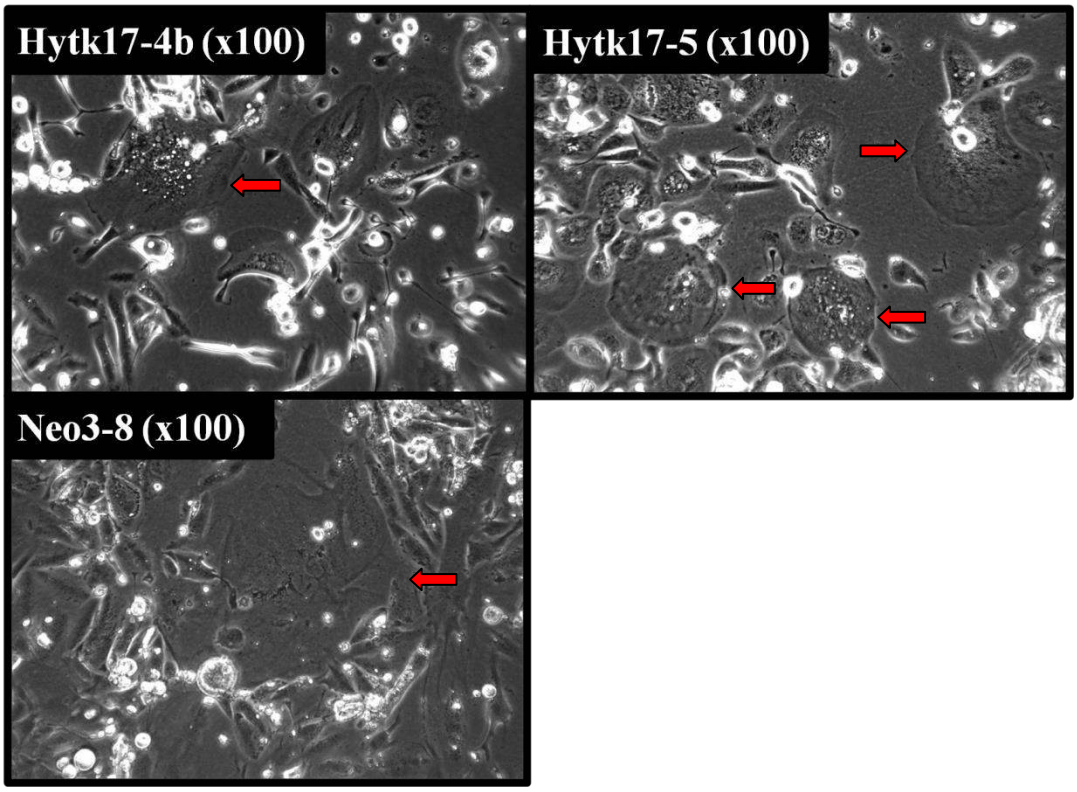


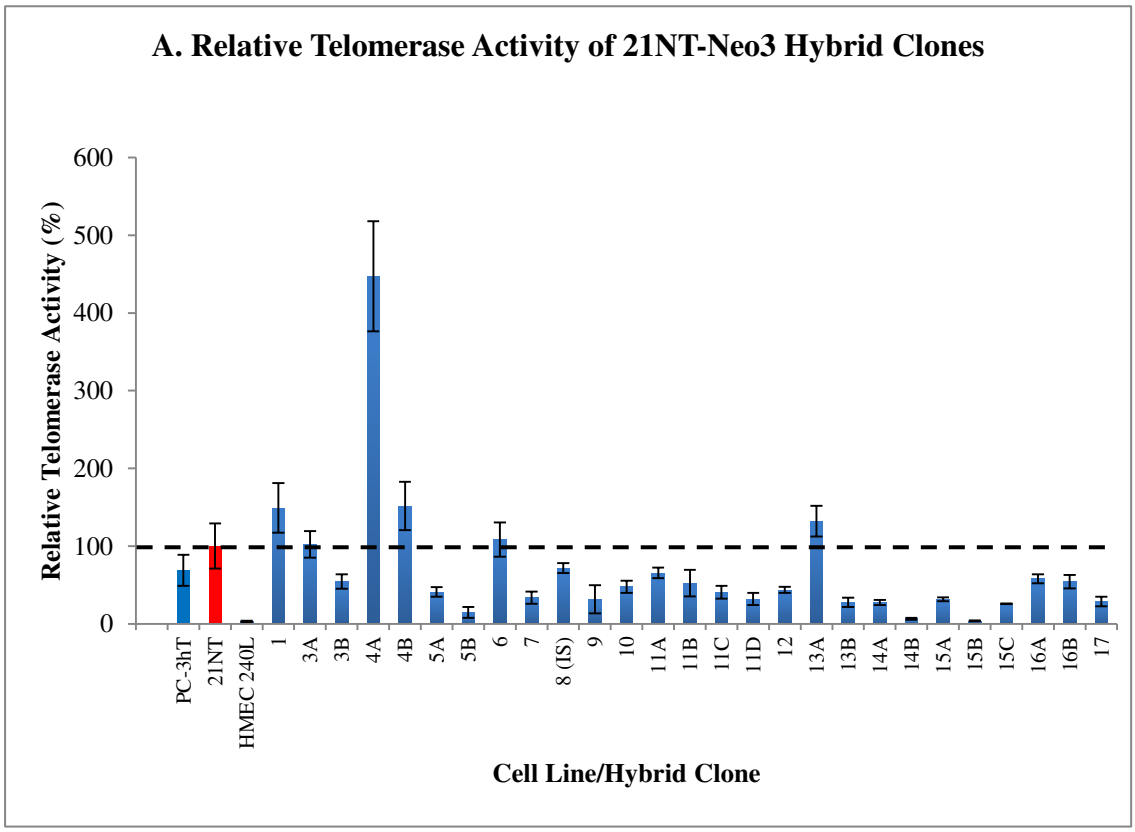
Figure 3.2 - *Representative photomicrographs (magnification x100) of two 21NT-Hytk17 and one 21NT-Neo3 hybrid clone(s) harvested in situ, that displayed morphological characteristics of senescence including enlarged, granular cytoplasms, as indicated by red arrows.*

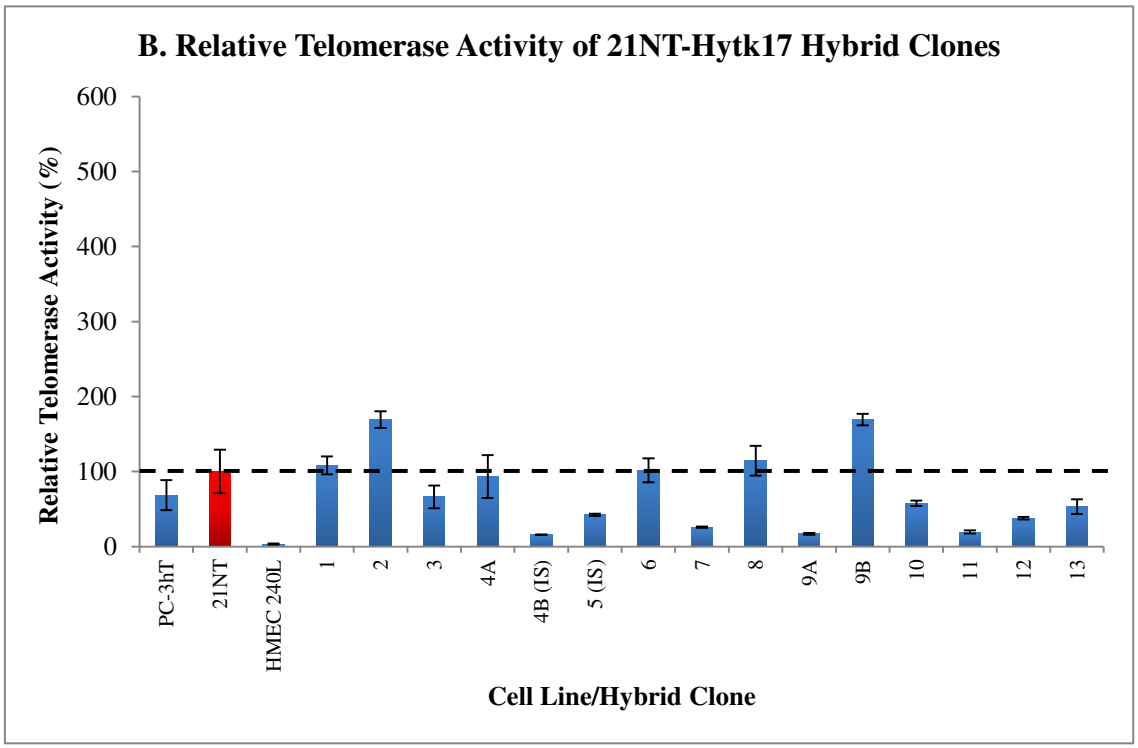
3.3.1 Telomerase Activity of 21NT-Neo3 and 21NT-HyTK17 hybrid colonies

The next aim of this study was to examine the effect of introducing a normal copy of chromosome 3 and 17 into the 21NT cell line on telomerase activity. Telomerase activity of hybrid colonies was determined using a quantitative, real-time PCR-based TRAP assay procedure (qRT-PCR). This two-step assay works by first allowing endogenous telomerase within protein sample extracts to add a number of telomere repeat sequences (TTAGGG) to a

telomerase substrate molecule (TS). The second step involves PCR amplification and real-time detection of extended TS substrate molecules using an anchored return primer (ACX) and SYBR green nucleic acid dye. The ACX primer caps the 3' end of an extended telomerase product after one PCR cycle, and prevents further elongation of the same substrate in subsequent cycles. In this way, PCR-amplification of telomerase products reflects the number of active telomerase enzyme molecules within sample extracts, and is therefore proportional to the amplification signal generated.

All twenty-seven 21NT-Neo3 and fifteen Hytk17 hybrid clones recovered during this study were either lysed *in situ,* if cells displayed a senescent phenotype 3-4 weeks after selection (Figure 3.2), or harvested 1-2 weeks after actively dividing colonies were picked and propagated as individual cell lines (Chapter 3.2.2). Following protein extraction and quantification, telomerase activity of all hybrid colonies was determined. The level of telomerase activity exhibited by untreated parental 21NT cells was also determined. In addition, the telomerase activity of the early-passage normal human mammary epithelial cell (HMEC) strain 240L, derived from a reduction mammoplasty tissue sample (Garbe *et al.,* 2009), was examined to determine the level of telomerase activity exhibited by normal breast epithelial cells. The HMEC 240L cell strain has been shown to enter growth arrest following approximately 15 population doublings (Garbe *et al.,* 2009). The human prostate cancer cell line (PC-3-*hTERT*) over-expressing exogenous *hTERT* (via cDNA transfection) was also included, to quantify the telomerase activity of sample extracts and provide an additional telomerase-positive control (Chapter 2.4.2). To determine the interclonal variability of telomerase activity within 21NT cells, single colonies were picked from a mass population of cells seeded at a low density, and propagated in individual P35 dishes. The telomerase activity of each colony was determined relative to PC-3-*hTERT*, and the standard deviation of the mean relative telomerase activity of all clones, was used to represent the interclonal variability.


A. Relative Telomerase Activity of 21NT-Neo3 Hybrid Clones
Relative Telomerase Activity (%)
600
500
400
300
200
100
0
PC-3hT
21NT
HMEC 240L
1
3A
3B
4A
4B
5A
5B
6
7
8 (IS)
9
10
11A
11B
11C
11D
12
13A
13B
14A
14B
15A
15B
15C
16A
16B
17
Cell Line/Hybrid Clone


B. Relative Telomerase Activity of 21NT-Hytk17 Hybrid Clones
Relative Telomerase Activity (%)
600
500
400
300
200
100
0
PC-3hT
21NT
HMEC 240L
1
2
3
4A
4B (IS)
5 (IS)
6
7
8
9A
9B
10
11
12
13
Cell Line/Hybrid Clone

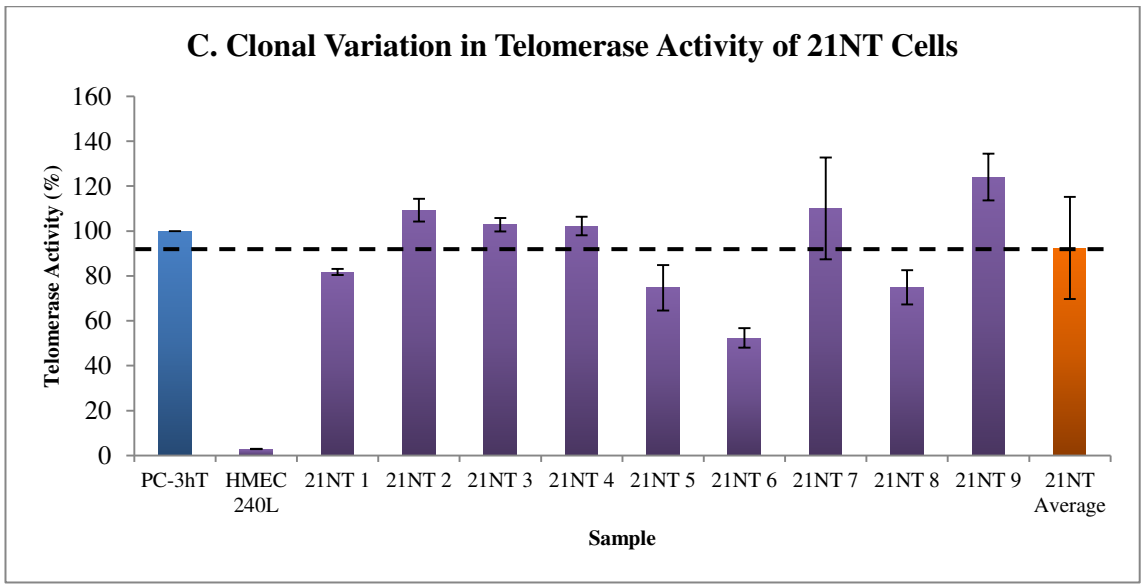


Figure 3.3 - *Telomerase activity within* ***(A)*** *21NT-Neo3 and* ***(B)*** *21NT-Hytk17 hybrid clones generated, expressed as a percentage of parental 21NT cells (100%, dotted line). A maximum of nine hybrid clones were analyzed across five separate assay runs and telomerase activity was calculated relative to the 21NT sample included in the same assay. Error bars for each hybrid colony represent the standard deviation (SD) of triplicate repeats. Mean ± SD telomerase activity of the most concentrated PC-3-hTERT standard from five assay runs, expressed as a percentage of 21NT cells is shown. 21NT sample error bars represent SD across all assay runs.* ***(C)*** *The telomerase activity of nine individual 21NT clones relative to PC-3-hTERT (100%), including the mean ±SD of all clones. (IS) denotes hybrid colonies that entered growth arrest in situ. Hybrid colonies picked from the same culture dish are labelled alphanumerically.*

As shown in Figure 3.3, telomerase activity was detected within parental 21NT and telomerized PC-3-*hTERT* protein extracts, which have been shown to exhibit telomerase activity within previous studies (Cuthbert *et al.,* 1999, Motevalli *et al.,* 2014). The telomerase activity of the normal HMEC 240L cell strain was approximately 97% lower than the 21NT cell line (Figure 3.3). As shown in Figure 3.3C, the standard deviation from the mean telomerase activity exhibited by nine individual 21NT cell colonies, was found to be 23%. Therefore, 21NT-Neo3 and 21NT-Hytk17 hybrid colonies exhibiting a greater than 23% change in telomerase activity relative to 21NT cells, were considered to be sufficiently different from the parental cells.

Table 3.3 - The proportion of 21NT-Neo3 and 21NT-Hytk17 hybrid colonies recovered that showed an increase or decrease in telomerase activity, outside the normal range of 21NT interclonal variability, including those that exhibited a greater than 90% reduction.

	Telomerase Activity		
Hybrid Colony	**Increase by >SD of 21NT clonal mean**	**Decrease by >SD of 21NT clonal mean**	**≥90% reduction**
21NT-Neo3	15% (4/27)	78% (21/27)	11% (3/27)
21NT-Hytk17	13% (2/15)	60% (9/15)	0% (0/15)

The standard deviation of mean 21NT clonal telomerase activity was 23% (Figure 3.3C). Abbreviations: SD (Standard Deviation).

As shown in Table 3.3, the majority of 21NT-Neo3 (78%) and 21NT-Hytk17 (60%) hybrid colonies recovered were found to exhibit a reduction in telomerase activity relative to parental 21NT cells. None of the 21NT-Hytk17 hybrids recovered were found to display a greater than 90% reduction in telomerase activity relative to 21NT cells, compared with 11% of 21NT-Neo3 hybrids. However, 4 out of 15 (27%) 21NT-Hytk17 hybrids demonstrated a marked (74-84%) reduction in telomerase activity (Figure 3.3B, 21NT-Hytk17 clones 4B, 7, 9A and 11). A larger proportion of 21NT-Neo3 hybrids were found to display a greater than 50% reduction in telomerase activity, compared to 21NT-Hytk17 hybrids. However, similar proportions of 21NT-Neo3 and 21NT-Hytk17 hybrids exhibited an increase in telomerase activity relative to parental 21NT cells.

The results presented here demonstrate that the introduction of a normal human copy of chromosome 3 and 17 into the 21NT cell line results in a reduction in telomerase activity relative to parental 21NT cells, in the majority of cases. A larger proportion of 21NT-Neo3 hybrids were found to exhibit a greater reduction in telomerase activity relative to parental 21NT cells, than 21NT-Hytk17 hybrids.

3.3.2 The Relationship Between 21NT-Neo3 and 21NT-Hytk17 Hybrid Proliferation and Telomerase Activity

In order to investigate whether the observed reduction in telomerase activity within 21NT-Neo3 and 21NT-Hytk17 hybrids is associated with a change in hybrid proliferation, the growth rate, division potential, and cellular morphology of a sub-set of 21NT-Neo3 and 21NT-Hytk17 hybrid colonies was examined and compared with telomerase activity. Eleven 21NT-Neo3 and six 21NT-Hytk17 hybrid colonies recovered from the first set of MMCT procedures carried out (Table 3.1), were re-established in culture after a cell sample was taken for protein extraction and analysis of telomerase activity. Hybrid colonies were then passaged a maximum number of five times to determine proliferative growth rate and potential. By counting the cells at each passage and re-seeding a fixed quantity of cells, the number of population doublings

(PD) that had occurred between each passage was ascertained (Chapter 2.8.1). Hybrid growth rate was determined by calculating the mean PD time over five passages. For comparison, the identical procedure was used to determine the growth rate of parental 21NT cells. Photomicrograph images of all hybrid colonies and 21NT cells were taken at regular intervals to monitor hybrid cell morphology.

Table 3.4 - The frequency of 21NT-Neo3 and 21NT-Hytk17 hybrids that entered growth arrest or proliferated continuously

Hybrid Colony	Hybrids Analysed	Growth arrest (IS)	Growth arrest after picking	Proliferated Continuously	
21NT-Neo3	11	9% (1/11)	27% (3/11)	Normal GR	27% (3/11)
				Reduced GR	36% (4/11)
21NT-Hytk17	6	33% (2/6)	17% (1/6)	Normal GR	17% (1/6)
				Reduced GR	33% (2/6)

Growth rate (GR) was determined as the mean PD time over five serial passages. A student's t-test was used to compare the mean PD time of hybrid colonies with parental 21NT cells. The GR was considered to be significantly different if p>0.05. (IS) denotes hybrids that entered growth arrest in situ.

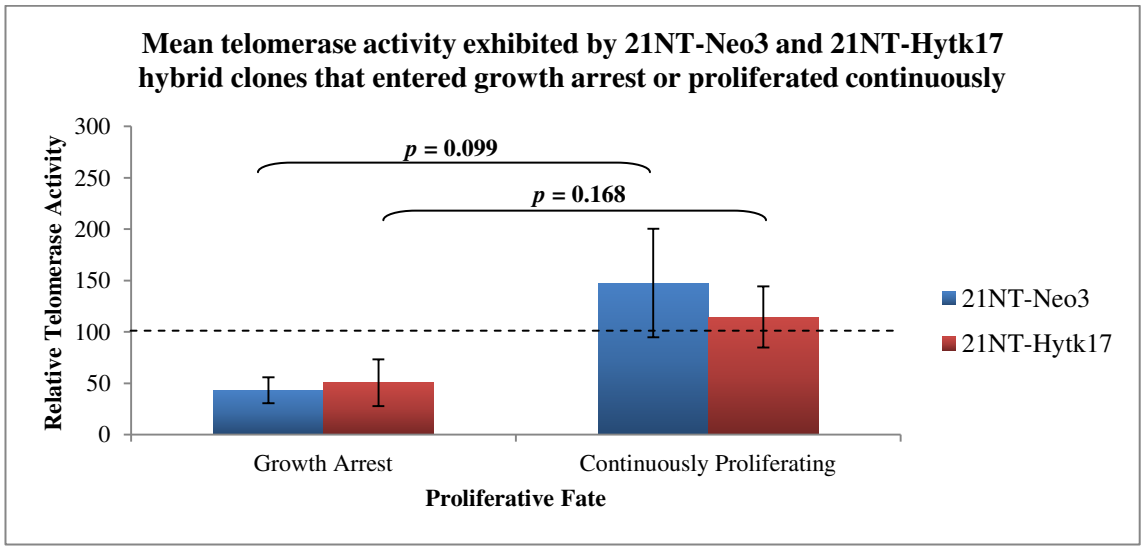


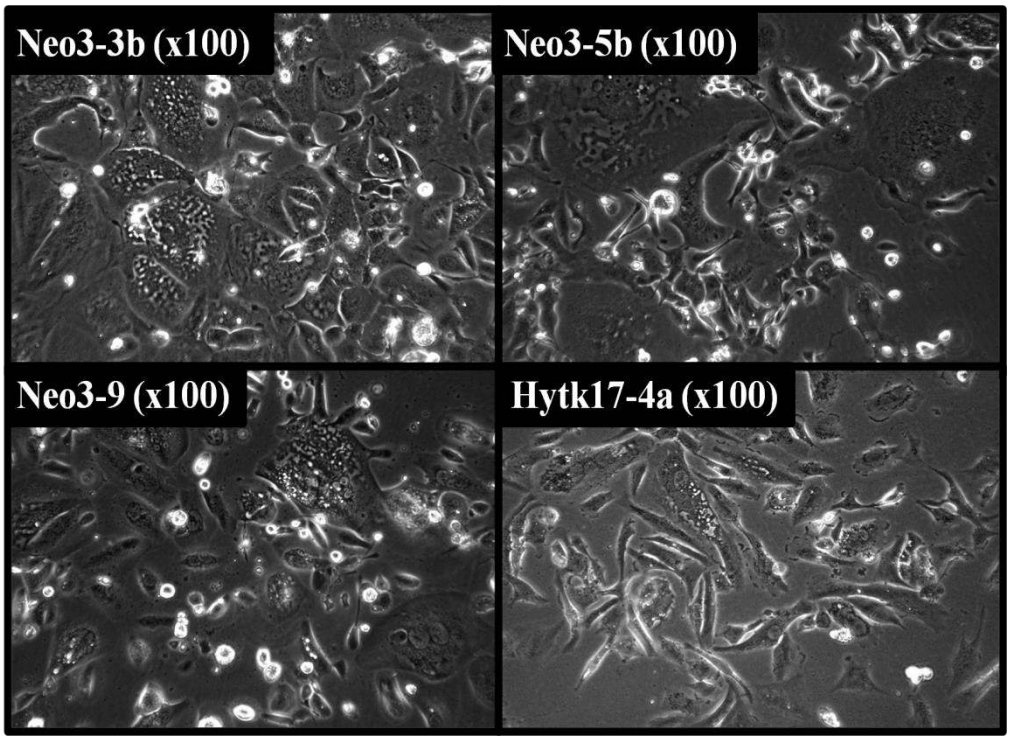
Figure 3.4 - *Average telomerase activity expressed as a percentage of wild-type 21NT cells (100%, dotted line), within 21NT-Neo3 and 21NT-Hytk17 hybrid clones that entered growth arrest (either in situ or after they were picked) or proliferated continuously for 12-15PD after they were picked. Error bars represent the standard error of the mean telomerase activity for each group. A student's t-test was used to compare the mean telomerase activity of hybrids that entered growth arrest with those that proliferated continuously.*

As shown in Table 3.4, 63% of 21NT-Neo3 hybrid colonies recovered from a single MMCT procedure, were found to proliferate continuously for 12-15 PD after they were picked, compared with 50% of 21NT-Hytk17 hybrids recovered. The majority of both continuously proliferating 21NT-Neo3 and 21NT-Hytk17 hybrids were found to display a significant reduction in growth rate compared to 21NT cells. 36% of 21NT-Neo3 and 50% of 21NT-Hytk17 hybrids entered growth arrest either *in situ,* or after they were picked and cultured as individual cell lines. No 21NT-Neo3 and 21NT-Hytk17 hybrids exhibited an increase in growth rate compared with parental 21NT cells.

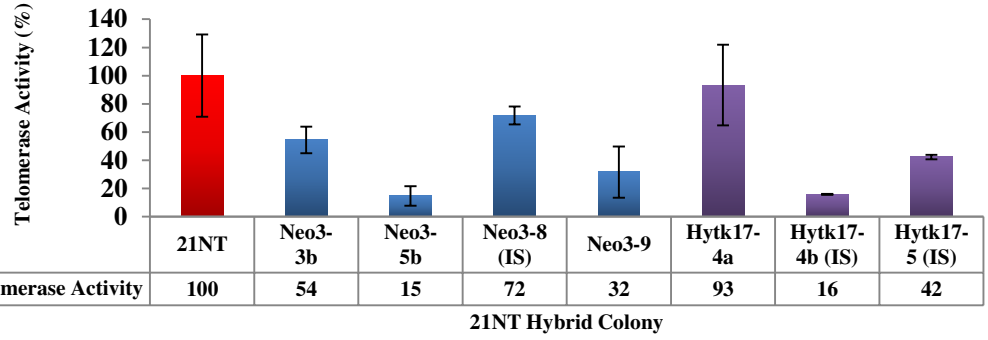
In 1999, Cuthbert and colleagues demonstrated a strong association between telomerase repression and induction of permanent growth arrest of 21NT-chromosome 3 hybrids. Therefore, in order to determine whether the proliferative fate of 21NT-Neo3 and 21NT-Hytk17 hybrids was linked to telomerase activity, the mean telomerase activity levels exhibited by growth arrested and continuously proliferating hybrids were compared. As shown in Figure 3.4, 21NT-Neo3 and 21NT-Hytk17 hybrids that entered growth arrest either *in situ* or after they were picked and propagated as individual cell lines, were found to display an average reduction of telomerase activity of 57% and 50% relative to wild-type 21NT cells respectively. On the other hand, 21NT-Neo3 and 21NT-Hytk17 hybrids that proliferated continuously after they were picked, showed either similar to or higher mean telomerase activity levels relative to wild-type 21NT cells. Therefore, growth arrested 21NT-Neo3 and 21NT-Hytk17 hybrids show a marked, but not statistically significant, reduction in average telomerase activity compared with continuously proliferating hybrids. Consistent with Cuthbert *et al.,* (1999), this could suggest a link between telomerase repression and induction of hybrid growth arrest within 21NT-Neo3 and 21NT-Hytk17 hybrids.

Cellular Morphology and Telomerase Activity of 21NT-Neo3 and 21NT-Hytk17 Hybrids that Entered Growth Arrest

A.



B. Telomerase Activity of 21NT-Neo3 and 21NT-Hytk17 hybrid clones that entered growth arrest



	21NT	Neo3-3b	Neo3-5b	Neo3-8 (IS)	Neo3-9	Hytk17-4a	Hytk17-4b (IS)	Hytk17-5 (IS)
Telomerase Activity	100	54	15	72	32	93	16	42

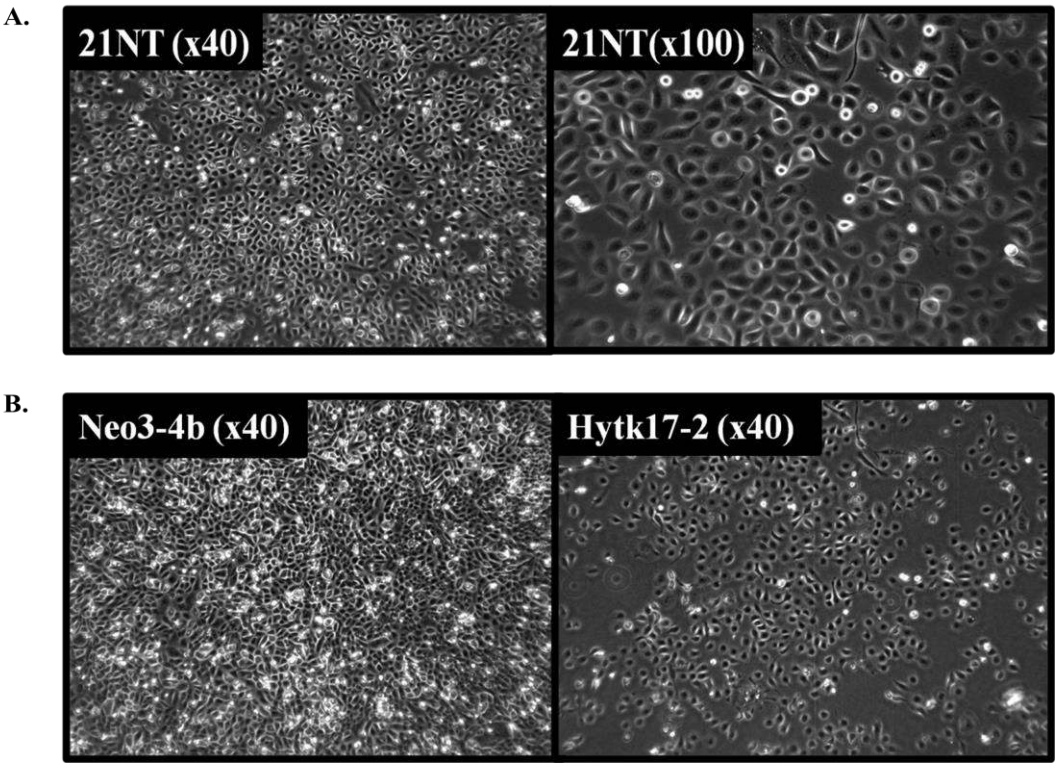
Figure 3.5 - *Analysis of 21NT-Neo3 and 21NT-Hytk17 hybrid colonies that emerged following two independent MMCT experiments and entered growth arrest either in situ or after they were picked and cultured as individual cell lines* ***(A)*** *Representative photomicrograph images of three 21NT-Neo3 and one 21NT-Hytk17 hybrid(s) that entered growth arrest after they were picked (magnification x100).* ***(B)*** *Telomerase activity of all hybrids that entered growth arrest both in situ (IS) or after they were picked, expressed as a percentage of wild type 21NT cells (21NT).*

Cuthbert *et al.,* (1999) found that 21NT-chromosome 3 hybrids that entered growth arrest displayed morphological and molecular features of senescence. Therefore, in order to

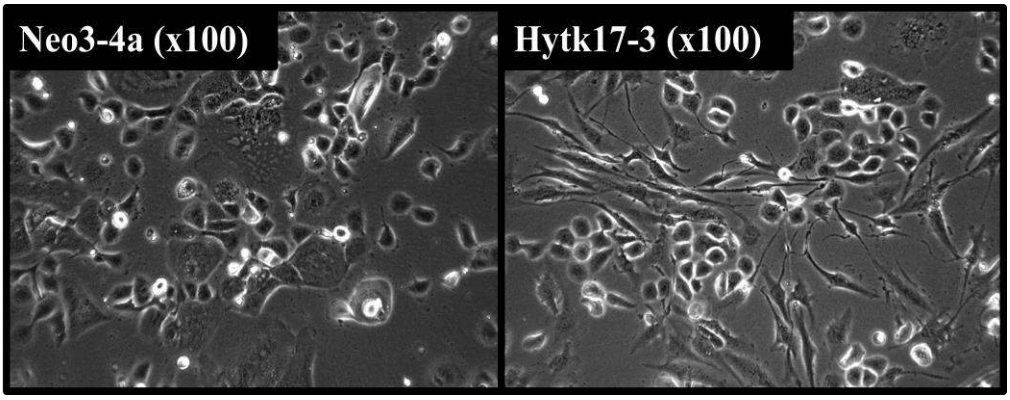
investigate the link between telomerase repression and induction of senescence pathways, the cellular morphology of 21NT-Neo3 and 21NT-Hytk17 hybrid clones that entered growth arrest was examined. Morphological features of senescence were observed within all four 21NT-Neo3 hybrids that entered growth arrest: enlarged, flattened and vacuolated cells (Figure 3.2, Neo3-8 and Figure 3.5, Neo3-3b, Neo3-5b, and Neo3-9). Out of these, 21NT-Neo3 hybrid colonies that entered growth arrest, one occurred *in situ,* two occurred within 2 weeks of picking (and before hybrid growth rate could be ascertained), and the remaining hybrid entered growth arrest following 6 PD after it was picked (Figure 3.5A, Clone Neo3-3b). Growth arrested 21NT-Neo3 hybrids, exhibited a reduction in telomerase activity by an average of 57%, relative to parental cells (Figure 3.4).

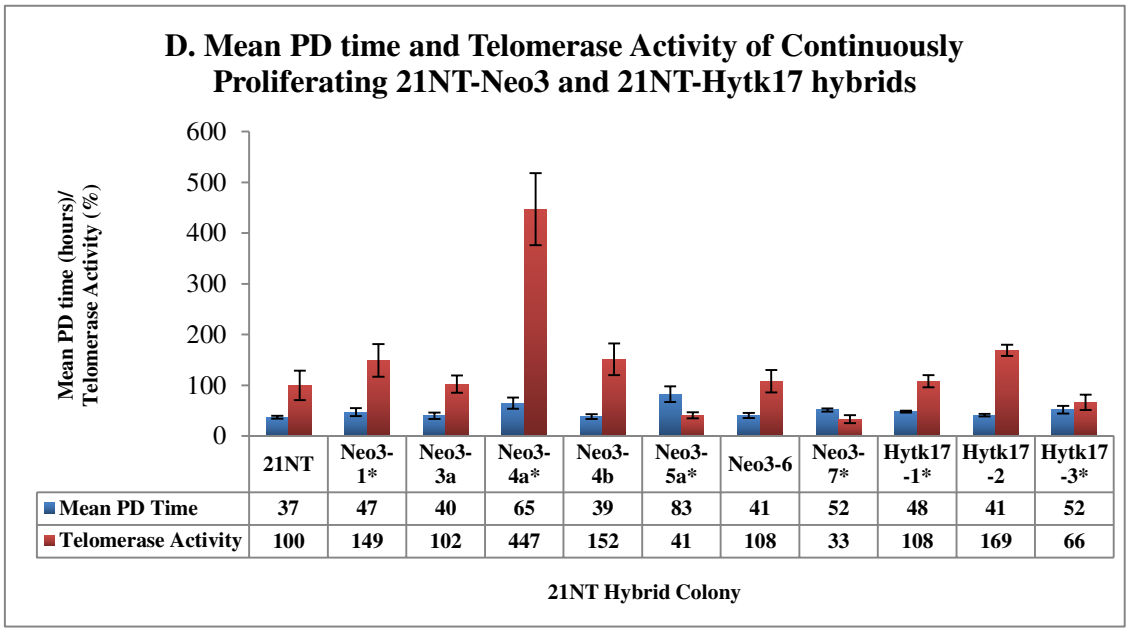
Three out of six (50%) 21NT-Hytk17 hybrids recovered, were found to enter growth arrest (Table 3.4). Two 21NT-Hytk17 hybrids entered growth arrest *in situ* and displayed morphological features of senescence (Figure 3.2, Hytk17-4b and Hytk17-5). These hybrids also displayed a reduction in telomerase activity relative to parental 21NT cells. The remaining hybrid proliferated slowly for 2 PD before entering growth arrest, and was not found to display a significant difference in telomerase activity compared to parental 21NT cells (Figure 3.5B, Hytk17-4a). This particular hybrid did not exhibit features of senescence (Figure 3.5A, Hytk17-4a), but cells appeared enlarged and vacuolated compared to parental 21NT cells (Figure 3.6A).

Cellular Morphology, Mean Population Doubling Time and Telomerase Activity of 21NT-Neo3 and 21NT-Hytk17 Hybrid Colonies that Proliferated Continuously



C.





	21NT	Neo3-1*	Neo3-3a	Neo3-4a*	Neo3-4b	Neo3-5a*	Neo3-6	Neo3-7*	Hytk17-1*	Hytk17-2	Hytk17-3*
Mean PD Time	37	47	40	65	39	83	41	52	48	41	52
Telomerase Activity	100	149	102	447	152	41	108	33	108	169	66

Figure 3.6 - *Analysis of 21NT-Neo3 and 21NT-Hytk17 hybrid colonies that emerged following two independent MMCT experiments and proliferated continuously after they were picked and cultured as individual cell lines. Representative photomicrographs showing the cellular morphologies of* ***(A)*** *parental 21NT cells,* ***(B)*** *one 21NT-Neo3 and one 21NT-Hytk17 continuously proliferating hybrid colony with similar growth rates to parental 21NT cells,* ***(C)*** *proliferating 21NT-Neo3 and 21NT-Hytk17 hybrid colonies with a significant reduction in growth rate compared with parental 21NT cells. The magnification (x40 or x100) is shown for each image.* ***(D)*** *The mean PD time over five serial passages and the relative telomerase activity of proliferating 21NT-Neo3 and 21NT-Hytk17 hybrid colonies. (*) denotes hybrids displaying a significant reduction in growth rate compared with parental 21NT cells.*

Over half of all continuously proliferating 21NT-Neo3 and 21NT-Hytk17 hybrids exhibited a significant reduction in growth rate compared with parental 21NT cells, which were found to proliferate with a mean PD time of 37 hours (Table 3.4 and Figure 3.6D). Out of seven (64%) continuously proliferating 21NT-Neo3 hybrids, three exhibited a similar growth rate and mean PD time to parental 21NT cells. These hybrids were also found to display a similar cellular morphology to parental cells (Figure 3.6B, Neo3-4b) and exhibited either similar to or increased levels of telomerase activity relative to 21NT cells (Figure 3.6D, Neo3-3a, Neo3-4b, Neo3-6). The remaining four proliferating 21NT-Neo3 hybrids displayed a significantly reduced growth rate compared to parental 21NT cells, and exhibited an up to four-fold higher level of telomerase activity, or a greater than 59% reduction. (Figure 3.6D, Neo3-1, Neo3-4a, Neo3-5a, Neo3-7). Three of these hybrid colonies were found to consist of both cells with senescent features, and those with a similar morphology to parental 21NT cells (Figure 3.6C, clone Neo3-4a).

Fifty percent of 21NT-Hytk17 hybrids were found to proliferate continuously for five serial passages (Table 3.4). Out of these three hybrids, only one was found to display a similar growth rate and cellular morphology to parental 21NT cells (Figure 3.6B, Hytk17-2). This hybrid also exhibited an increase in telomerase activity relative to parental cells (Figure 3.6D). The remaining two hybrids demonstrated a significant reduction in growth rate, but unlike 21NT-Neo3 hybrids, neither were found to exhibit morphological features of senescence (Figure 3.6C, Hytk17-3). One of these hybrids demonstrated a 44% reduction in telomerase activity, whilst the remaining hybrid exhibited a similar level to parental 21NT cells (Figure 3.6D, Hytk17-1, Hytk17-3).

In order to investigate the relationship between telomerase activity and growth rate of proliferating 21NT-Neo3 and 21NT-Hytk17 hybrids, the mean PD time of all proliferating hybrids was plotted against relative telomerase activity. The Pearson Correlation Coefficient was used to determine the strength of the correlation between both variables. A negative correlation between relative telomerase activity and mean PD time was observed for both 21NT-Neo3 and 21NT-Hytk17 hybrid colonies, indicating that a decrease in telomerase activity was associated with a decrease in hybrid growth rate (Figure 3.7A and B). A stronger correlation between telomerase activity and hybrid growth rate was observed for 21NT-Neo3 compared with 21NT-Hytk17 hybrids.

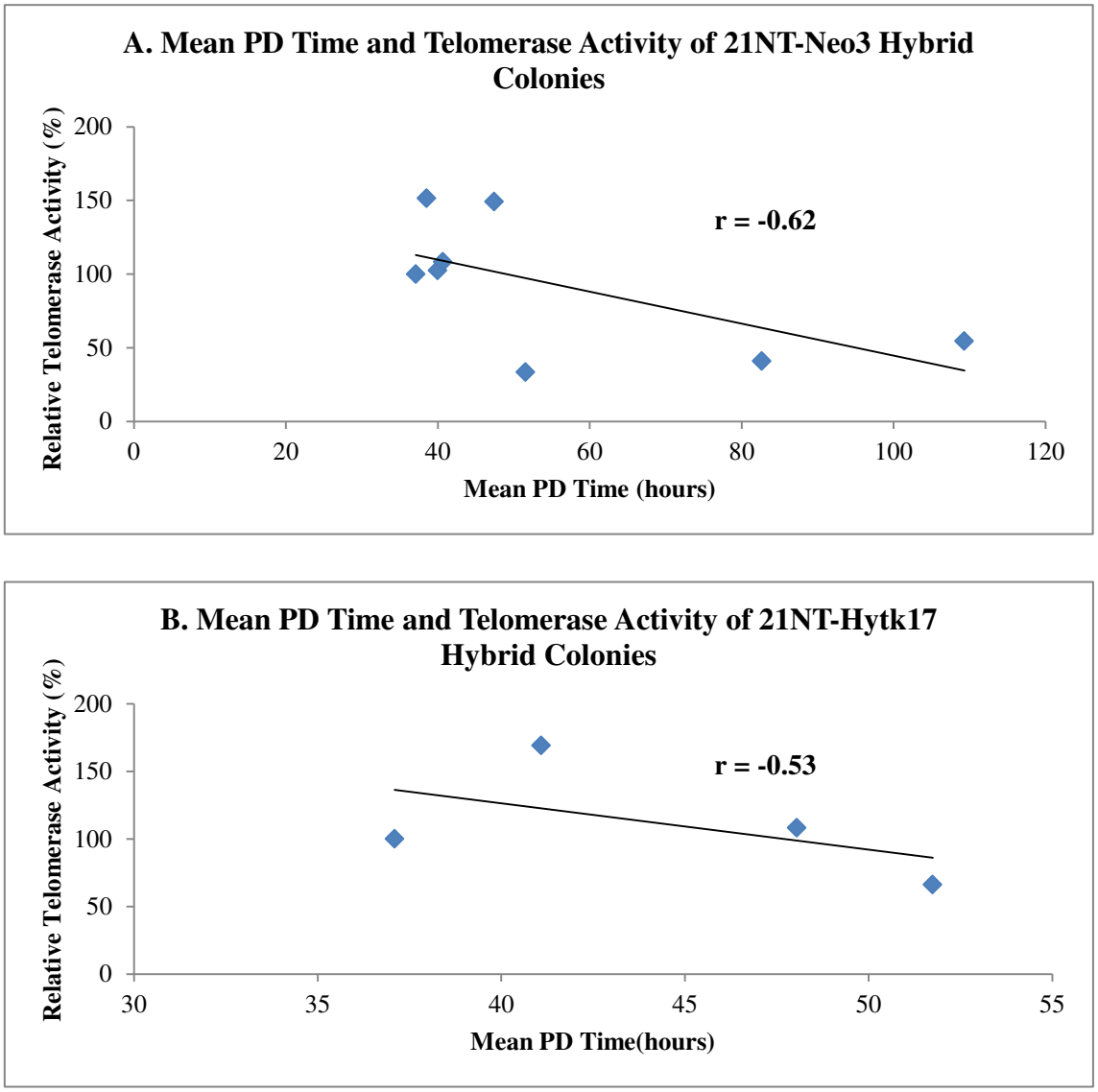


Figure 3.7 - *Scatter plots showing the relationship between the mean population doubling time and relative telomerase activity of continuously proliferating* ***(A)*** *21NT-Neo3 and* ***(B)*** *21NT-Hytk17 hybrid clones. The Pearson Correlation Coefficient (r) was used to quantify the strength of the relationship between both variables. Hybrid Neo3-4a was omitted from the analysis.*

3.4 SUMMARY

The bypass of normal barriers to proliferation and cellular immortalization are critical steps that have been thought to occur during the transformation of normal human somatic cells into tumour cells. The high level of telomerase activity observed within the majority of human tumours, but not within most normal cells, is thought to permit both cellular immortality and prevent induction of cellular senescence pathways, through maintenance of telomeric repeat sequences. Reactivation of telomerase activity is thought to occur due to the functional loss of *hTERT* transcriptional regulatory sequences during carcinogenesis.

Using the microcell-mediated monochromosome transfer (MMCT) technique and human: mouse monochromosomal hybrid panels, several independent studies have identified multiple chromosomes that are able to repress telomerase activity within different cancer types (Chapter 1.3.5.2.2.3, Oshimura and Barrett., 1997, Ducrest *et al.,* 2002, Tanaka *et al.,* 2005). Introduction of a normal copy of chromosome 3 into the 21NT breast cancer cell line and the RCC23 and KC12 renal cell carcinoma cell lines, has been shown to repress telomerase activity and induce senescence (Cuthbert *et al.,* 1999, Ohmura *et al.,* 1995, Tanaka *et al.,* 1998 respectively). Introduction of chromosomes 3 and 4 into the HeLa cervical carcinoma cell line and chromosomes 2 and 6 into the SiHa cervical carcinoma cell line, have also been found to repress telomerase activity (Backsch *et al.,* 2001, Steenbergen *et al.,* 2001, Uejima *et al.,* 1995). These observations provide evidence that multiple telomerase repressor sequences can function within the same cancer cell line. Consistent with these observations, the results presented within my study showed that introduction of both chromosome 3 and 17 into the 21NT breast cancer cell line resulted in a significant reduction in telomerase activity (within 78% and 60% of hybrids respectively). In addition, 73% and 83% of chromosome 3 and 17-21NT hybrids respectively, either entered growth arrest and displayed morphological features of senescence, or exhibited a significant reduction in growth rate compared with parental 21NT cells. A reduction in telomerase activity was associated with a reduction in hybrid growth rate for both chromosome 3 and 17 hybrids.

Previously, Cuthbert et al (1999) found that 67%-78% of 21NT-chromosome 3 hybrids generated by MMCT-mediated transfer, displayed a greater than 90% reduction in telomerase activity compared to wild-type 21NT cells. By comparison, only 11% of 21NT-chromosome 8 and 8% of 21NT-chromosome 20 hybrid colonies generated in the same study, exhibited a greater than 90% reduction in telomerase activity. These findings suggest that chromosome 3 harbours sequences that repress telomerase activity within the 21NT cell line. Even though 78% of 21NT-chromosome 3 hybrids generated within my study were found to display a significant reduction in telomerase activity relative to wild-type 21NT cells, I found that only 11% of

hybrids exhibited a greater than 90% reduction in telomerase activity relative to wild-type 21NT cells, which contrasts findings presented by Cuthbert *et al.,* (1999).

Cuthbert *et al.,* (1999) found that 90% of 21NT-chromosome 3 hybrid colonies recovered from multiple MMCT procedures, entered delayed permanent growth arrest. The majority of these hybrids (~90%) displayed an increased mean PD time of 50 hours and proliferated for 10-12 PD (3-4 weeks in selection) before exhibiting a further reduction in growth rate prior to growth arrest. At this point, cells displayed morphological features of senescence. A small proportion (~10%) of 21NT-chromosome 3 hybrids that entered growth arrest, entered crisis following 15-18 PD (4-6 weeks in selection). After a prolonged crisis period, immortal variants emerged from approximately 40% of these hybrids, whilst the remaining hybrids remained in a state of permanent growth arrest. By comparison, all 21NT hybrid colonies containing a single copy of chromosomes 8, 12 or 20 that were recovered, proliferated with a similar growth rate to parental cells and did not display morphological features of senescence. In contrast to these findings, only 36% of 21NT-Neo3 hybrid colonies recovered from a single MMCT procedure within my study were found to enter growth arrest, while the remaining proliferated continuously for 12-15PD after they were picked and propagated as individual cell lines. However, growth arrested hybrids were found to display a marked reduction in telomerase activity relative to wild-type 21NT cells and continuously proliferating hybrids, which suggests that telomerase repression was associated with induction of growth arrest. The growth rate of the majority of these hybrids was not determined during this study, however most were found to enter growth arrest after a similar period of time in selection (4-6 weeks), as 21NT-chromosome 3 hybrids generated by Cuthbert *et al.* (1999). Only one 21NT-Neo3 hybrid was found to proliferate for approximately 9 weeks in selection prior to growth arrest, and displayed a significantly higher mean PD time of 109 hours. Similarly to Cuthbert *et al.* (1999), all 21NT-Neo3 hybrids that entered growth arrest within my study, were found to display morphological features of senescence.

Introduction of chromosome 3 into the RCC23 and KC12 renal cell lines, resulted in hybrids that exhibited undetectable levels of telomerase activity, which were found to enter growth arrest with shortened telomere lengths (Horikawa *et al.,* 1998, Tanaka *et al*., 1998). Cuthbert *et al.,* (1999) observed that the majority of chromosome 3 hybrids displayed a reduction in growth rate and entered growth arrest after 10-12 or 15-18 population doublings (PD), compared with hybrids recovered by Horikawa *et al., (*1998) and Tanaka *et al.,* (1998), which exhibited longer division potentials of 31-41PD and 10-30PD prior to growth arrest. The number of population doublings that were found to occur prior to growth arrest may have been different within each study, due to the telomere length of the parental cell lines. For example, the 21NT and RCC23 cell lines have been found to have mean telomere restriction fragment lengths (TRF) of 3 and 6 kilobases (kb) respectively, and 21NT hybrids were found to enter

growth arrest within fewer population doublings, than RCC23 hybrids (Cuthbert *et al.,* 1999, Horikawa *et al.,* 1998). The number of PD(s) and mean telomere restriction fragment (TRF) lengths of chromosome 3 and 17-21NT hybrids that entered growth arrest within my study, were not ascertained due to an insufficient number of cells. The 21NT-chromosome 3 hybrids recovered within my study, entered growth arrest either *in situ* (3-4 weeks following microcell fusion), within two weeks of being picked, or following two serial passages after they were picked. Based on these observations, together with the lack of strong telomerase repression, it is unclear whether 21NT-chromosome 3 hybrids within my study, entered telomere length-dependent growth arrest.

The findings presented within my study contrast those presented by Cuthbert *et al.,* (1999) in that only 11% of 21NT-chromosome 3 hybrids were found to exhibit a 90% reduction in telomerase activity relative to wild-type 21NT cells and only 36% of hybrids were found to enter growth arrest. Interestingly, introduction of a normal copy of human chromosome 6 into the cervical cancer cell line (SiHa), carried out by two independent research groups, has also been shown to yield contrasting results (Steenbergen *et al.,* 2001, Uejima *et al.,* 1995). Steenbergen and colleagues, found that the majority of SiHa-chromosome 6 hybrids exhibited a significant reduction in the level of *hTERT* mRNA and telomerase activity. These hybrids were also found to enter growth arrest associated with progressive telomere shortening. On the other hand, introduction of chromosome 6 into the same cell line by Uejima *et al.,* (1995), resulted in no observable effect on the telomerase activity, cellular morphology or growth potential of SiHa cells. Instead, introduction of normal copy of chromosome 2, was found to induce cellular senescence in 93% of hybrid colonies recovered. Both studies obtained hybrid donor cell lines containing a single copy of human chromosome 6 from two different human: mouse monochromosome donor libraries, which may have been responsible for the contrasting results observed. Human chromosomes within human: rodent monochromosome donors have been found to undergo structural rearrangements with host chromosomes (Tanabe *et al.* 2000). These observations demonstrate the importance of ensuring that human chromosomes maintained within human: rodent hybrid donor cell lines, are structurally intact before they are used as donors for functional gene transfer studies. Cytogenetic analysis of human monochromosomes within A9-Neo3 and A9-Hytk17 hybrid donor cell lines was not carried out during this study, therefore the structural integrity of these chromosomes was unknown prior to microcell fusion.

Alternatively, there is evidence to show that propagation of cell lines in culture for an extended period of time, are susceptible to genetic drift due to genetic instability, cross-contamination with other cell lines or exposure to different culture conditions (Osborne *et al.,* 1987, Bahia *et al.,* 2002). Differences in tumorigenicity, cloning efficiency and growth rate, have been observed within variants of the same cell line cultivated in different laboratories, which indicates that culture conditions have a substantial affect on the biological properties of

cells and may explain why conflicting results are sometimes obtained (Osborne *et al.,* 1987). The 21NT primary breast cancer cell line, originally established by Band *et al.,* (1990), has been cultivated for use in multiple *in vitro* studies at Brunel since the late 1990s. Therefore, it is possible that serial propagation of this cell line over an extended time period, may have resulted in genetic drift and selection of cells with different genetic abnormalities to early-passage cells. This may provide an explanation as to why introduction of a normal copy of human chromosome 3 into the 21NT cell line, did not result in the same phenotypic effect observed previously by Cuthbert *et al.,* (1999).

Introduction of a normal copy of human chromosome 17 has been shown to cause cell growth inhibition of colorectal and breast cancer cells (Goyette *et al.,* 1992, Negrini *et al.,* 1994, Plummer *et al.,* 1997, Yang *et al.,* 1999) and growth suppression of peripheral neuroepithelial cells and prostate cancer cells (Lin *et al.,* 1995, Yoshinori *et al.,* 1995). Following transfer of chromosome 17 into MDA-MB-231 and MCF-7 breast cancer cells, no viable hybrids were found to possess chromosome 17p-arm material (Negrini *et al.,* 1994). However, one proliferating MDA-MB-231 hybrid with chromosome 17q-arm material was found to exhibit reduced tumorigenicity *in vivo.* This suggests that distinct growth regulatory sequences may exist on both the p and q arm of chromosome 17. Transfection of a wild-type *p53* cDNA vector into these cell lines resulted in a 50% reduction in the number of colonies observed compared with a control vector, which suggested that *p53* (located on 17p13.1) may have been responsible for mediating growth inhibition of both hybrids (Negrini *et al.,* 1994). In contrast, Plummer *et al.,* (1997) observed delayed growth arrest of MCF-7 cells following transfer of a chromosome 17 fragment with a 17p13.1pter deletion, which indicated that *p53* did not function as a tumour suppressor gene within this cell line. Only MCF-7 hybrids with 17q-arm material were found to enter growth arrest following 10-12PD, and deletion mapping of proliferating hybrids identified a putative tumour suppressor sequence localized to 17q24-q25. Similarly, transfer of 17q into the PPC-1 prostate cancer cell line was found to produce two hybrids, both of which exhibited a significant reduction in growth rate and reduced tumorigenicity in athymic nude mice (Yoshinori *et al.,* 1995). This study also found that sequences within the 17q12-q22 subchromosomal region mediated repression of tumorigenicity of PPC-1 prostate cancer cells. These observations suggest that multiple tumour suppressor sequences present on chromosome 17, function to regulate the growth of human cells originating from different tissue types.

A putative mechanism by which chromosome 17 mediates cell growth inhibition was proposed by Yang *et al.,* (1999), who observed strong telomerase repression and a 90% reduction in cell growth, following introduction of chromosome 17 into the benzo(a)pyrene-transformed MCF-10F breast epithelial cell line. This study provided evidence to show that chromosome 17 may harbour sequences that regulate telomerase activity. In my study, 60% of 21NT-chromosome 17 hybrids recovered, exhibited a significant reduction in telomerase

activity relative to wild-type 21NT cells, with 27% of hybrids displaying strong (74%-84% reduction) telomerase repression. In addition 50% of hybrids entered growth arrest with a 50% average reduction in telomerase activity. The identification of a causal link between a reduction in telomerase activity and hybrid growth arrest was unclear, due to the lack of sufficient cell numbers to determine telomere lengths within 21NT-chromosome 17 clones. However, the majority of hybrids were found to either enter growth arrest, or display a significant reduction in growth rate, which provides additional evidence that chromosome 17 harbours sequences that inhibit/suppress the growth of breast cancer cells. The mechanisms by which these sequences mediate a tumour suppressive function and their relative positions along the chromosome, have yet to be fully elucidated. However, I have shown that at least two sequences are present within the genome that function to repress telomerase activity within the 21NT breast cancer cell line.

CHAPTER 4

4 FUNCTIONAL INVESTIGATION OF THE 3P21-LINKED SEQUENCES, *SETD2* AND *FLJ*, AS TELOMERASE REPRESSOR GENES IN BREAST CANCER CELLS

4.1 INTRODUCTION

Repression of human telomerase activity and induction of cell growth arrest has been observed following introduction, of a normal copy of human chromosome 3 into the 21NT breast cancer cell line by MMCT (Cuthbert *et al.,* 1999). The proposed mechanism by which chromosome 3 mediates this effect, is thought to be through transcriptional repression of the *hTERT* gene, which encodes the reverse transcriptase sub-unit of telomerase (Ducrest *et al.,* 2001). In support of this, telomerase-repressed 21NT-chromosome 3 hybrids have been found to contain less than 0.004 molecules/cell of immature intron-containing *hTERT* transcripts, compared with parental 21NT cells, which contain approximately 30-fold higher levels of *hTERT* molecules/cell (Ducrest *et al.,* 2001). Interestingly, the same study also showed that telomerase-negative normal human lung fibroblasts (HLF) and SV40-transformed fibroblast cells, exhibited 50-1500 lower levels of spliced *hTERT* mRNA transcript molecules than telomerase-positive cancer-derived cell lines. This reinforces the link between *hTERT* expression and telomerase activity. Similarly, Horikawa *et al.,* (1998) found that telomerase repression within RCC23-chromosome 3 renal cell carcinoma hybrids, was associated with a marked reduction in *hTERT* mRNA levels, which provides further evidence that *hTERT* regulatory sequences exist on chromosome 3. However, the recovery of telomerase-repressed hybrids produced by whole cell fusion of RCC23 and 21NT cells (i.e. somatic genetic complementation) indicates that more than one *hTERT* transcriptional repressor sequence may be present on chromosome 3 (Tanaka *et al.,* 2005).

In order to determine whether chromosome 3-mediated *hTERT* repression within the 21NT breast cancer cell line involves regulatory sequences that exist upstream of the *hTERT* transcription start site, Ducrest *et al.,* (2001) transfected multiple hTERT-GFP (green fluorescent protein) reporter constructs containing 1.3-7.4kb of upstream 5'-*hTERT* flanking sequences into 21NT-chromosome 3 hybrids and parental cells. Despite observing a substantial reduction in immature pre-spliced *hTERT* mRNA levels, no difference in the level of GFP expression for all reporter constructs were found between 21NT-chromosome 3 hybrids and parental cells. These results indicate that regulatory sequences located up to 7.4kb upstream of

the *hTERT* transcription start site (TSS) may not be responsible for chromosome 3-mediated transcriptional repression. However, it is important to note that telomerase positive GM369 and telomerase-negative GM847 SV40-transformed fibroblast cell lines, expressing detectable and undetectable levels of *hTERT* transcripts respectively, also exhibited the same level of GFP expression following transfection with the same reporter constructs. This could signify the presence of important *hTERT* regulatory sequences further upstream or downstream of the 7.4kb region flanking the 5'-*hTERT* TSS, and which may be involved in chromosome 3-mediated repression.

In 2001, Ducrest and colleagues explored a putative link between chromosome 3-mediated *hTERT* repression and the expression levels of *c-Myc* and c-Myc-target genes within 21NT-chromosome 3 hybrids (Ducrest *et al.,* 2001). Amplification of the *c-Myc* proto-oncogene has been found to occur within a 17-32% of primary breast cancers and is associated with more progressive disease (Nesbit *et al.,* 1999). c-Myc over-expression has also been found to induce *hTERT* gene expression within normal human cells, and increase *hTERT* mRNA levels within telomerase-positive cancer cell lines (see Chapter 1.3.5.2.1.1, Takakura *et al.,* 1999, Wu *et al.,* 1999). In contrast, the Mad protein has been found to repress *hTERT* transcription in normal human cells and competes with c-Myc for dimerization with Max proteins and binding to consensus sequences within the *hTERT* promoter (see Chapter 1.3.5.2.2.1, Oh *et al.,* 2000). Taking these observations into account, Ducrest *et al.,* (2001) observed similar mRNA and protein levels of c-Myc and its antagonist Mad within 21NT-chromosome 3 hybrids and parental cells. In addition, no difference between the mRNA levels of five c-Myc target genes *(CAD, ODC, GADD45, eIF4E* and *LDHA)* were observed between chromosome 3 hybrids and parental cells, providing evidence that sequences on human chromosome 3 do not mediate *hTERT* transcriptional repression through indirect regulation of the c-Myc pathway.

By determining the micrococcal nuclease (MNase) sensitivity pattern of chromatin incorporating genomic DNA 10kb upstream and 16kb downstream of the *hTERT* transcription start site, Szutorisz *et al.,* (2003) explored the chromatin structure of this region within normal human cells and cancer cells. Two independent sites located within the first 1kb of the second intron of the *hTERT* gene, were found to be susceptible to MNase digestion within cancer cell lines expressing *hTERT* mRNA, but not within a normal human lung fibroblast or an alternative lengthening of telomeres (ALT)-positive SV40-transformed fibroblast cell line with undetectable *hTERT* mRNA levels. These results indicated that an 'open' chromatin conformation was associated with the presence of *hTERT* transcripts, which were detectable within cancer cells and not *hTERT*-negative normal human cells or ALT-positive cancer cells. Telomerase-positive (and *hTERT*-positive) 21NT cells were shown to exhibit a similar MNase-sensitivity pattern to other *hTERT*-positive cancer cell lines, whereas two 21NT-chromosome 3 hybrids were found to display the same digestion pattern as normal human fibroblasts. Szutorisz

et al., (2003) also discovered that 21NT-chromosome 3 segregants that had lost the 3p14.1-21.2 region containing the putative telomerase-repressor sequence, exhibited an identical MNase-sensitivity pattern to parental 21NT cells. However, not all 21NT segregant clones were found to re-express *hTERT* mRNA to the same level of parental cells, which could imply that a change in chromatin structure within the second intron of *hTERT* to an 'open' conformation, is necessary but insufficient to induce *hTERT* expression. These findings not only provided evidence of a causal relationship between chromatin structure and *hTERT* expression, but also demonstrated a possible mechanism by which the chromosome 3-encoded sequence mediates *hTERT* transcriptional repression.

Subsequent experiments, carried out by a research group led by Professor Newbold at Brunel University London, have focused on narrowing the search for an *hTERT* repressor sequence located on chromosome 3, that plays a functional role in chromatin remodelling. To identify a smaller region of chromosome 3 encompassing the repressor sequence, chromosome 3 fragments generated by radiation exposure, were introduced into the 21NT cell line by MMCT (unpublished data). Following selection of hybrid colonies, analysis of *hTERT* mRNA levels was carried out and a single *hTERT*-repressed colony was identified. The chromosome 3 fragment from this colony was transferred into an A9 mouse fibroblast cell line by MMCT and mapped using a series of polymorphic marker sequences spanning the length of human chromosome 3. The fragment was found to consist of discreet regions of chromosome 3 short-arm (3p23, p21 and p11) and long-arm material (3q12 and q26). A single A9-chromosome 3 fragment hybrid colony was found to have lost a 490kb sequence, which mapped to the 3p21.3 region of chromosome 3. Frequent loss of this region was observed within segregant 21NT-chromosome 3 hybrids that exhibited high levels of telomerase activity within the study by Cuthbert *et al.,* (1999). Based on these observations, the 3p21.3 region was considered a strong candidate to harbour the putative *hTERT* repressor sequence.

The identified 490kb region of chromosome 3p21.3 encompassed six genes; *TESSP2* (testis serine protease 2), *MYL3* (myosin light chain 3), *PTHR1* (parathyroid hormone receptor 1), *CCDC12* (coiled-coil domain containing 12), *NBEAL2* (neurobeachin-like 2) and *SETD2* (SET-domain containing 2), one pseudogene *MRP63P3* (mitochondrial ribosomal protein 63 pseudogene 3) and one non-coding RNA (ncRNA) *FLJ* (also known as *KIF9-AS1*). Out of these genes, *SETD2* has been shown to function as an epigenetic regulator of gene transcription through histone H3 lysine 36 trimethylation (H3K36) (Sun *et al.,* 2005). Levels of H3K36 trimethylation have been found to increase within the coding regions of actively transcribed genes and SETD2 has been shown to be a direct binding partner of ser2/ser5 phosphorylated RNA polymerase II (RNA polII) *in vitro* and *in vivo*, which indicates a functional role of SETD2 in transcriptional elongation (Sun *et al.,* 2005, Edmunds *et al.,* 2007).

A putative tumour suppressive role of *SETD2* in breast cancer was suggested by Sarakbi *et al.,* (2009), who showed that *SETD2* mRNA levels within malignant breast cancer tissue were significantly lower than within normal breast tissue samples. Breast cancer samples derived from patients with more progressive disease were also found to have significantly lower levels of *SETD2* mRNA than those who were disease-free for greater than 10 years. Similarly, *SETD2* mRNA levels were found to be significantly lower within tumour samples than matched adjacent non-cancerous tissue (ANCT) samples from 25 breast cancer patients (Newbold and Mokbel., 2010). Immunohistochemical staining for SETD2 protein expression also revealed higher protein levels within ANCT than tumour samples. These findings provided valuable evidence to show that *SETD2* may function as a tumour suppressor in breast cancer.

The 490kb region of the chimeric chromosome 3 fragment was also found to possess the non-protein coding gene *FLJ/KIF9-AS1* (referred to as *FLJ* from herein), which is 79.7kb in length and is located approximately 400bp upstream from the 5' coding region of *SETD2*. The final 16kb of the 3' *FLJ* genomic sequence is shared with part of the 3' coding region of *KIF9* (Kinesin family member 9). Unlike *SETD2* and *KIF9, FLJ* is transcribed from the antisense DNA strand. According to current records, only one non-coding splice variant of *FLJ* has been identified, which is 4.9kb in length and consists of seven exons (NCBI and Ensembl). The biological role of *FLJ* has not yet been elucidated; however, it is possible that *FLJ* belongs to a group of long non-coding RNA (lncRNA) antisense sequences. It is thought that several thousands of lncRNA sequences are expressed within the human genome that are involved in regulating gene expression through alternative splicing, mRNA decay and chromatin remodelling, and which influence a wide range of cellular processes including cell cycle regulation, metabolism and migration (reviewed by Zhang *et al.,* 2013). Much like protein-encoding genes, deregulated lncRNAs have been implicated in different cancer types and have been found to exert either oncogenic or tumour suppressive functions (Gutschner *et al.,* 2013, Mourtada Maarabouni *et al.,* 2009, Zhang *et al.,* 2013).

Independent studies have found that lncRNA sequences can bind to and guide chromatin remodelling complexes *in cis* and *trans* to regulate the expression of multiple genes involved in disease pathogenesis (Rinn *et al.,* 2007, Gupta *et al.,* 2011). The HOTAIR antisense long interfering (linc) RNA is transcribed from within the *HOXC* locus on 12q13.3 and is expressed at high levels within breast cancer metastases and some primary breast and colorectal tumours (Gupta *et al.,* 2010, Kogo *et al.,* 2011). The HOTAIR lincRNA was found to bind to the polycomb repressive complex 2 (PRC2) and repress the transcription of the tumour suppressor gene *HOXD* on 2q31.1 by mediating trimethylation of histone H3K27 (Rinn *et al.,* 2007). A subsequent study found that HOTAIR was responsible for global re-targeting of the PCR2 complex leading to transcriptional repression of other important tumour suppressor genes

to promote metastasis (Gupta *et al.,* 2010). Therefore, lncRNAs can play important roles in the transcriptional regulation of many target genes through chromatin remodelling.

The aim of this study was to investigate the possible role of *SETD2* and *FLJ* as tumour suppressor genes in breast cancer cells and to determine whether these candidate genes are involved in mediating transcriptional repression of *hTERT* within the 21NT breast cancer cell line.

4.2 MATERIALS AND METHODS

4.2.1 CHARACTERISATION OF BREAST CANCER CELL LINES AND HMEC CELL STRAINS

Eight breast cancer cell lines 21NT, 21MT, BT474, BT20, H5S78T, HCC1143, GI101 and MCF-7 and one SV40-immortalized normal breast epithelial cell line MTSV, were cultured under normal growth conditions as detailed previously (Chapter 2.1.2). Pre-stasis normal human mammary epithelial cell strains HMEC 184, 240L and the post-stasis HMEC LONZA cell strain were cultured by my colleague Dr. Hemad Yasaei, and provided to me upon request as cell pellets derived from cells growing in log-phase. They were immediately stored at -80^{o}C.

4.2.1.1 *SETD2, FLJ* and *hTERT* gene expression analysis

Cell lines growing in log-phase, were cultured under normal growth conditions in P100 dishes until sub-confluent. Cells were then harvested as described in Chapter 2.1.3 and pelleted by centrifugation at 15,000xg for 5 minutes and stored at -80^{o}C. All cell lines/strains underwent RNA extraction, DNAseI-treatment and cDNA synthesis as detailed in Chapter 2.3.

4.2.1.1.1 GeNorm™ Analysis

In order to quantify the expression levels of a target gene within a sample set, it is important to control for differences in starting cDNA concentrations, reaction enzyme efficiencies and the general level of transcriptional activity within each sample (Vandesompele *et al.,* 2002). This can be achieved through normalization of target gene mRNA levels against an endogenous control gene that is expressed constitutively and at a stable level within all samples. In order to identify a suitable endogenous control gene that is stably expressed within the above samples, a geNorm kit (Primer Design) and the qBase^{PLUS} software package was used to determine the expression stability of seven endogenous control genes (Table 4.1).

cDNA samples were first diluted at a ratio of 1:10 with nuclease-free water. Reaction mixtures for each endogenous control gene consisted of: 1µl primer mix (300nM final concentration), 10µl of 10X iTaq™ Universal SYBR® Green Supermix (BioRad, US), 4µl of

nuclease-free water and 5µl of diluted sample cDNA. Reaction mixtures without cDNA were first pipetted into wells of a 96-well microplate (MicroAmp®, ABI, US). 5µl of sample cDNA was then added to appropriate wells before sealing microplates with optical adhesive film (MicroAmp®, ABI, US). Following centrifugation at 1000xg for 30 seconds, sealed microplates were loaded into the ABI Prism 7900HT Sequence Detection System. Cell line/strain cDNA samples were analysed in duplicate and non-targeting controls (NTC) were included for each endogenous control gene analysed. Thermal cycling conditions for all genes apart from *GAPDH* consisted of an initial enzyme activation step for 10 minutes at 95°C, followed by 50 cycles of 15 seconds at 95°C, 30 seconds at 50°C and 15 seconds at 72°C. The same thermal cycling conditions for *GAPDH* were used as outlined in Table 2.4, Chapter 2.3.4, except the number of amplification cycles was changed to 50.

Table 4.1 - Details of the endogenous control genes evaluated for expression stability across HMEC strains and breast cancer cell lines

Gene Symbol	Name	Genomic Location	Function
B2M	Beta-2-microglobulin	15q21-q22	Serum protein associated with major histocompatability complex I.
EIF2	Eukaryotic translation initiation factor 2	14q23.3	Promotes binding of tRNA to ribosome to initiate protein synthesis.
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	12p13	Oxidoreductase enzyme involved in carbohydrate metabolism.
RPL13A	Ribosomal protein L13a	19q13.3	A component of the 60S ribosomal subunit involved in translation of mRNA into proteins.
SDHA	Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	5p15	Catalytic subunit of succinate-ubiquinone oxidoreductase involved in mitochondrial respiration.
TOP1	Topoisomerase I	20q12-q13.1	Catalyzes the breaking and rejoining of DNA strands during transcription.
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta	8q23.1	Interacts with phosphoserine-containing proteins to mediate signal transduction

Information for each endogenous control gene was obtained from Vandesompele et al., (2002) and the NCBI database.

Amplification data was imported into the qbase^{PLUS} software package, which employs a unique analysis algorithm (Vandesompele *et al.,* 2002) to determine the gene expression stability (M value) of each endogenous control gene within the sample set. This is achieved by

calculating the average pairwise variation of each endogenous control gene with all other control genes. Genes with high M-values therefore show the greatest pairwise variation and exhibit the least stable expression across all samples. Stepwise exclusion of highest scoring genes and re-calculation of new M-values for the remaining genes, distinguishes those with the lowest average pairwise variation and therefore the most stable expression. As shown in Figure 4.1, *TOP1* and *YWHAZ* were found to have the most stable expression across all samples analysed, and were therefore used to normalize expression levels of target genes *SETD2, FLJ* and *hTERT*. Target gene expression levels were determined using the same methods, primers, thermal cycling parameters and data analysis software package detailed in Chapter 2.3.4.

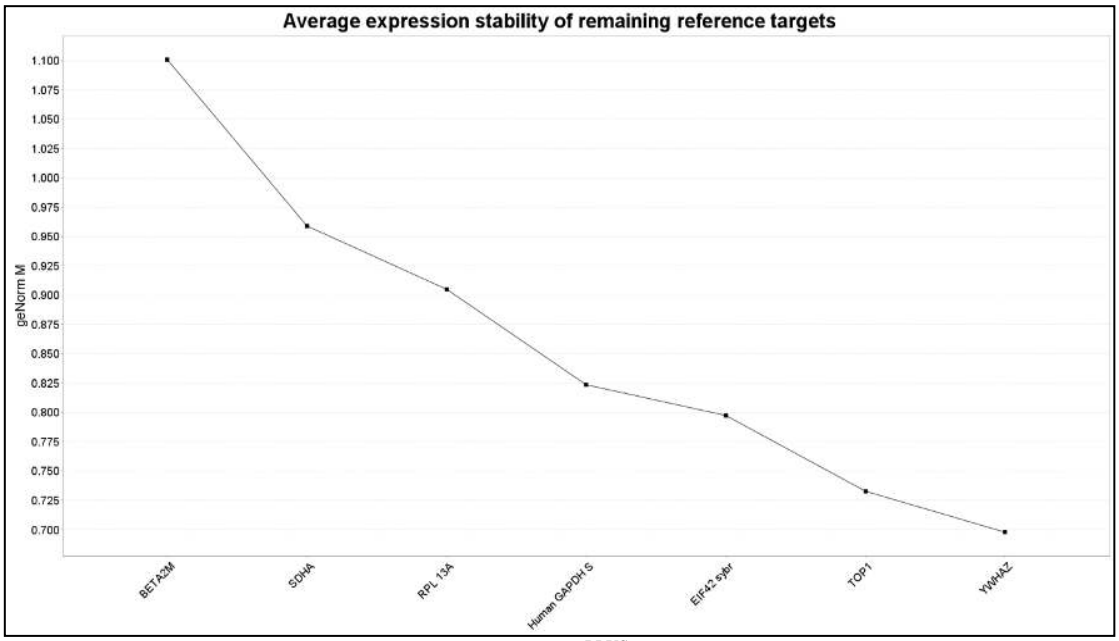


Figure 4.1 - *A line graph exported from qBase^{PLUS} showing the average expression stability (geNorm M) of seven endogenous control genes within all HMEC strains and breast cancer cell lines, following stepwise exclusion of the least stable endogenous control gene.*

4.2.2 SETD2 AND FLJ COPY NUMBER VARIATION (CNV) ANALYSIS

All breast cancer cell lines and normal HMEC strains were cultured under normal conditions until subconfluent before they were harvested, pelleted and stored at -80^{o}C. Genomic DNA (gDNA) was extracted from cell lines and HMEC cell strains as outlined in Chapter 2.2. Sample DNA extracts were diluted to 5ng/µl using nuclease-free water. Gene copy number variation analysis of *SETD2* and *FLJ* within breast cancer cell lines and normal HMEC strains was carried out as described in Chapter 2.5. Details of each pre-designed primer set (ABI, US) for *SETD2* and *FLJ* are detailed in Table 2.6 (Chapter 2.5). The normal HMEC 184 cell strain was used to calibrate gene copy number within all samples.

4.2.3 *OVER-EXPRESSION OF SETD2 WITHIN THE 21NT CELL LINE*

The 8452bp-coding region of the human *SETD2* gene was cloned into the pCMVNeo mammalian expression vector (OriGene) and subsequently transformed into One Shot® TOP10 Chemically Competent E.coli (Invitrogen, US) by my colleague Dr. Terry Roberts. Frozen (-80ºC) stock solutions of pCMVNeo and pCMVNeo-*SETD2*-transformed E.coli in 85% LB-broth and 15% glycerol, were kindly provided to me upon request.

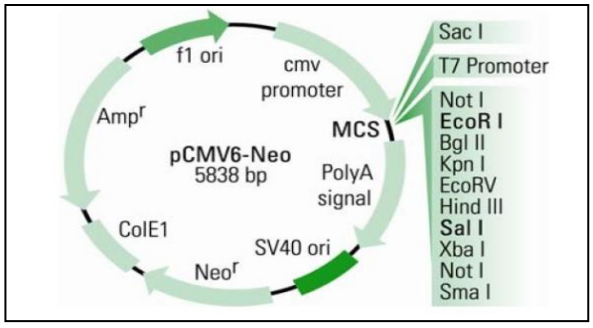


Figure 4.2 - *Physical map of the pCMVNeo vector (OriGene), obtained from http://www.origene.com/assets/Documents/OTIProductManual-pCMVApplicationGuide.pdf*

The pCMVNeo plasmid vector is 5838bp in length and contains a cytomegalovirus (CMV) early promoter sequence upstream of the multiple cloning site (MCS), which permits expression of inserted cDNA clones within mammalian cells (Figure 4.2). The downstream human growth hormone (*hgh*) poly A signal enables the production of mature mRNA sequences. Following transfection of the plasmid construct into mammalian cells, the presence of a neomycin resistance gene (*Neo*^{r}) facilitates positive selection of cells that have undergone stable integration of the plasmid construct within the host genome.

Chemically competent OneShot® TOP10 E.coli transformed with pCMVNeo and pCMVNeo-*SETD2* plasmid constructs were propagated using the procedure described in Chapter 2.6.3. Plasmid DNA was extracted using the QIAfilter Midi Plasmid Purification Kit (Qiagen, NL) according to manufacturer's guidelines outlined in Chapter 2.6.4.

4.2.3.1 Linearization of pCMVNeo and pCMVNeo-*SETD2* vector constructs

Stable integration of plasmid vectors into host cell gDNA involves genetic recombination events. In order to reduce the risk of these events occurring within important regions required for the expression of cDNA in mammalian cells, both pCMVNeo and pCMVNeo-*SETD2* plasmids were linearized by restriction enzyme digestion of a single targeted site. In order to identify a single restriction enzyme recognition sequence within a non-essential region of both plasmid constructs, plasmid sequences were entered into the NEBCutter software program (Posfai *et al.,* 2005). This program can be used to detect many restriction enzyme cleavage recognition sites within any DNA sequence, and provide information about their relative positions within the sequence. pCMVNeo and pCMVNeo-*SETD2* constructs were

found to contain a single DraIII restriction site (Figure 4.3) that maps to the filamentous phage origin of replication known as *f1 ori* (Figure 4.2), which is not required for expression of *SETD2* within mammalian cells.

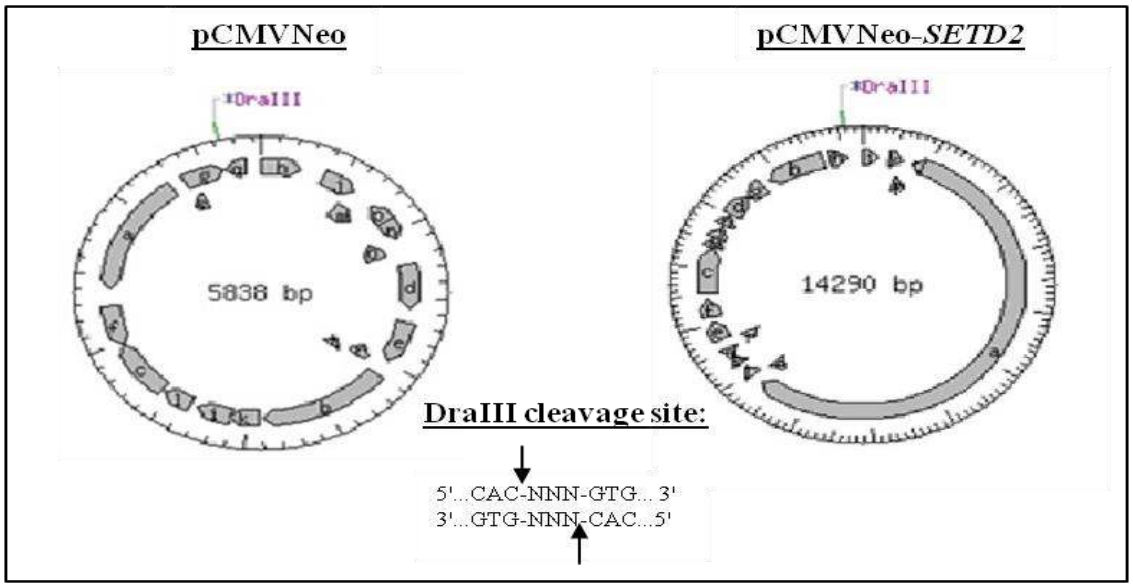


Figure 4.3 - *The position of DraIII restriction enzyme cleavage sites within pCMVNeo and pCMVNeo-SETD2 circular plasmid vector constructs (NEBcutter, Posfai et al., 2005).Grey arrows indicate open reading frames.*

Restriction enzyme reactions consisted of 50µg of plasmid DNA, 20µl of 10X CutSmart™ Buffer, 5µl of 20U/µl DraIII-HF® (High Fidelity) Restriction Enzyme and nuclease-free water to a total volume of 200µl. The reaction mixture was then incubated at 37ºC for 2 hours. Enzyme activity was inhibited by transferring samples to wet ice. To ensure complete digestion and linearization of circular plasmid DNA, the migration pattern of digested and undigested plasmid DNA was determined by gel electrophoresis. 250ng of plasmid DNA was added to wells of a 0.75% agarose gel made with 1X Tris-Borate-EDTA buffer (Sigma-Aldrich, US) and 0.5µg/ml ethidium bromide. After submerging in 1X TBE buffer, the gel was run at 60-70V for around 1 hour 30 minutes. The TrackIt™ 1Kb Plus DNA Ladder (Invitrogen, US) was used to estimate DNA fragment size. Resolved DNA fragments were visualized using an Alphaimager under UV light. As expected, the supercoiled undigested plasmids migrated faster than DraIII-digested plasmids, indicating that the plasmid had been linearized (Figure 4.4). Expected fragment lengths were also observed for both pCMVNeo (5838bp) and pCMVNeo-*SETD2* (14,290bp) following digestion, which confirmed the presence of a single DraIII-cleavage site within both plasmid constructs.

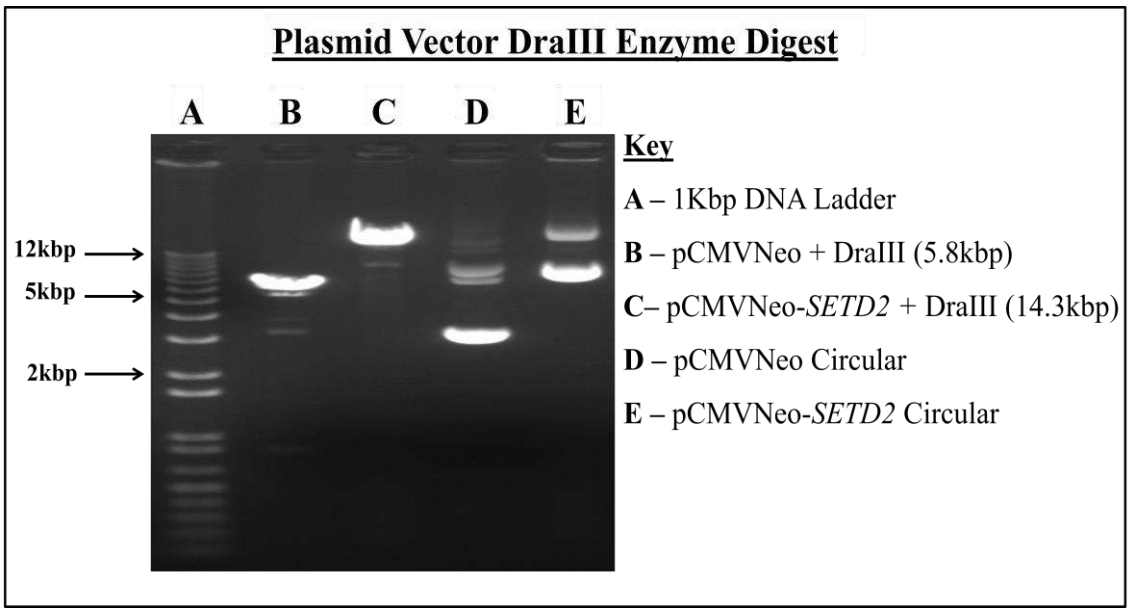


Figure 4.4 - *Gel electrophoresis of DraIII-digested (B and C) and undigested circular (D and E) pCMVNeo and pCMVNeo-SETD2 vector constructs.*

4.2.3.2 Phenol-chloroform extraction and ethanol precipitation of linearized plasmid DNA

Plasmid DNA was purified from the restriction digest reaction mixture using a phenol-chloroform method of DNA extraction. Following the addition of 1 volume of phenol: chloroform (1:1) to plasmid DNA reaction mixtures, solutions were thoroughly mixed and centrifuged at 16,000xg for 5 minutes at room temperature. Upper aqueous phases were carefully transferred to fresh 1.5ml Eppendorf tubes and 1 volume of chloroform was added before mixtures were centrifuged at 16,000xg for 5 minutes. To ensure complete removal of contaminating proteins from the reaction mixture, the previous step was repeated once more. Purified DNA was precipitated by adding 0.1 volumes of 3M sodium acetate (Sigma, US) and 2.5 volumes of 100% ethanol (Sigma, US), followed by incubation of samples at -80ºC for 1 hour or -20ºC overnight. DNA was then pelleted by centrifugation at 16,000xg for 30 minutes at 4ºC. DNA pellets were washed twice by adding 70% ethanol (diluted in nuclease-free water) and centrifuging at 16,000xg for 2 minutes at 4ºC. After carefully decanting sample supernatants, pellets were air-dried for 5-10 minutes before they were re-dissolved in nuclease-free water. The DNA concentration was ascertained before storing samples at -20ºC.

4.2.3.3 Transient transfection of pCMVNeo and pCMVNeo-*SETD2* plasmids into the 21NT cell line

Transient transfection of *SETD2* was carried out in order to examine the effect of short-term *SETD2* overexpression within the 21NT cell line. 24 hours prior to transfection, 21NT

cells were seeded at $2.5x10^5$ cells/dish into thirty P60 dishes and maintained in complete culture media. 21NT cells were divided into three individual groups of ten dishes. The following day, one group of cells were transfected with a negative control empty vector pCMVNeo plasmid, while the second group were transfected with target pCMVNeo-*SETD2* plasmid constructs, using the same methods described in Chapter 2.7.1. The remaining ten dishes of cells remained untreated and were maintained under normal growth conditions throughout the course of the experiment. 48 hours post-transfection, two dishes from each group were harvested, pelleted and stored at -80ºC for downstream analysis. This process was repeated every 24 hours for a further four days.

4.2.3.4 Selection of stable 21NT-pCMVNeo and 21NT-pCMVNeo-*SETD2* transfection clones

In order to examine the long-term effect of *SETD2* overexpression within the 21NT cell line, positive selection of 21NT cells that had undergone stable integration of plasmid vector constructs was carried out. 48 hours post-transfection with control pCMVNeo and target pCMVNeo-*SETD2* constructs, P60 dishes containing 21NT cells were passaged at a ratio of 1:8 into four P100 dishes, using methods described in Chapter 2.1.3. Cells were maintained in complete medium supplemented with 400µg/ml geneticin (G418) for approximately two weeks and monitored regularly for the emergence of stable colonies. 5-8 stable 21NT-pCMVNeo and 21NT-pCMVNeo-*SETD2* colonies were isolated at random and propagated as individual cell lines as described in Chapter 2.1.5. Colonies were maintained under antibiotic selection and propagated until three sub-confluent P100 dishes were obtained. Out of these, one dish of stable cells were harvested for cryopreservation (Chapter 2.1.4), and the remaining dishes were harvested, pelleted and stored at -80ºC for downstream analysis.

4.2.3.5 Colony Forming Unit (CFU) assay

A CFU assay was carried out following transfection of pCMVNeo and pCMVNeo-*SETD2* plasmid constructs into the 21NT cell line to determine the effect of *SETD2* over-expression on cell growth as described in Chapter 2.7.3.

4.2.4 TRANSFECTION OF FLJ INTO THE 21NT CELL LINE

The 2540bp transcript sequence of the non-coding *FLJ* (*KIF9*-AS1) gene, was cloned into the pCMVNeo expression vector described in Figure 4.2, and both control pCMVNeo and pCMVNeo-*FLJ* constructs were transfected into the 21NT cell line by Dr. T. Roberts. Cryopreserved aliquots of stable 21NT-pCMNeo and 21NT-pCMVNeo-*FLJ* cells were

provided to me upon request and were recovered using methods described in Chapter 2.1.4. Stable colonies were propagated in complete culture media supplemented with 400µg/ml G418. Subconfluent dishes of stable cell colonies growing in log-phase were harvested as described in Chapter 2.1.3 and pelleted by centrifugation at 15,000xg for 5 minutes, before immediately storing at -80ºC for downstream analysis.

4.2.5 siRNA-Mediated Knockdown of SETD2

RNA-interference (RNAi) was used to determine the effect of transiently inhibiting *SETD2* expression within a single *hTERT*-repressed 21NT-chromosome 3 hybrid colony (H3.5E). This was achieved by transfecting four pre-designed FlexiTube siRNA constructs (Qiagen, NL), that each target specific untranslated regions (UTRs) of the 8452bp *SETD2* transcript (Figure 4.5). As a non-targeting control, the AllStars siRNA Negative Control (Qiagen, NL) was also transfected into the H3.5E hybrid cell line. The transfection procedure and downstream analysis was carried out by both Dr. T. Roberts and myself.

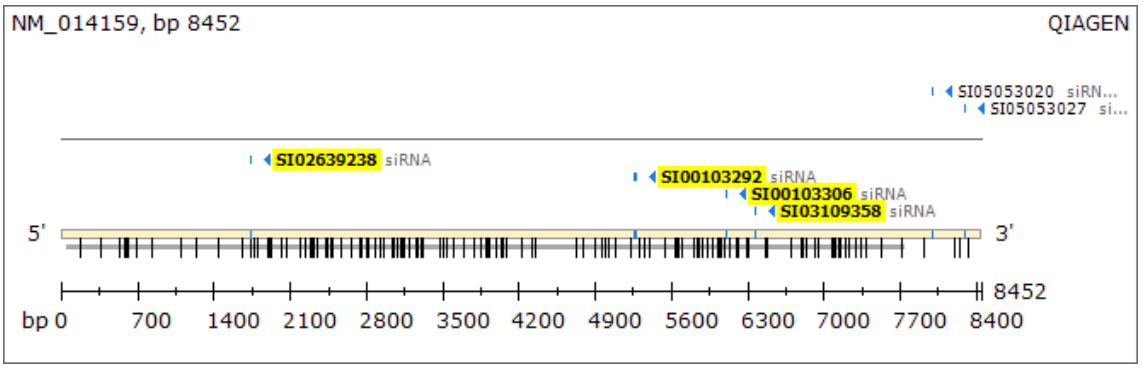


Figure 4.5 - *A physical map of the SETD2 transcript sequence and the relative positions of UTR sequences targeted by four siRNA constructs developed by Qiagen, NL. Diagram obtained from:http://www.qiagen.com/gb/products/catalog/assay-technologies/rnai/flexitube-sirna?catno=GS29072#geneglobe*

On the day of transfection, three subconfluent P100 dishes of H3.5E cells growing in log-phase were harvested and counted using methods described in Chapter 2.1.6. An aliquot of $1x10^6$ cells was pelleted by centrifugation and stored at -80ºC, to be used as an untreated control sample. The remaining cells were re-plated at $4.5x10^5$ cells/dish into P60 dishes. Following the addition of 1.3ml of complete medium supplemented with 400U/ml of hygromycin B (Calbiochem, DE), cells were incubated for three hours at 37ºC and 5% CO_2. Each of the four FlexiTube *SETD2* siRNA construct solutions were diluted to a final concentration of 250ng/µl in nuclease-free water, before transferring 6µl of each solution into a single sterile 1.5ml Eppendorf tube. The AllStars Negative Control siRNA mixture was also diluted to 250ng/µl in nuclease-free water. For each P60 dish of H3.5E cells to be tranfected, 1000ng of either AllStars or pooled FlexiTube *SETD2* siRNA constructs were combined with 96µl of Opti-MEM I

Reduced Serum Medium (Invitrogen, US) and 20µl of HiPerfect Transfection Reagent. To enable the formation of transfection reagent-siRNA complexes, reaction mixtures were incubated at room temperature for 10 minutes. Transfection reagent-siRNA complexes were then added drop-wise onto cells, and dishes were gently swirled to ensure even distribution. Individual P60 dishes of H3.5E cells were transfected with either transfection reagent-FlexiTube *SETD2* siRNA complexes or transfection reagent-AllStars Negative Control siRNA complexes. 3 hours post-transfection, 2.8ml of complete medium supplemented with 400U/ml hygromycin B was added to each dish. Transfected cells were harvested, pelleted and stored at -80ºC for downstream analysis at 48, 72 and 96 hours post-transfection.

4.2.6 GENE EXPRESSION ANALYSIS OF TREATED 21NT CELLS

21NT cells that had undergone transient or stable transfection of target gene-vector constructs and H3.5E hybrids transfected with small-interfering RNA (siRNA) molecules, underwent the same procedure of RNA extraction, DNAse I-treatment and cDNA synthesis as outlined in Chapter 2.3. Gene expression analysis of target genes *SETD2, FLJ* or *hTERT* was carried out using primer/probe concentrations and thermal cycling parameters described in Chapter 2.3.4. Target gene expression levels were normalized to the *GAPDH* endogenous control gene. The qbase^{PLUS} data analysis software program (Biogazelle) was used to calculate normalized relative quantities (NRQ) for each sample outlined in Chapter 2.3.5.

4.2.7 DETERMINATION OF TELOMERASE ACTIVITY

Protein extraction and determination of telomerase activity within breast cancer cell lines, normal HMEC cell strains and stable 21NT transfection clones, was carried out using the same procedure described in Chapter 2.4.

4.2.8 CHROMOSOME 3 ARM-SPECIFIC PAINTING BY FLUORESCENCE IN SITU HYBRIDIZATION

4.2.8.1 Preparation of metaphase chromosomes and chromosome spreading

Cell monolayers growing in log-phase, were cultured under normal growth conditions until 70% confluent on P100 dishes. Cells were arrested in metaphase by adding colcemid (Sigma Aldrich, US) to a final concentration of 0.1µg/ml and incubating at 37ºC and 5% CO_2 for 2 hours. Cells were then harvested as described in Chapter 2.1.3, transferred to 15ml Falcon tubes and pelleted by centrifugation at 15,000xg for 5 minutes. Following aspiration of supernatants, cell pellets were resuspended in 10ml of 75mM pre-warmed (37ºC) hypotonic

potassium chloride (KCl) solution, to initiate nuclear swelling. Cell suspensions were then incubated at 37ºC for 15 minutes. To arrest hypotonic cell swelling and inhibit metabolic processes, 0.5ml of fresh fixative solution consisting of methanol: acetic acid (3:1), was added to cell suspensions and mixed by inverting the tube several times. Cells were then pelleted by centrifugation at 15,000xg for 5 minutes. Following aspiration of the supernatant, 1ml of fixative solution was added to cell pellets drop-wise along the side of tubes. Solutions were immediately vortexed to prevent cell clustering, before a further 9ml of fixative solution was added. Cell suspensions were then incubated at room temperature to 10 minutes, before centrifuging at 15,000xg for 5 minutes. The previous three steps were repeated twice more, before finally resuspending cell pellets in 1ml of fixative solution.

On the same day chromosome spreading was carried out by dropping 7µl of cell suspension onto clean 25 x 75 x 1mm poly-lysine-coated glass microscope slides (Menzel Glaeser). After complete evaporation of the fixative solution, slides were visualized under a phase contrast microscope to confirm the presence of chromosome spreads before proceeding to the next stage. Remaining cell suspensions were either stored at 4ºC for 1 month or at -20ºC for up to 1 year.

4.2.8.2 Chromosome 3-specfic probe denaturation and hybridization

XCAP 3 short and long arm-specific partial chromosome 3 painting probes (Metasystems), labelled with green and red fluorochromes respectively, were used to visualize chromosome 3 structure within cell samples according to manufacturer's guidelines. For each slide to be analysed, 5µl of XCAP3 short arm and 5µl of XCAP3 long arm probe solutions were mixed together in a sterile microcentrifuge tube. The 10µl solution was then carefully spotted onto the centre of each slide and a 22 x 50mm borosilicate glass cover slip was placed over the top. Metaphase chromosomes and probe mixtures were denatured simultaneously by heating slides on a hotplate, pre-heated to 75ºC for 2 minutes. To facilitate probe hybridization, slides were incubated at 37ºC overnight in a humidified chamber.

4.2.8.3 Post-hybridization washes, counterstaining and image capture

The following day, glass cover slips were carefully removed and slides were washed in pre-heated (72ºC) 0.4X saline-sodium citrate solution (SSC) for 2 minutes. Slides were then washed in 2X SSC solution for 30 seconds at room temperature, they were rinsed under tap water for a few seconds. Slides were then serially dehydrated by sequentially transferring 1ml of 70%, 90% and 100% ethanol solution onto slide surfaces and incubating for 2 minutes at room temperature. Slides were left to air-dry for 2-3 minutes. metaphase chromosomes were counterstained by transferring 10µl of blue fluorescent DAPI (Vectashield) stain onto each slide, overlaying a 22 x 50mm borosilicate cover slip and incubating slides for 10 minutes at room temperature. The edges of cover slips were then sealed using rubber cement.

Chromosomes were visualized using a digital fluorescence microscope (Zeiss Axioplan 2 Imaging) and images were taken at 100x magnification using an attached CCD camera (Photometrics) and the Smart Capture (Digital Scientific, UK) software package.

4.3 Results and Discussion

4.3.1 Exploring the Functional Role of SETD2 and FLJ in Breast Cancer Cells

The initial aim of this study was to investigate the putative tumour suppressive role of target genes *SETD2* and *FLJ* in breast cancer by examining their expression levels and allele copy number at the *SETD2-FLJ* genomic locus, within a panel of breast cancer cell lines and normal human mammary epithelial (HMEC) cell strains. To determine whether these genes may have suffered loss of function in connection with malignant transformation and/or telomerase activation, the relationship between *SETD2* and *FLJ* expression levels, gene copy number and telomerase activity within breast cancer cell lines, was also examined.

The majority of breast cancer cell lines employed for the purposes of this study, were previously derived from seven female Caucasian patients aged between 36-74 years who were diagnosed with primary breast cancer (Chapter 2.1.2.1, Table 2.1). As shown in Table 4.2, the majority of breast cancer cell lines were derived from primary intraductal/invasive ductal carcinomas (IDC), most of which have also been found to harbour *p53* mutations. The presence of somatic *p53* mutations within breast tumours is associated with lower rates of disease-free survival (Pharoah *et al.,* 1999). Over half of all breast cancer cell lines employed during this study were previously found to exhibit a tumorigenic phenotype, characterized by the ability to form tumours in nude mice (Table 4.2 and Chapter 2.1.2.1, Table 2.1). The H5S78T cell line is derived from a rare form of aggressive breast cancer known as carcinosarcoma, which is responsible for around 0.1% of all breast cancer cases and is associated with a poor prognosis (Hackett *et al.,* 1977, Abbasi *et al.,* 2012). H5S78T is the only non-tumorigenic cell line harbouring a *p53* mutation within the entire panel (Table 4.2) The 21MT and MCF7 breast cancer cell lines were previously derived from the metastatic lung deposits of two female patients diagnosed with primary breast IDC and adenocarcinoma respectively (Band *et al.,* 1990, Soule *et al.,* 1973). The 21MT cell line has been found to harbour the same *p53* mutation as the matched primary tumour-derived cell line 21NT, from the same patient (Liu *et al.,* 1994). In contrast to other breast cancer cell lines employed during this study, MCF7 has been found to harbour wild-type copies of *p53* (Table 4.2). The BT474, 21NT and 21MT cell lines have been found to be *HER2*-positive (Kao *et al.,* 2009, Band *et al.,* 1990). *HER2*-positive breast cancers are associated with a poorer patient prognosis and a higher disease recurrence rate compared

with *HER2*-negative breast cancers (reviewed by Burstein, 2005). The MTSV cell line is a non-tumorigenic SV40-immortalized luminal epithelial cell cultured from breast milk (Bartek *et al.,* 1990).

Table 4.2 - Characteristics of all breast cancer cell lines employed during this study

Breast Cancer Cell Line	Breast Cancer Type	Primary Tumour (PT)/Metastatic Deposit (MD)	Tumorigenic (Y/N)	p53 mutation status	*HER2/neu* positive
BT20	IDC	PT	Y	MUT	N
BT474	IDC	PT	Y	MUT	Y
GI101	IDC	PT	Y	UD	N
HCC1143	IDC	PT	UD	MUT	N
H5S78T	CS	PT	N	MUT	N
MCF7	AC	MD	N	WT	N
MTSV*	N/A	N/A	N	N/A	N/A
21NT	IDC	PT	Y	MUT	Y
21MT	IDC	MD	Y	MUT	Y

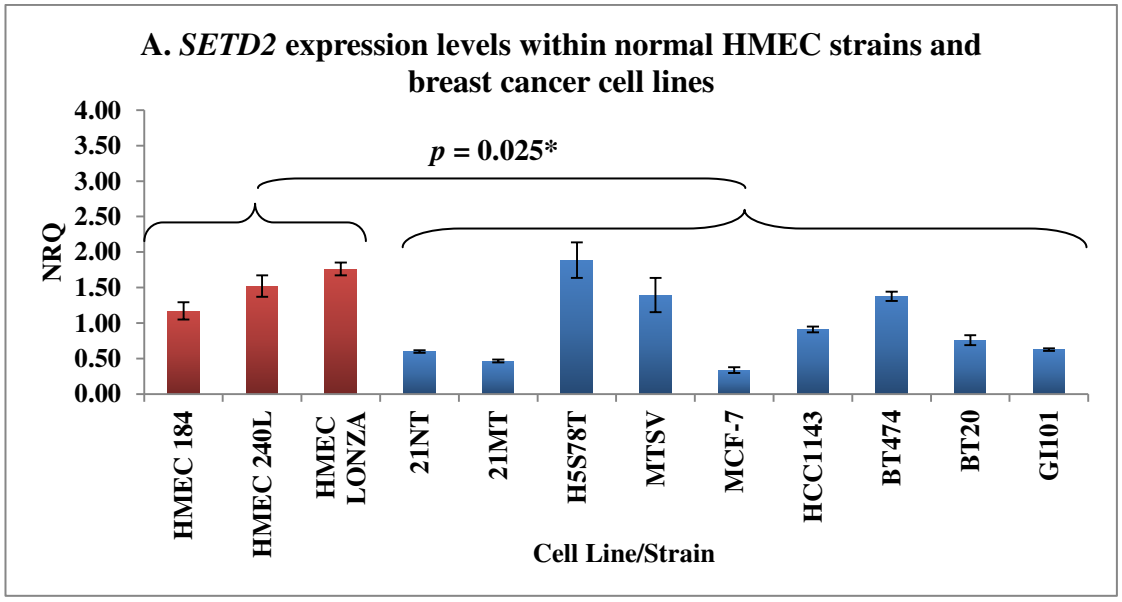
*The International Agency for Research on Cancer (IARC) TP53 Database (http://p53.iarc.fr/CellLines.aspx) was used to determine the p53 mutation status of the majority of breast cancer cell lines. The p53 mutation status of the H5S78T cell line was determined using the Universal Mutation Database, Edlund et al., (2012). Information for human epidermal growth factor receptor (HER2/neu) status of breast cancer cell lines was obtained from Kao et al., (2009), Band et al., (1990) and Hurst et al., (1993). Abbreviations: IDC (intraductal/invasive ductal carcinoma), CS (carcinosarcoma), AC (adenocarcinoma), MUT (mutation), WT (wild-type), UD (undetermined). * Denotes that MTSV is an SV40-immortalized breast luminal epithelial cell line.*

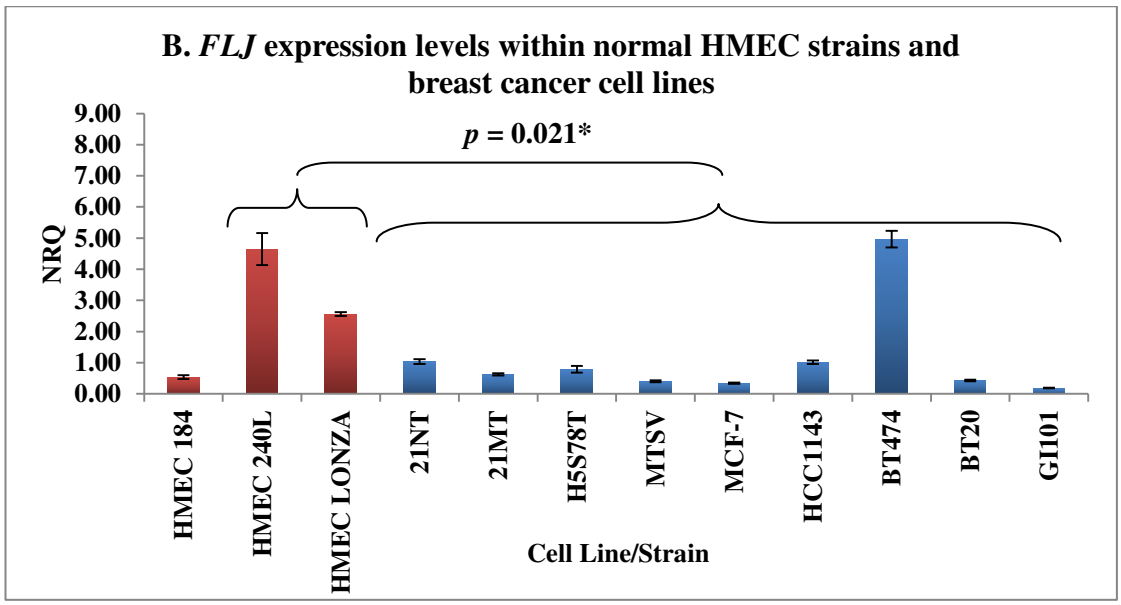
Normal HMEC cell strains (184, 240L and LONZA) derived from breast reduction mammoplasty tissue of three Caucasian females aged between 19-21 years, were included as normal human controls within this study (Chapter 2.1.2.1, Table 2.1). HMEC 184 and 240L pre-stasis cell strains were originally isolated and characterized by Dr. Martha Stampfer and colleagues at the Lawrence Berkeley National Laboratory, University of California. These cell strains have been found to enter stasis with a normal karyotype following 13-16 PD in continuous culture (HMEC Cell Bank, Stampfer., 2003, Garbe *et al.,* 2009). The primary HMEC LONZA cell strain, developed by Lonza Group Ltd, has bypassed the stasis barrier (stage M0, *p16*-negative) to proliferation and enters replicative senescence (termed agonescence if *p53* is functional) after approximately 15 PD in continuous culture. All HMEC strains used within this study were cultivated by my Brunel colleague Dr. H. Yasaei.

4.3.1.1 *SETD2* and *FLJ* gene expression analysis

The expression levels of candidate genes *SETD2* and *FLJ* were first examined within all breast cancer cell lines and HMEC cell strains. Figure 4.6A shows that on average, breast cancer cell lines were found to exhibit significantly lower levels of *SETD2* than HMEC cell strains (p = 0.025). Six out of nine breast cancer cell lines examined, showed 40-80% lower *SETD2* gene expression levels compared with HMEC strains and two out of nine breast cancer cell lines

exhibited a small reduction in relative *SETD2* expression levels of 6%-7%. Only the single carcinosarcoma-derived cell line H5S78T, was found to display 27% higher *SETD2* expression levels, relative to normal HMEC strains. Interestingly, the lowest *SETD2* expression levels were observed within 21MT and MCF7 breast cancer cell lines derived from metastatic deposits. Similar levels of *SETD2* expression were observed within the primary tumour-derived 21NT cell line and metastatic deposit-derived 21MT cell line from the same patient. This suggests that a reduction in *SETD2* expression levels may occur at an early point during disease pathogenesis, prior to metastatic spread. The results presented here are consistent with those obtained by Sarakbi *et al.,* (2009) and Newbold and Mokbel., (2010), which showed that breast cancer tissue samples were found to exhibit lower *SETD2* gene expression levels compared with normal and matched adjacent non-cancerous tissue (ANCT) samples. Together, these data provide additional evidence to show that *SETD2* may function as an important tumour suppressor gene in breast cancer.





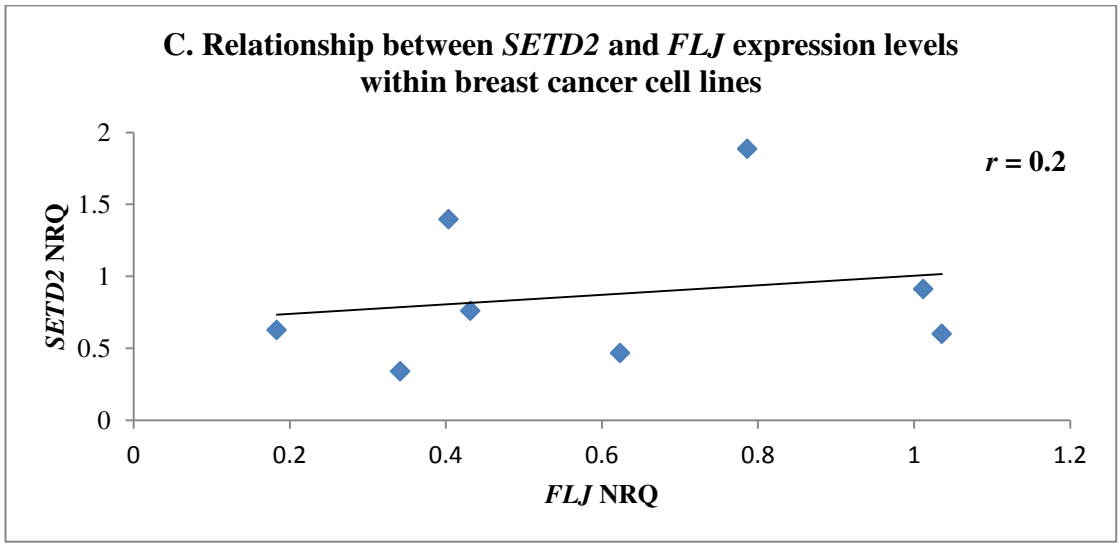


Figure 4.6 - *The normalized relative quantities (NRQ) of* ***(A)*** *SETD2 and* ***(B)*** *FLJ mRNA within a panel of nine breast cancer cell lines (blue) and three normal HMEC cell strains (red). NRQ values are expressed relative to the mean NRQ value of all samples. Error bars represent the standard error of the mean NRQ values from triplicate repeats for each sample. A student's t-test was carried out on log-transformed NRQ values to compare mean SETD2 and FLJ gene expression levels of HMEC cell strains and breast cancer cell lines.* ***(C)*** *A scatter graph showing the relationship between SETD2 and FLJ expression levels within breast cancer cell lines (excluding BT474). The Pearson Correlation Coefficient (r) was used to quantify the strength of the relationship between both variables.*

As shown in Figure 4.6B, a wide range in *FLJ* gene expression levels was observed within HMEC cell strains. In particular, the HMEC 184 cell strain was found to exhibit considerably lower *FLJ* expression levels compared with HMEC 240L or HMEC LONZA strains. Despite this observation, eight out of nine breast cancer cell lines examined exhibited

60-90% lower *FLJ* gene expression levels relative to HMEC cell strains. Upon exclusion of the HMEC 184 cell strain, breast cancer cell lines were found to exhibit significantly lower *FLJ* expression levels compared with normal HMECs (p = 0.021). The expression levels of *FLJ* within 21NT and 21MT cell lines were reduced by 60% and 76% respectively relative to HMEC strains 240L and LONZA, which indicates that, similarly to *SETD2,* a reduction in *FLJ* expression levels may occur at an early point during disease pathogenesis. Out of all breast cancer cell lines examined, only the BT474 cell line was found to exhibit higher *FLJ* expression levels relative to HMEC cell strains (Figure 4.6B). This particular cell line shares many features with other breast cancer cell lines examined (Table 4.2). Therefore, due to the unknown biological function of *FLJ* it is currently unclear as to why this particular cell line was found to exhibit high levels of *FLJ* expression relative to normal HMEC strains and other breast cancer cell lines. However, the results presented here demonstrate that the majority of breast cancer cell lines examined within this study, display lower expression levels of *FLJ* compared with normal HMEC strains, which would perhaps be expected of a novel putative tumour suppressor lncRNA gene in breast cancer.

In humans, *SETD2* and *FLJ* genomic sequences are separated by a small, 393bp non-transcribed DNA sequence, and are actively transcribed from opposing DNA strands and in opposite directions. Due to the observed reduction in both *SETD2* and *FLJ* expression levels within breast cancer cell lines relative to normal HMEC strains, it is possible that the expression of these genes may be co-regulated, perhaps due to the presence of a shared promoter region. As shown in Figure 4.6A and B, normal HMEC cell strains do not appear to show any correlation between *SETD2* and *FLJ* expression levels. Similarly, a very weak positive correlation between *SETD2* and *FLJ* gene expression levels were observed within breast cancer cell lines (r = 0.2), suggesting that the expression of these genes may not be co-regulated.

4.3.1.2 *SETD2* and *FLJ* Copy Number Variation (CNV) Analysis

Molecular and cytogenetic profiling studies have shown that many different cancer types acquire somatic copy number alterations at whole chromosome arms or foci, some of which are thought to promote cancer development and progression through dysregulation of gene expression (Baudis., 2007, Beroukhim *et al.,* 2010). Through parallel DNA copy number profiling and microarray gene expression analysis of primary breast tumours and cell lines, Pollack *et al.,* (2002) found that at least 12% of gene expression variation could be directly linked to gene copy number alterations. Similar studies have identified recurrent regions of somatic copy number gains and losses that harbour functionally characterized oncogenes and tumour suppressor genes respectively (Baudis., 2007, Beroukhim *et al.,* 2010). In breast cancer, amplification of a particular region of chromosome 17q is associated with the overexpression of the HER2/neu oncogene, which is thought to promote the growth of breast cancer cells through

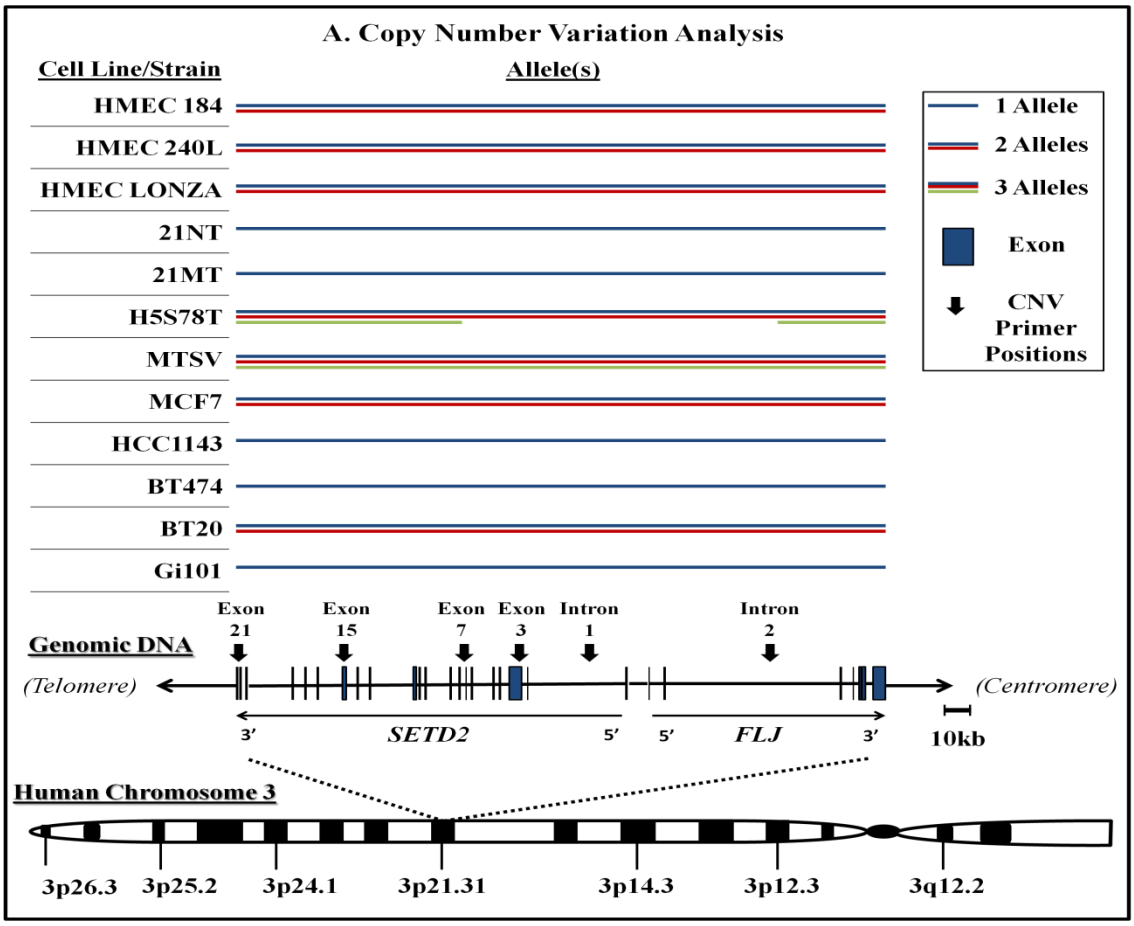
activation of mitogenic signalling pathways (Slamon *et al.,* 1987, Baerlund *et al.,* 1997, Nahta *et al.,* 2006). Based on these observations, copy number variation analysis may be a useful tool in the search for genes involved in cancer pathogenesis.

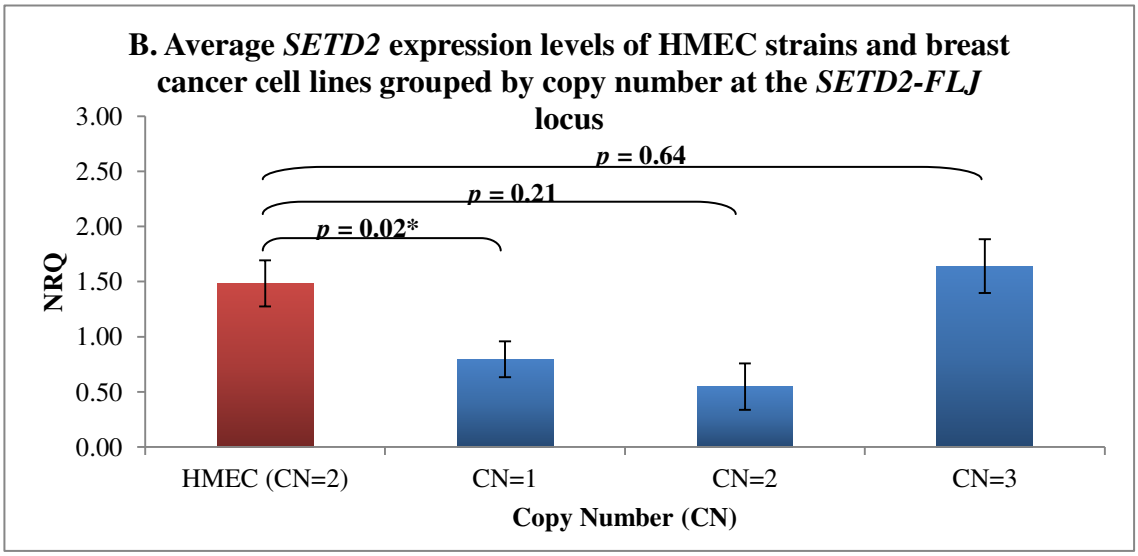
Independent studies have shown that allele loss within the short arm of chromosome 3 occurs within 65-87% of breast cancer cell lines, tumour samples and around 45% of pre-neoplastic lesions (Maitra *et al.,* 2001, Chen *et al.,* 1994, De Oliveira *et al.,* 2012). This indicates that chromosome 3p loss is a common and early event in breast carcinogenesis, and could signify the presence of important tumour suppressor genes within this region. The most common site of allele loss in breast cancer has been found to occur within the 3p21.3 region (Maitra *et al.,* 2001), which is known to harbour *SETD2* and *FLJ* genes.

In order to determine whether the observed reduction in *SETD2* and *FLJ* expression levels within breast cancer cell lines relative to normal HMECs may be linked to gene copy number loss, detailed copy number variation (CNV) analysis of the *SETD2-FLJ* genomic locus was carried out. The number of alleles present at six distinct genomic loci mapping to intronic and exonic sequences of *SETD2* and *FLJ* was examined within the same panel of HMEC strains and breast cancer cell lines detailed previously. As shown in Figure 4.7, all HMEC strains were found to harbour two alleles at each loci, which indicates that this locus is present as two copies within normal human mammary cells. In contrast, only two out of nine breast cancer cell lines were found to harbour two alleles at each loci, while over half of breast cancer cell lines (5/9) were found to have undergone single copy number loss at all loci. Breast cancer cell lines that had undergone copy number loss were derived from patients with breast IDC and almost all had been previously found to exhibit a tumorigenic phenotype and harbour *p53* mutations. However, the presence of two alleles at all loci within the BT20 cell line, implies that these features are not always associated with copy number loss at the *SETD2-FLJ* genomic locus. The remaining two breast cancer cell lines were found to have gained a single allele at the majority of loci examined, which included the rare carcinosarcoma-derived H5S78T cell line and the SV40-immortalized MTSV cell line. This indicates that the *SETD2-FLJ* locus can also undergo copy number gain in breast cancer.

In order to examine the relationship between copy number variation and the gene expression levels of *SETD2* and *FLJ*, breast cancer cell lines were grouped by gene copy number at the *SETD2-FLJ* genomic locus and the mean expression levels of both genes were calculated for each group. As shown in Figure 4.7B and C, significantly lower levels of both *SETD2* and *FLJ* were observed within breast cancer cell lines that had undergone allele loss at this locus, compared with normal HMEC strains ($p = 0.02$ and $p = 0.03$ respectively). Interestingly, lower expression levels of both genes were also observed within breast cancer cell lines that had two alleles compared with HMEC cell strains and breast cancer cell lines with

only one allele at this locus. In addition, breast cancer cell lines that had undergone gene amplification at the *SETD2-FLJ* genomic locus, were found to exhibit substantially lower *FLJ* expression levels relative to HMEC strains (Figure 4.7C). These results indicate that copy number alterations at the *SETD2-FLJ* locus may be responsible for some, but not all cases of *SETD2* and *FLJ* expression downregulation in breast cancer, and that alternative mechanisms of transcriptional silencing may be involved. These observations provide additional evidence that *SETD2* and *FLJ* may function as important tumour suppressor genes in breast cancer.





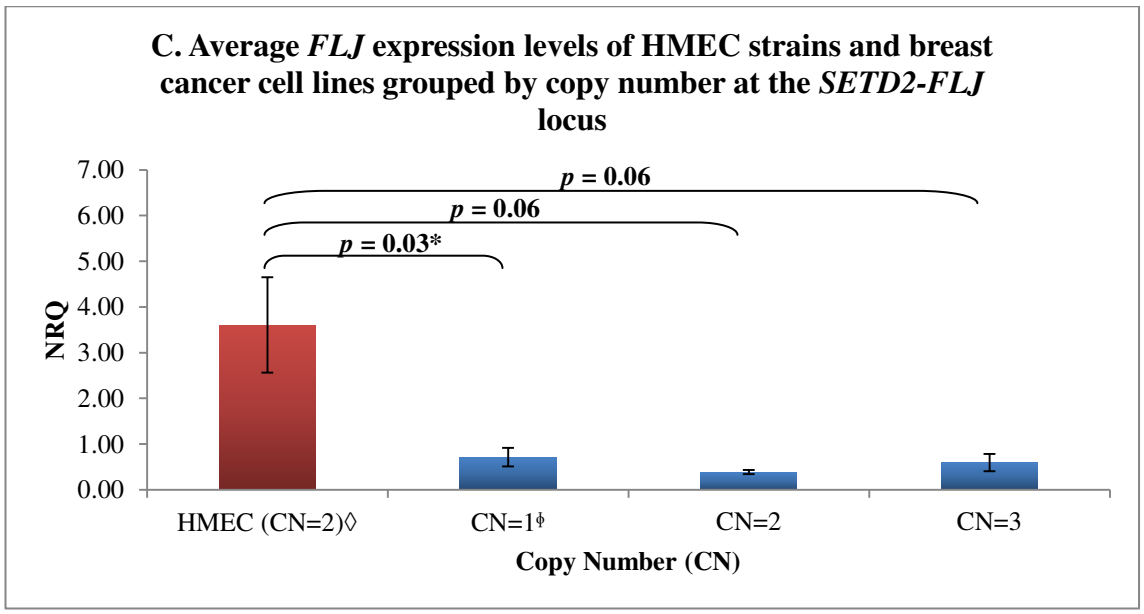
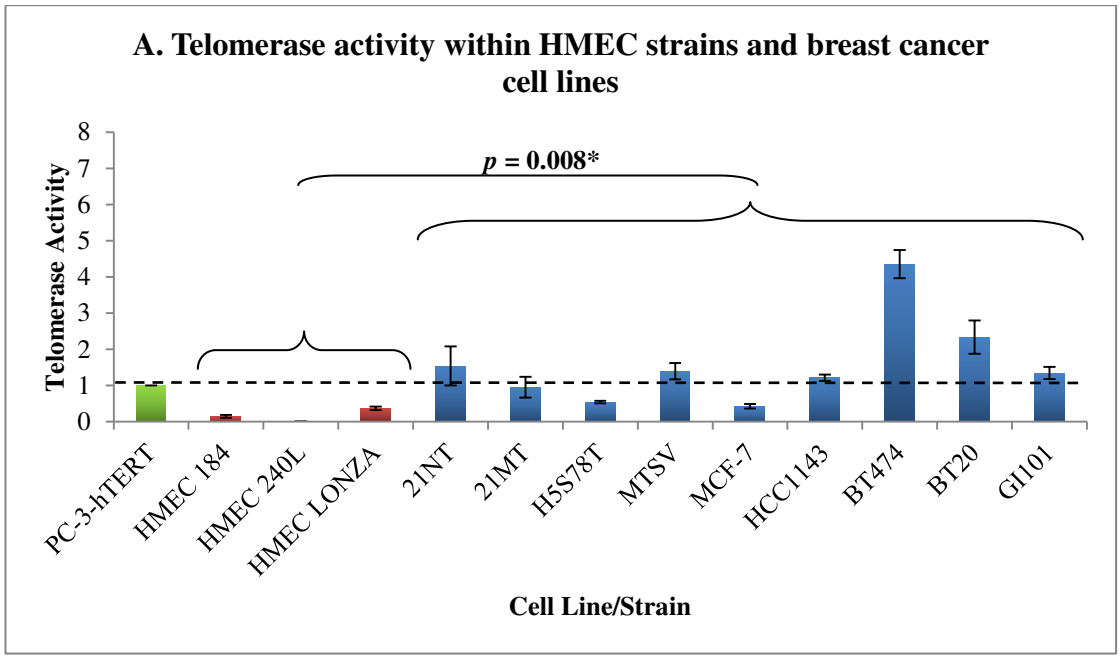


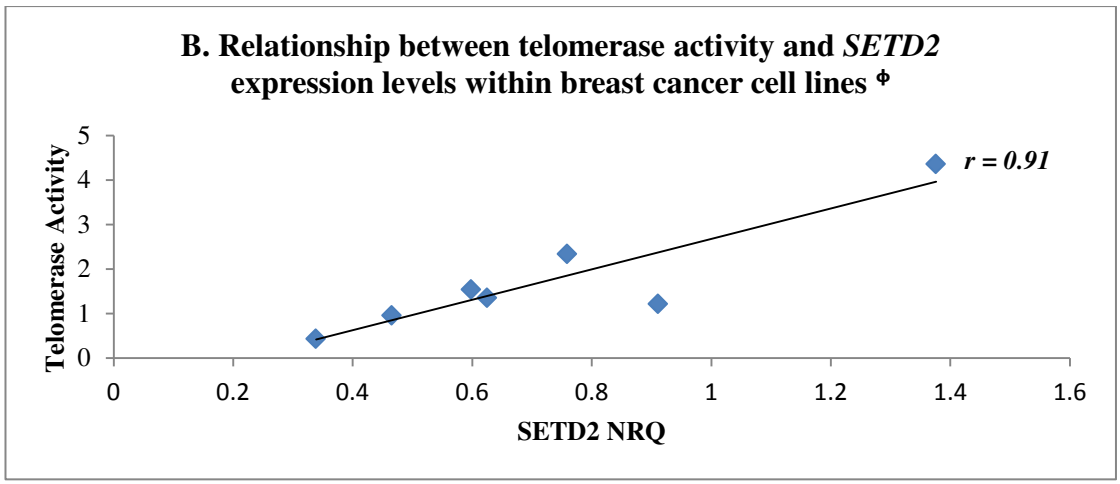
Figure 4.7 *- **(A)** Copy Number Variation (CNV) analysis of SETD2 and FLJ gene loci within nine breast cancer cell lines and three normal HMEC cell strains. The number of alleles at six genomic loci mapping to exonic and intronic regions of SETD2 and FLJ was determined as indicated above. An ideogram of chromosome 3 shows the relative positions of both target genes. The H5S78T cell line was found to harbour 3 alleles at 4/6 genomic loci examined and was therefore considered to have undergone copy number gain at this locus. Mean NRQ values of **(B)** SETD2* and ***(C)** FLJ mRNA within HMEC cell strains and breast cancer cell lines grouped by overall copy number at the SETD2-FLJ gene locus. Error bars represent the standard error of the mean NRQ values for each group. A student's t-test was used to compare the log-transformed mean NRQ values of breast cancer copy number groups with mean HMEC NRQ values for both SETD2 and FLJ. ◊ and ϕ denotes the exclusion of HMEC 184 and BT474 from sample groups respectively.*

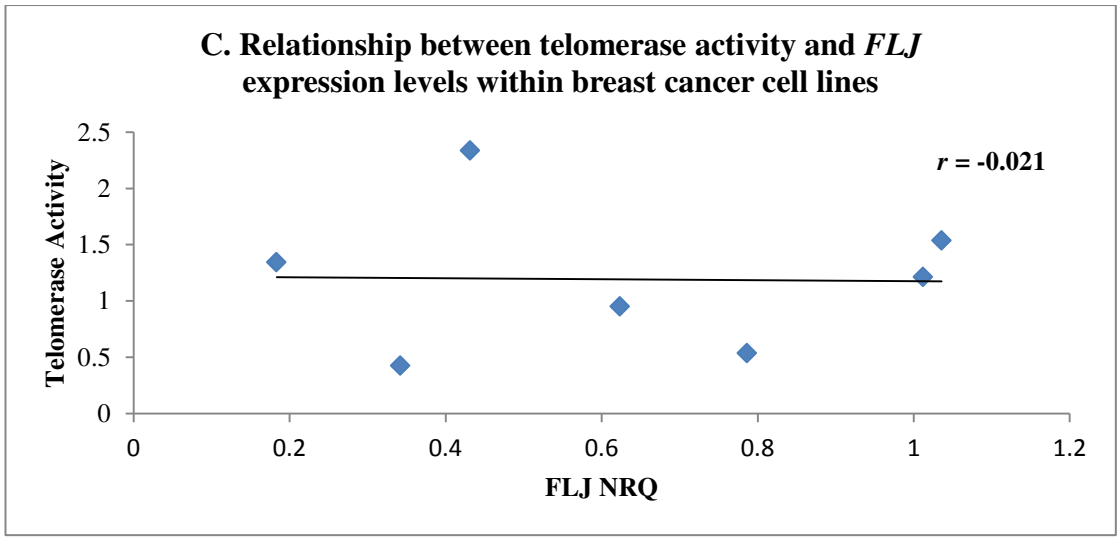
4.3.1.3 Telomerase activity within HMEC strains and breast cancer cell lines

The next aim of this study was to explore the relationship between *SETD2* and *FLJ* gene expression levels and allele copy number variation at the *SETD2-FLJ* locus, with telomerase activity of breast cancer cell lines. As shown in Figure 4.8A, breast cancer cell lines were, as expected, found to exhibit significantly higher levels of telomerase activity compared with HMEC strains (p = 0.008). The highest levels of telomerase activity were observed within BT474, BT20 and 21NT breast cancer cell lines, derived from patients with breast IDC, which have been previously shown to exhibit a tumorigenic phenotype and harbour *p53* mutations. In addition, BT474 and 21NT cell lines have been found to be *HER2*-positive, which may suggest that higher levels of telomerase activity are associated with a more aggressive disease phenotype. Breast cancer cell lines 21MT and MCF7, derived from metastatic deposits were

found to exhibit lower levels of telomerase activity compared to breast cancer cell lines derived from primary tumours.







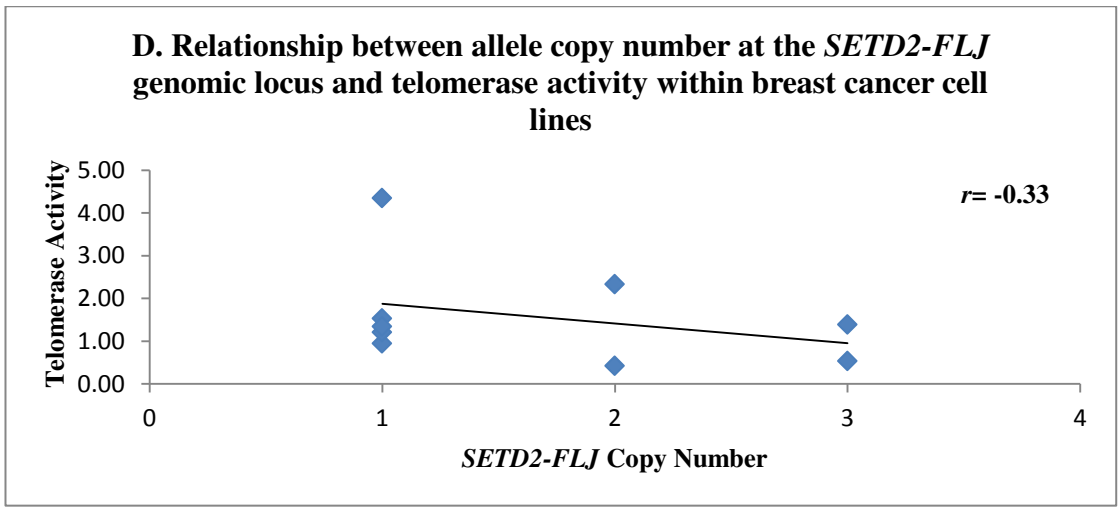


Figure 4.8 - (A) *Telomerase activity within HMEC cell strains and breast cancer cell lines relative to a telomerized prostate cancer cell line PC-3-hTERT. Error bars represent the standard deviation of the mean telomerase activity from triplicate repeats for each sample. A student's t-test was used to calculate the difference between mean telomerase activity of HMEC strain and breast cancer cell lines. Scatter plots showing the relationship between telomerase activity and* ***(B)*** *SETD2 NRQ values,* ***(C)*** *FLJ NRQ values and* ***(D)*** *allele copy number within the SETD2-FLJ locus within breast cancer cell lines examined. The Pearson Correlation Coefficient (r) was used to measure the strength of the correlation between variables.*$^{\phi}$ *denotes exclusion of the MTSV and H5S78T cell lines.* ***(B)*** *shows a strong positive correlation between SETD2 gene expression and telomerase activity levels within breast cancer cell lines, which suggests that SETD2 may function as a positive regulator of telomerase activity in breast cancer cells.*

The relationship between *SETD2* and *FLJ* expression levels with telomerase activity levels exhibited by breast cancer cell lines was then explored further. A very strong positive correlation was observed between telomerase activity and *SETD2* gene expression levels within the majority of breast cancer cell lines examined (Figure 4.8B, r = 0.91). These observations contrast the hypothesis that *SETD2* functions as a negative regulator of telomerase activity in breast cancer. On the other hand, no correlation between *FLJ* gene expression levels and telomerase activity was observed (Figure 4.8C, r = -0.021), which suggests that *FLJ* may not function as a regulator of telomerase in breast cancer.

A weak negative correlation was observed between allele copy number at the *SETD2-FLJ* genomic locus and telomerase activity within breast cancer cell lines (Figure 4.8D, r = -0.33). Due to the lack of association between *SETD2* and *FLJ* gene expression levels and the number of alleles present at the *SETD2-FLJ* locus (Figure 4.7B and C), together with the observed relationship between *SETD2* expression levels and telomerase activity, this observation does not indicate a functional link between either candidate gene and telomerase

activity within breast cancer cells. However, due to the frequency and extent of chromosome 3p loss in breast cancer (Maitra *et al.,* 2001), it is likely that the observed change in copy number within the majority of breast cancer cell lines examined extends beyond the *SETD2-FLJ* gene locus. This would provide an explanation as to why a negative correlation was observed between *SETD2-FLJ* copy number and telomerase activity, but not between candidate gene expression levels and telomerase activity. Further investigation into the extent of chromosome 3p loss within breast cancer cell lines and telomerase activity levels would be required in order to explore this possibility further.

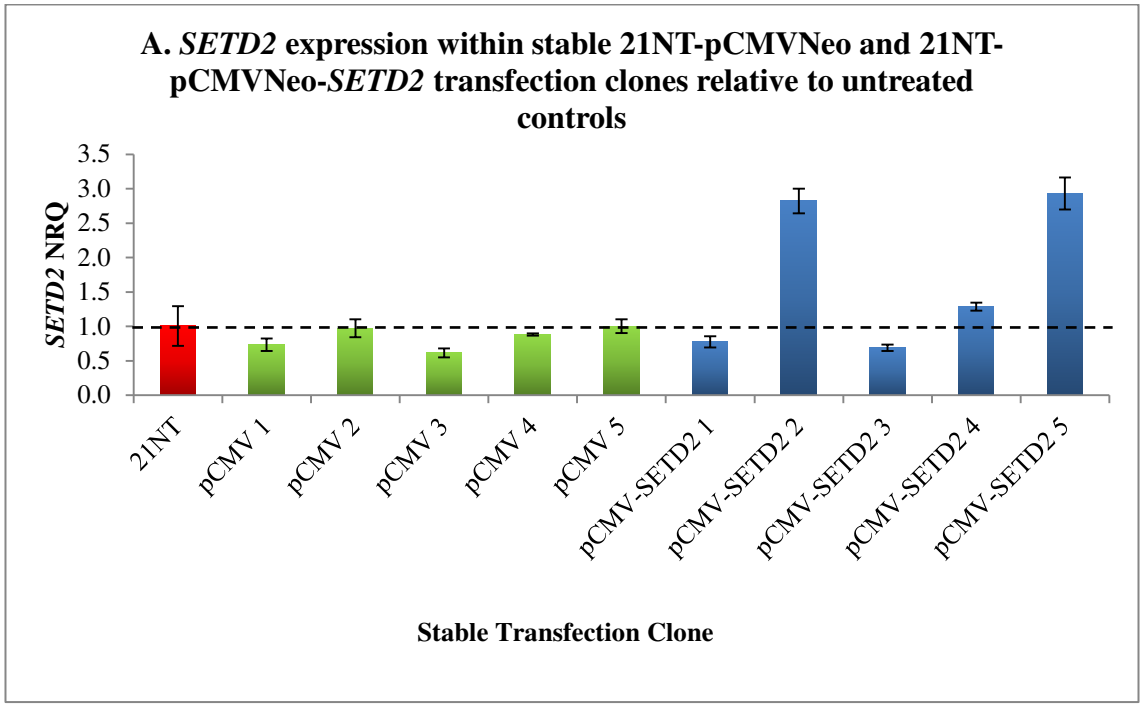
4.3.2 Forced Overexpression of SETD2 within the 21NT Breast Cancer Cell Line

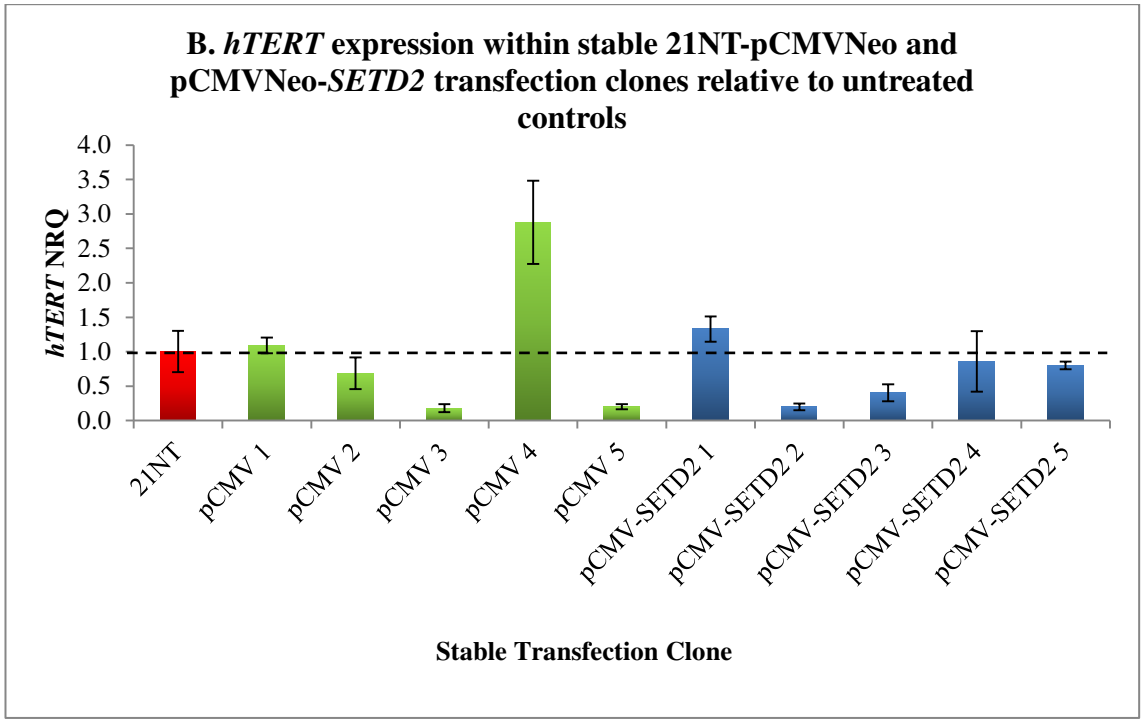
The results presented in this Chapter so far are consistent with previous studies demonstrating a putative role of *SETD2* as a tumour suppressor gene in breast cancer (Sarakbi *et al.,* 2009, Newbold and Mokbel., 2010). Despite observing a positive correlation between *SETD2* gene expression levels and telomerase activity within breast cancer cell lines (Figure 4.8B), *SETD2* was identified within a chimeric chromosome 3 fragment that caused telomerase repression when introduced into the 21NT breast cancer cell line (unpublished data, Newbold group). Results presented by Ducrest *et al.,* (2001) and Szutorisz *et al.,* (2003) demonstrating that the chromosome 3-encoded repressor sequence mediates telomerase repression through chromatin remodelling and silencing of the *hTERT* promoter, together with the known function of *SETD2* as a histone methyltransferase enzyme, warranted further investigation into the functional role of *SETD2* in telomerase regulation. In order to achieve this, the effect of forced *SETD2* overexpression on *hTERT* gene transcription was examined within the 21NT cell line, known to exhibit telomerase repression and growth arrest following introduction of a normal copy of chromosome 3. The human *SETD2* 8452bp coding region was cloned into the pCMVNeo mammalian expression vector by Dr. T. Roberts. Both target gene pCMVNeo-*SETD2* and vector only pCMVNeo negative control plasmid constructs were linearized and transfected into the 21NT cell line using a chemical-based method of gene delivery.

4.3.2.1 Stable overexpression of *SETD2* within the 21NT breast cancer cell line

In order to examine the effect of stable long-term *SETD2* overexpression on the transcriptional activity of *hTERT* and telomerase activity levels, 21NT cells transfected with negative control pCMVNeo and target gene pCMVNeo-*SETD2* constructs were cultured in the presence of neomycin for two weeks to select for cells that had undergone stable integration of plasmid constructs. Two sets of five individual 21NT-pCMVNeo and 21NT-pCMVNeo-*SETD2*

transfection clones were isolated and propagated as individual cell lines, until sufficient cells were obtained for *SETD2* and *hTERT* expression analysis.





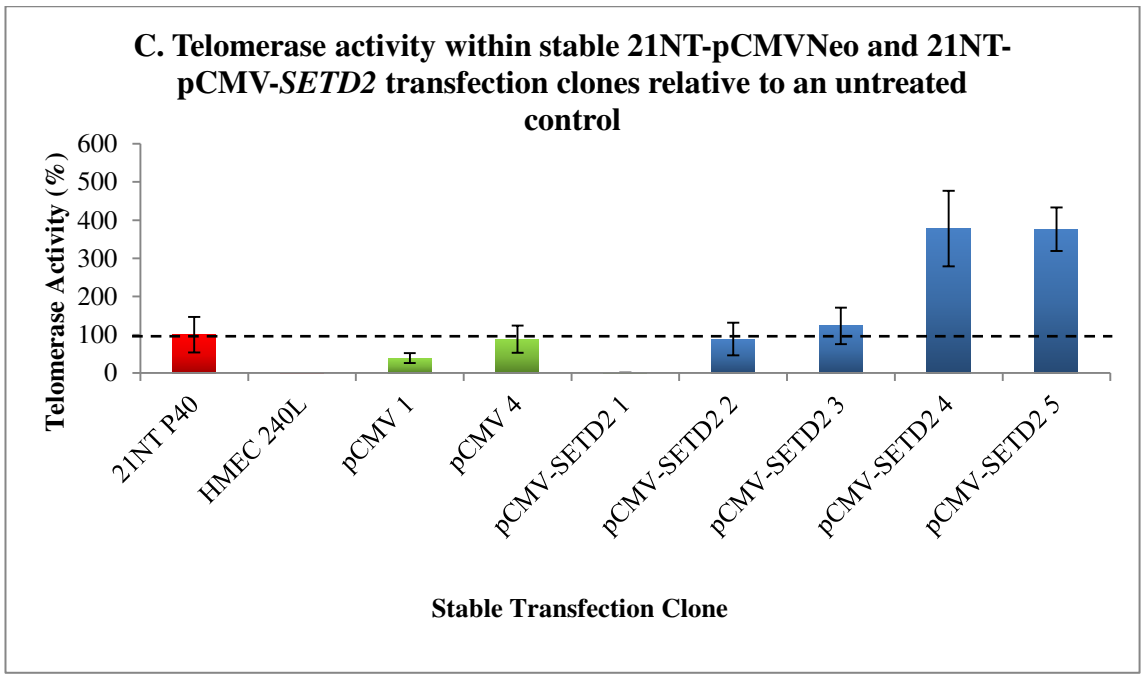


Figure 4.9 *- The normalized relative quantities (NRQ) of* ***(A)*** *SETD2* and ***(B)*** *hTERT mRNA within stable 21NT-pCMVNeo (green) and 21NT-pCMVNeo-SETD2 (blue) colonies relative to the mean expression levels of two untreated 21NT cell samples (red). Error bars for stable clones represent the standard error of the mean NRQ values obtained from triplicate repeats for each sample. Error bars for the 21NT cell line represent the standard error of the mean NRQ values of two individual untreated 21NT samples included within the same assay.* ***(C)*** *Telomerase activity within stable 21NT-pCMVNeo and 21NT-pCMVNeo-SETD2 clones expressed as a percentage of untreated 21NT cells (100%). Error bars represent the standard error of the mean telomerase activity levels obtained from triplicate repeats for each sample.*

Out of five stable 21NT-pCMVNeo-*SETD2* stable clones, two clones were found to exhibit around 3-fold higher *SETD2* expression levels relative to untreated 21NT cells (Figure 4.9A, pCMV-*SETD2* clones 2 and 5). These clones were found to display 80% and 20% lower expression levels of *hTERT* mRNA relative to untreated 21NT cells (Figure 4.9B). However, the same clones were found to exhibit either similar to or 4-fold higher levels of actual telomerase activity relative to untreated 21NT cells (Figure 4.9C). This suggests that the observed reduction in *hTERT* expression levels was insufficient to lead to telomerase repression within 21NT cells overexpressing *SETD2*. Out of all 21NT-pCMV-*SETD2* stable clones, only clone 1 was found to exhibit substantially lower levels of telomerase relative to untreated 21NT cells (Figure 4.9C). This particular clone was found to display similar levels of *SETD2* and *hTERT* expression relative to untreated 21NT cells, therefore repression of telomerase activity within this clone, does not appear to be linked to *SETD2* expression or deregulation of *hTERT* transcription. It is formally possible that the pCMVNeo-*SETD2* vector may have integrated into an important site responsible for post-transcriptional telomerase regulation.

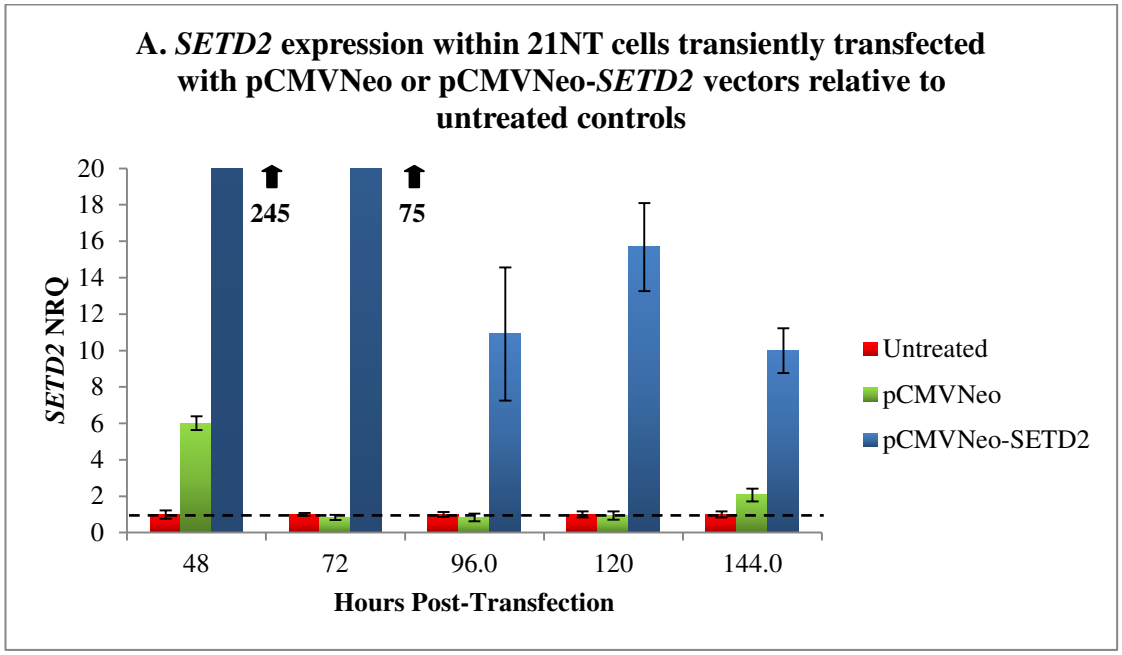
It is important to note that three out of five negative control 21NT-pCMVNeo stable clones were found to exhibit an 80% reduction or a 3-fold increase in *hTERT* expression levels relative to untreated 21NT cells (Figure 4.9B, pCMVNeo clones 3, 4 and 5). This could either suggest that the plasmid vector has integrated into important *hTERT* regulatory regions or, similar to telomerase activity, 21NT cells exhibit a normal degree of clonal variation in *hTERT* expression levels. Therefore, it is unclear whether the observed reduction in *hTERT* expression levels within stable 21NT-pCMVNeo-*SETD2* clones is directly linked to *SETD2* overexpression.

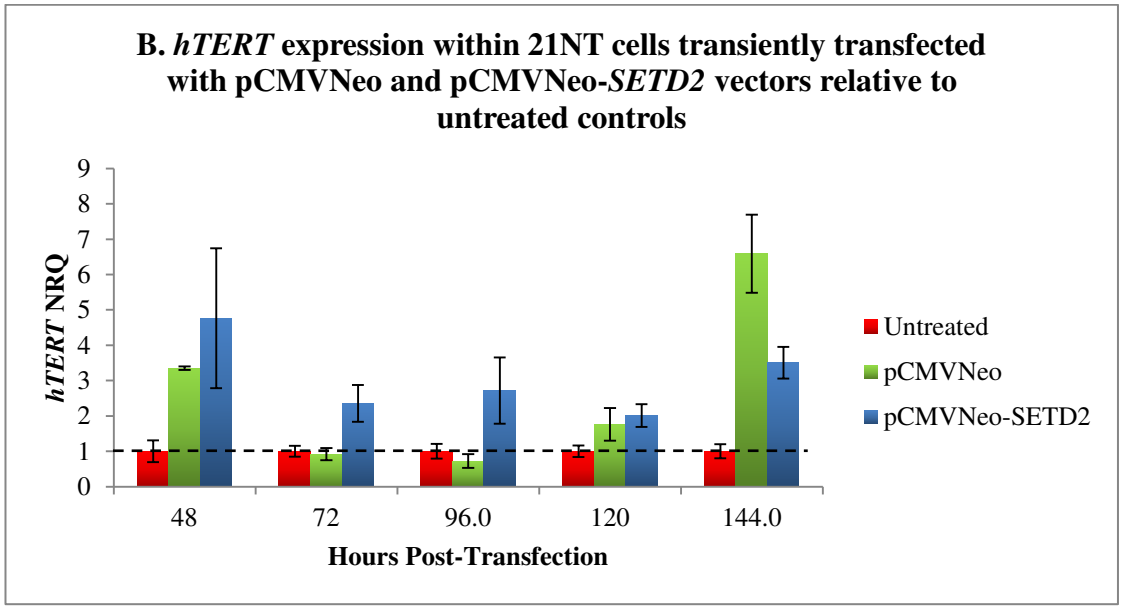
The results presented here do not provide persuasive evidence to show that *SETD2* may function as a negative transcriptional regulator of *hTERT*. It is possible that a greater than 3-fold higher level of *SETD2* expression may be required to influence *hTERT* transcriptional activity. These findings also suggest that stable integration of pCMVNeo plasmid vector constructs influences *hTERT* expression regulation. Therefore, this particular plasmid vector may not be suitable for gene transfer studies to investigate *hTERT* transcriptional regulation.

4.3.2.2 Transient overexpression of *SETD2* within the 21NT cell line

In order to achieve high-level *SETD2* overexpression and explore the possibility that higher levels of *SETD2* expression may influence *hTERT* transcriptional activity, 21NT cells were transiently transfected with the pCMVNeo-*SETD2* plasmid construct. The expression levels of *SETD2* and *hTERT* were determined at 24-hour intervals, over a period of six days following transfection of 21NT cells with control pCMVNeo or pCMVNeo-*SETD2* plasmid constructs. As shown in Figure 4.10A, 21NT cells transfected with pCMVNeo-*SETD2* plasmid constructs maintained a greater than 10-fold higher level of *SETD2* expression relative to untreated cells over the course of six days post-transfection. Unexpectedly, *hTERT* expression levels were found to be consistently higher within 21NT cells transfected with pCMVNeo-*SETD2* plasmids compared with both untreated and pCMVNeo-transfected 21NT cells at all time points, apart from Day 6 (Figure 4.10B). Additionally, a strong positive correlation was observed between *SETD2* and *hTERT* gene expression levels within 21NT cells transiently transfected with pCMVNeo-*SETD2* vector constructs (Figure 4.10C, $r = 0.99$). These results suggest that high levels of *SETD2* expression may actually induce *hTERT* transcription within breast cancer cells. However, *hTERT* expression levels exhibited by 21NT cells transfected with control pCMVNeo plasmids, varied between 73% - 660% of untreated 21NT cells, which may reflect the normal degree of *hTERT* gene expression variation exhibited by 21NT cells. Taking this into account, high levels of *SETD2* expression may either have no effect or a positive effect on *hTERT* transcriptional activity. These findings are consistent with the observed relationship between *SETD2* expression levels and telomerase activity within breast cancer cell lines (Figure

4.8B), but contradict the hypothesis that *SETD2* may function as a transcriptional repressor of *hTERT*.





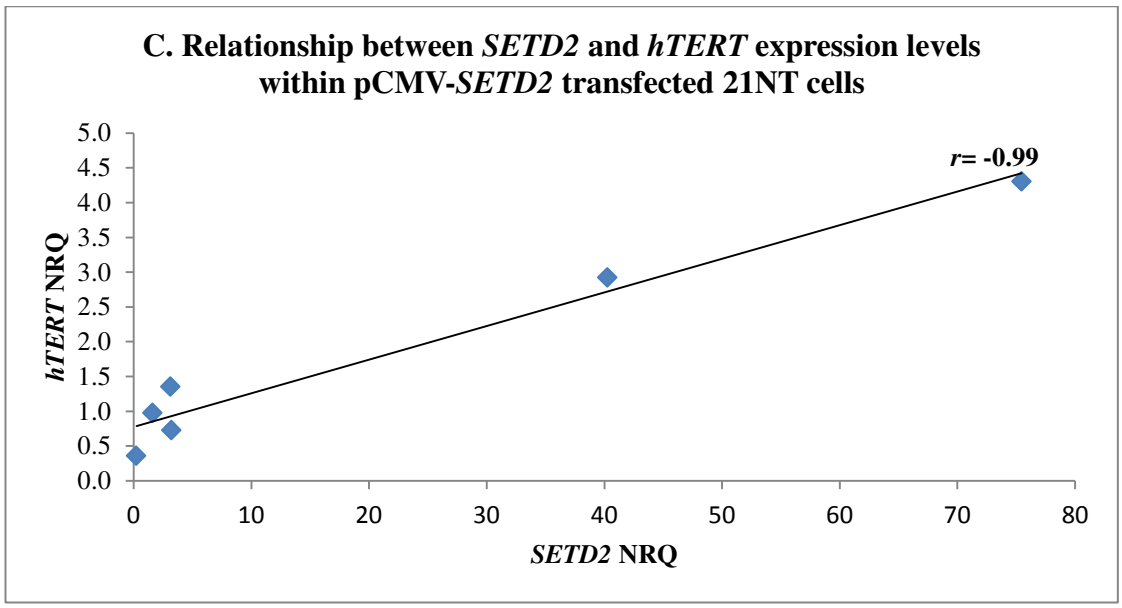


Figure 4.10 - *The normalized relative quantities (NRQ) of* ***(A)*** *SETD2 and* ***(B)*** *hTERT mRNA within 21NT cells 2-5 days following transient transfection of 21NT cells with pCMVNeo and pCMVNeo-SETD2 vector constructs, relative to untreated 21NT cells harvested on the same day. NRQ values represent the mean NRQ values of two samples harvested from each group on each day. Error bars show the standard error of the mean NRQ values for each duplicate sample* ***(C)*** *A scatter graph showing the relationship between SETD2 and hTERT gene expression levels within 21NT cells transiently transfected with the pCMVNeo-SETD2 plasmid. The Pearson Correlation Coefficient (r) was used to measure the strength of the correlation between SETD2 and hTERT expression levels.*

4.3.2.3 The effect of forced *SETD2* expression on 21NT cell growth

The results obtained from transient *SETD2* transfection within the 21NT cell line demonstrate that *SETD2* may function as a *positive* transcriptional regulator of *hTERT*. Due to the important role of *hTERT* de-repression and subsequent telomerase re-activation in cancer development and progression, these results suggest that *SETD2* may actually enhance tumorigenicity. These observations strongly contradict results presented in Figure 4.6A and by Sarakbi *et al.,* (2009) and Newbold and Mokbel (2010), which provided evidence to show that *SETD2* may exhibit a tumour suppressive function.

In order to understand the functional role of *SETD2* in promoting/inhibiting breast cancer cell growth, the effect of stable *SETD2* overexpression on the clonogenic survival of 21NT cells was determined. To do this, the number of stable, neomycin-resistant cell colonies that arose two weeks following transfection of 21NT cells with control pCMVNeo and target gene pCMVNeo-*SETD2* plasmid constructs was compared. As shown in Figure 4.11, the number of stable 21NT-pCMVNeo-*SETD2* colonies was consistently and significantly higher than the number of stable 21NT-pCMVNeo colonies that arose following two weeks of

neomycin selection (p = 3x10^{-4}). These results therefore suggest that *SETD2* may enhance the clonogenic survival of breast cancer cells.

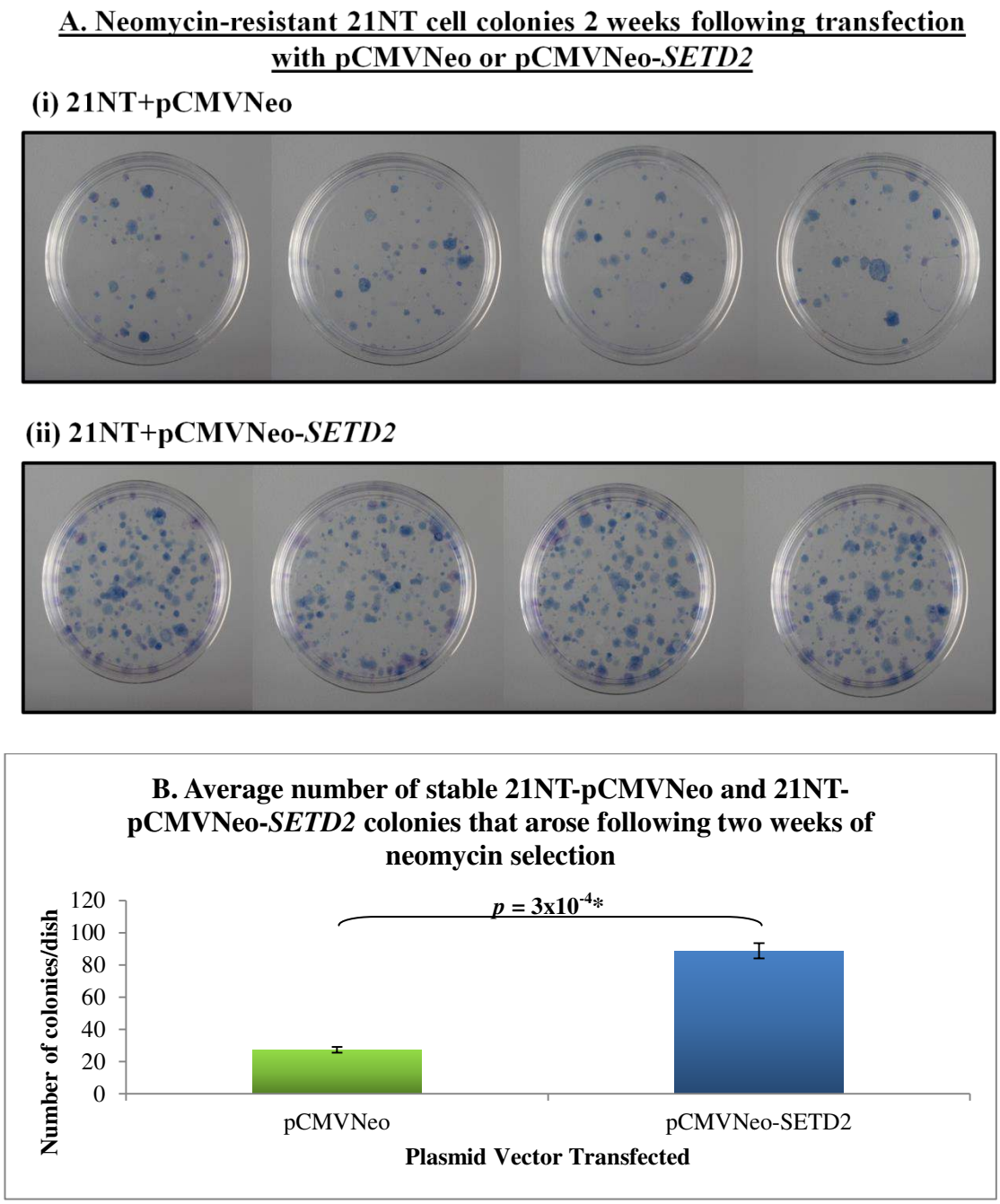


Figure 4.11 *- **(A)** Photographs of eight P60 dishes containing fixed and stained stable **(i)** control 21NT-pCMVNeo and **(ii)** target gene 21NT-pCMVNeo-SETD2 colonies that arose following two weeks of neomycin selection. **(B)** The mean number of 21NT-pCMVneo (green) and 21NT-pCMVNeo-SETD2 (blue) colonies present across four individual dishes for each plasmid construct. Error bars represent the standard deviation of the mean. A student's t-test was used to compare the mean of both groups.*

4.3.3 KNOCKDOWN OF SETD2 EXPRESSION WITHIN A SINGLE 21NT-CHROMOSOME 3 HYBRID CLONE (H3.5)

The findings presented so far, provide evidence to show that forced overexpression of *SETD2* within the 21NT breast cancer cell line may induce *hTERT* expression and promote cell growth. However, the transfection method employed within this study may have induced non-physiological levels of *SETD2* expression. Therefore, the observed phenotype may not reflect the true function of *SETD2 in vitro*.

In order to explore the functional role of *SETD2* in *hTERT* transcriptional regulation further, the effect of RNA-interference (RNAi)-mediated repression of *SETD2* on *hTERT* expression was examined within a single telomerase-repressed 21NT-chromosome 3 hybrid clone (H3.5). This hybrid was generated from a previous MMCT experiment carried out in our laboratory by Mrs. Alison Marriott (Senior Cell Culture Technician), whereby a hygromycin phosphotransferase-thymidine kinase (Hytk) - tagged normal copy of human chromosome 3 was introduced into the 21NT cell line. H3.5 was initially characterized as telomerase-repressed but later underwent a crisis event whereby telomerase was re-activated by a step-change, generating an immortal variant. As a result, both pre-crisis (H3.5E) and post-crisis (H3.5L) forms of this hybrid clone were available for further characterization.

4.3.3.1 Characterization of pre-crisis H3.5E and post-crisis H3.5L hybrid clones

In order to confirm previous observations demonstrating de-repression of *hTERT* and re-activation of telomerase activity following crisis, the expression levels of *hTERT* were examined within both pre-crisis (H3.5E) and post-crisis (H3.5L) forms of this hybrid clone. As shown in Figure 4.12, H3.5E hybrid clones exhibited an 88% reduction in *hTERT* expression levels relative to parental 21NT cells. This observation is consistent with findings by Ducrest *et al.,* (2001), demonstrating that telomerase-repressed 21NT-chromosome 3 hybrids exhibit substantially lower levels of *hTERT* transcript molecules compared with telomerase-positive parental cells. By comparison, H3.5L hybrid cells were found to display 54% higher levels of *hTERT* expression compared to H3.5E cells, indicating that an increase in *hTERT* expression levels had occurred following the crisis event.

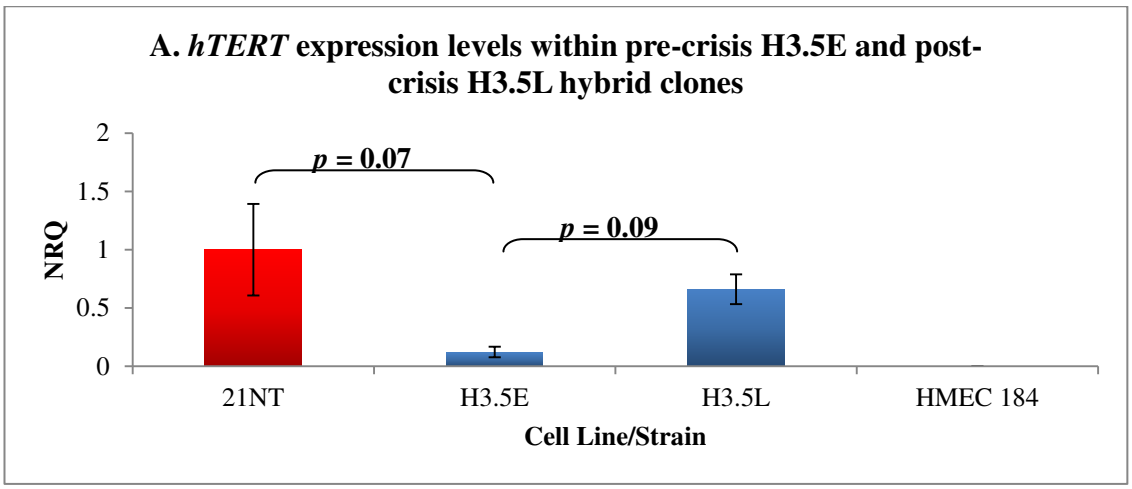


Figure 4.12 - *Normalized relative quantities (NRQ) of hTERT mRNA within pre-crisis H3.5E and post-crisis H3.5L hybrid clones relative to parental 21NT cells. HMEC 184 was included as a negative control. NRQ values represent the mean NRQ of two independent qPCR runs for each sample and error bars represent the standard error of the mean. A student's t-test was used to compare mean NRQ values of samples.*

As previously demonstrated by Cuthbert *et al.,* (1999), allelotype analysis of chromosome 3 within telomerase-positive segregant 21NT-chromosome 3 hybrid clones, revealed two commonly deleted regions at 3p21.3-p22 and 3p12-21.1. These results provided evidence of a strong link between chromosome 3p deletions and the presence of active telomerase within 21NT-chromosome 3 hybrid clones. Taking these observations into account, the structure of chromosome 3 within pre-crisis H3.5E and post-crisis H3.5L hybrid clones was examined by fluorescence *in situ* hybridization (FISH).

Chromosome 3 arm-specific Fluorescence *in situ* Hybridization (FISH) Analysis of HMEC 184, 21NT and H3.5E and H3.5L Hybrid Clones

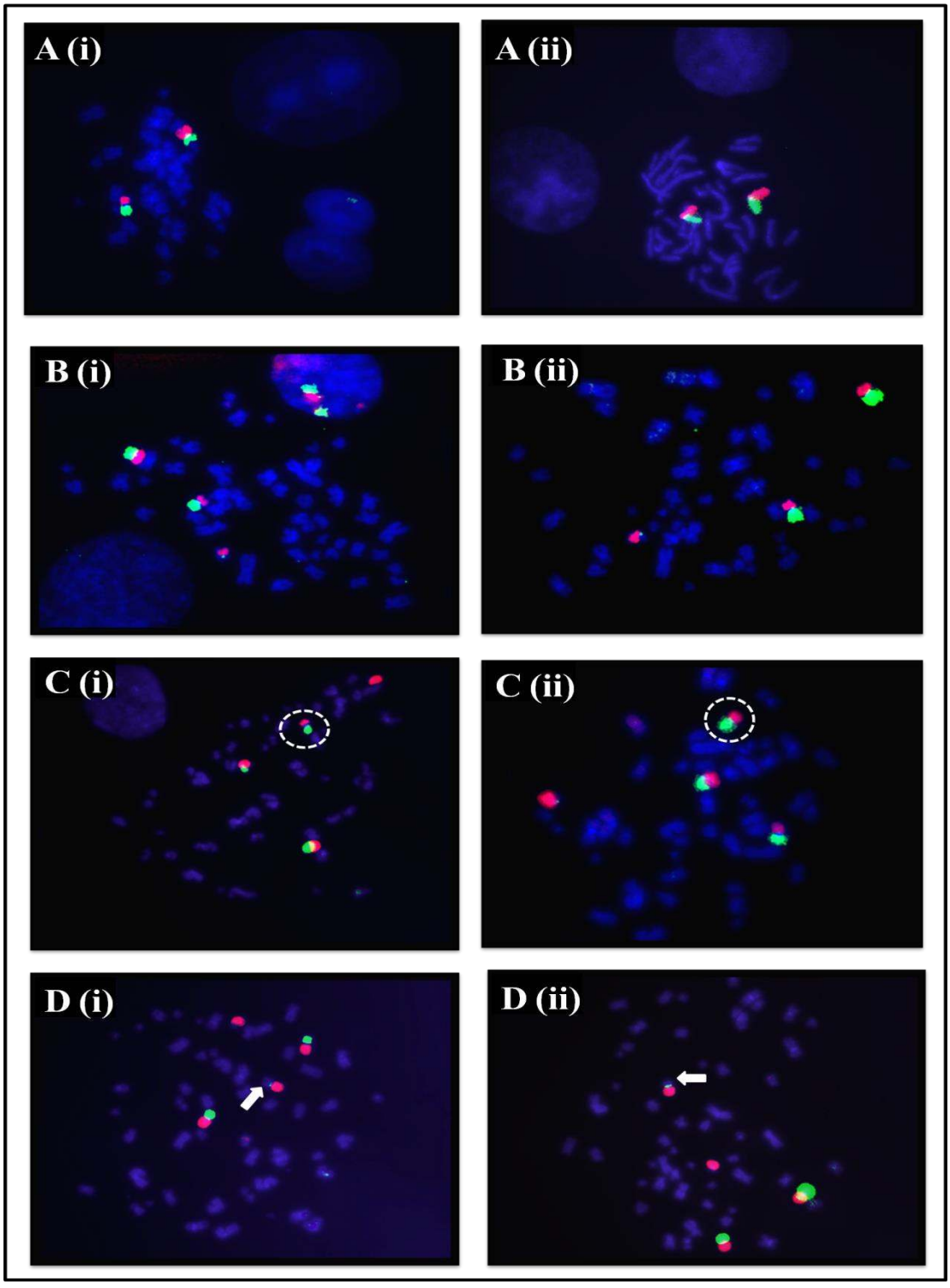
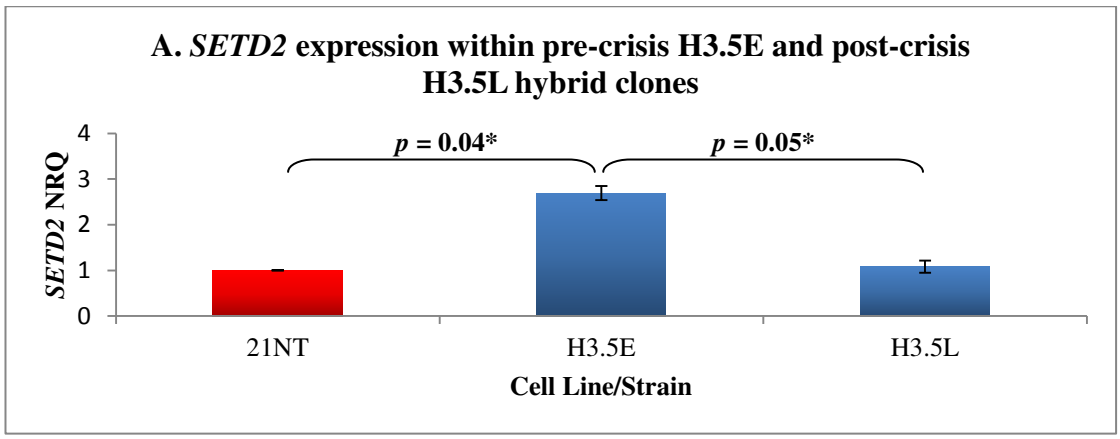


Figure 4.13 - *Representative fluorescence images (x100 magnification) of* ***(A. i, ii)*** *HMEC 184,* ***(B. i, ii)*** *21NT,* ***(C. i, ii)*** *H3.5E and* ***(D. i, ii)*** *H3.5L cell lines/strain metaphase spreads hybridized with chromosome 3 p-arm (green) and q-arm (red)-specific probes and counterstained with DAPI (blue). White circles indicate an extra chromosome 3 copy and white arrows indicate a consistent deletion of chromosome 3p observed within H3.5L hybrid metaphase spreads.*

HMEC 184, 21NT, H3.5E and H3.5L metaphase chromosomes were hybridized with fluorescently labelled chromosome 3 p-arm (green) and q-arm (red) specific probes. A minimum of five metaphase spreads were analysed for each cell line/strain. Representative images shown in Figure 4.13A indicate the presence of two intact copies of human chromosome 3 within all HMEC 184 cell strain metaphase spreads. This provides a reference for normal chromosome 3 structure and confirms the specificity of the chromosome 3 painting probe. Representative images of 21NT metaphase chromosomes demonstrate the presence of two whole chromosome 3 copies and a chromosome 3q translocation, indicated by an additional q-arm signal within another chromosome (Figure 4.13B). This was observed within five individual metaphase spreads. Similarly, FISH analysis of chromosome 3 structure within 21NT cells carried out by Cuthbert *et al.,* (1999), also demonstrated the presence of two submetacentric chromosome 3 copies, a single translocation and small regions of chromosome 3 material within two other chromosomes. Karyotypic analysis of 21NT cells within the study by Band *et al.,* (1990), showed gross chromosomal rearrangements and aneuploidy. Together these results demonstrate that 21NT cells exhibit a high level of genetic instability, including structural changes in chromosome 3.

The presence of an additional whole chromosome 3 copy was observed within four out of seven pre-crisis H3.5E hybrid clone metaphase spreads, which confirms the existence of a single intact exogenous chromosome 3 copy introduced by MMCT within the majority of hybrids (Figure 4.13C). In contrast, all five post-crisis H3.5L metaphase chromosome spreads revealed a consistent deletion of the short-arm within a single copy of chromosome 3 (Figure 4.13D). The remaining chromosome 3q fragment appears to have fused with endogenous chromosome material, demonstrated by the presence of DAPI signal at one end of the fragment. The observed difference in chromosome 3 structure between H3.5E and H3.5L hybrid clones, provides additional evidence of a functional link between chromosome 3 and *hTERT* gene regulation. These results also suggest that the *hTERT* regulatory sequence may be located on the short-arm of chromosome 3, which is consistent with findings by Cuthbert *et al.,* (1999), Ducrest *et al.,* (2001) and Szutorisz *et al.,* (2003).

The presence of *SETD2* within a chimeric chromosome 3 fragment, previously found to cause telomerase repression within the 21NT cell line, provided some evidence to show that this gene may function as a regulator of telomerase. The results presented here and by Cuthbert *et al.,* (1999), demonstrate an important link between chromosome 3p loss, increased *hTERT* expression levels and telomerase re-activation. Therefore, in order to explore the possibility that the observed chromosome 3p loss and increase in *hTERT* expression levels within the post-crisis H3.5L hybrid also involved loss of *SETD2* gene function, the expression levels and gene copy number were examined within pre-crisis H3.5E and post-crisis H3.5L hybrid clones.



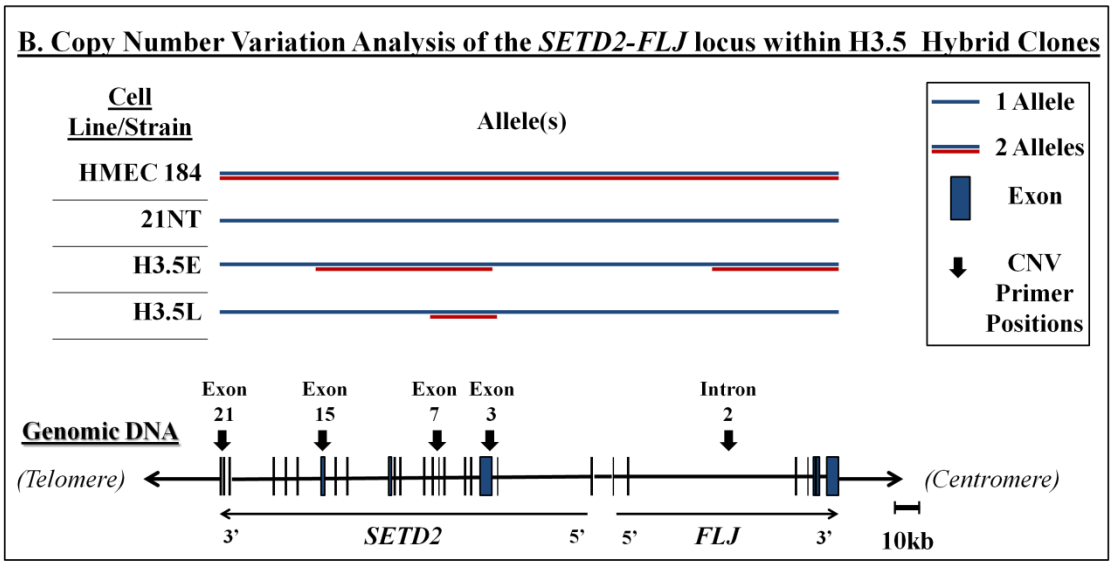


Figure 4.14 - (A) *Normalized relative quantities (NRQ) of SETD2 mRNA within pre-crisis H3.5E and post-crisis H3.5L hybrid clones relative to parental 21NT cells. NRQ values represent the mean of two independent qPCR runs for each sample and error bars represent the standard error of the mean. A student's t-test was used to compare log-transformed mean NRQ values of all samples.* ***(B)*** *Copy number variation analysis of the SETD2-FLJ genomic locus within HMEC 184, parental 21NT cells, H3.5E and H3.5L hybrid clones. The number of alleles at five genomic loci mapping to exonic and intronic regions of SETD2 and FLJ was determined as indicated above.*

As shown in Figure 4.14A, a significant increase in *SETD2* expression levels was observed within H3.5E hybrid clones compared with 21NT cells, which suggests that the introduction of a normal copy of chromosome 3 is associated with increased *SETD2* expression levels. A significant reduction in *SETD2* expression levels was observed within H3.5L hybrids compared with H3.5E hybrids, which suggests that the observed post-crisis chromosome 3p loss may have also involved loss of *SETD2*. In support of this, three out of five loci within the *SETD2-FLJ* genomic locus were present at two copies within the H3.5E hybrid, compared with only one out of five within the H3.5L hybrid, which indicates loss of chromosome 3p arm

material at the *SETD2-FLJ* genomic locus after the H3.5 clone entered crisis (Figure 4.14B). These results suggest that introduction of an intact copy of human chromosome 3 within the 21NT cell line was associated with an increase in *SETD2* expression and a reduction in *hTERT* expression levels. Following a crisis event, this hybrid clone underwent chromosome 3p loss involving the *SETD2-FLJ* genomic locus, which was associated with reduced levels of *SETD2* expression and increased *hTERT* expression. Overall, these findings provide evidence of a functional link between *SETD2* and *hTERT* gene regulation. However, taking into account the effects of stable *SETD2* overexpression on *hTERT* transcription levels and 21NT cell clonogenic survival described previously, it is possible that another chromosome 3p-encoded sequence may have been responsible for the observed telomerase repression within the H3.5E hybrid clone.

4.3.3.2 Knockdown of *SETD2* within the H3.5E hybrid clone

In order to explore further the putative functional link between *SETD2* and transcriptional regulation of *hTERT,* the expression levels of both genes were examined following RNA interference (RNAi)-mediated inhibition of *SETD2* expression within pre-crisis H3.5E hybrid clones. Hybrids were transfected with a mixture of *SETD2*-siRNA construct molecules, that target four distinct untranslated regions (UTRs) within the *SETD2* transcript sequence. As a negative control, H3.5E cells were also transfected with a non-targeting (NT) mixture of siRNA molecules. Treated H3.5E cells were harvested for gene expression analysis at 24-hour intervals, 48-96 hours post-transfection with both constructs. As shown in Figure 4.15A, a gradual reduction in *SETD2* expression levels from 37% to 8% relative to untreated H3.5E hybrids, were observed 48 and 96 hours post-transfection with *SETD2* siRNA molecules respectively. Unexpectedly, H3.5E hybrids transfected with NT-siRNA were found to exhibit a 54% and 80% reduction in *SETD2* expression levels relative to untreated cells, 72 and 96 hours post-transfection respectively (Figure 4.15A). This could suggest that the NT-siRNA constructs either directly or indirectly repressed the expression of *SETD2*, and therefore do not provide a reliable negative control. H3.5E hybrids transfected with *SETD2*-siRNA constructs were found to exhibit a similar level of *hTERT* expression relative to untreated cells, indicating that knockdown of *SETD2* expression is not associated with an observable change in *hTERT* transcriptional activity (Figure 4.15B). These results failed to provide evidence supporting the notion that *SETD2* may function as a transcriptional repressor of *hTERT* in breast cancer.

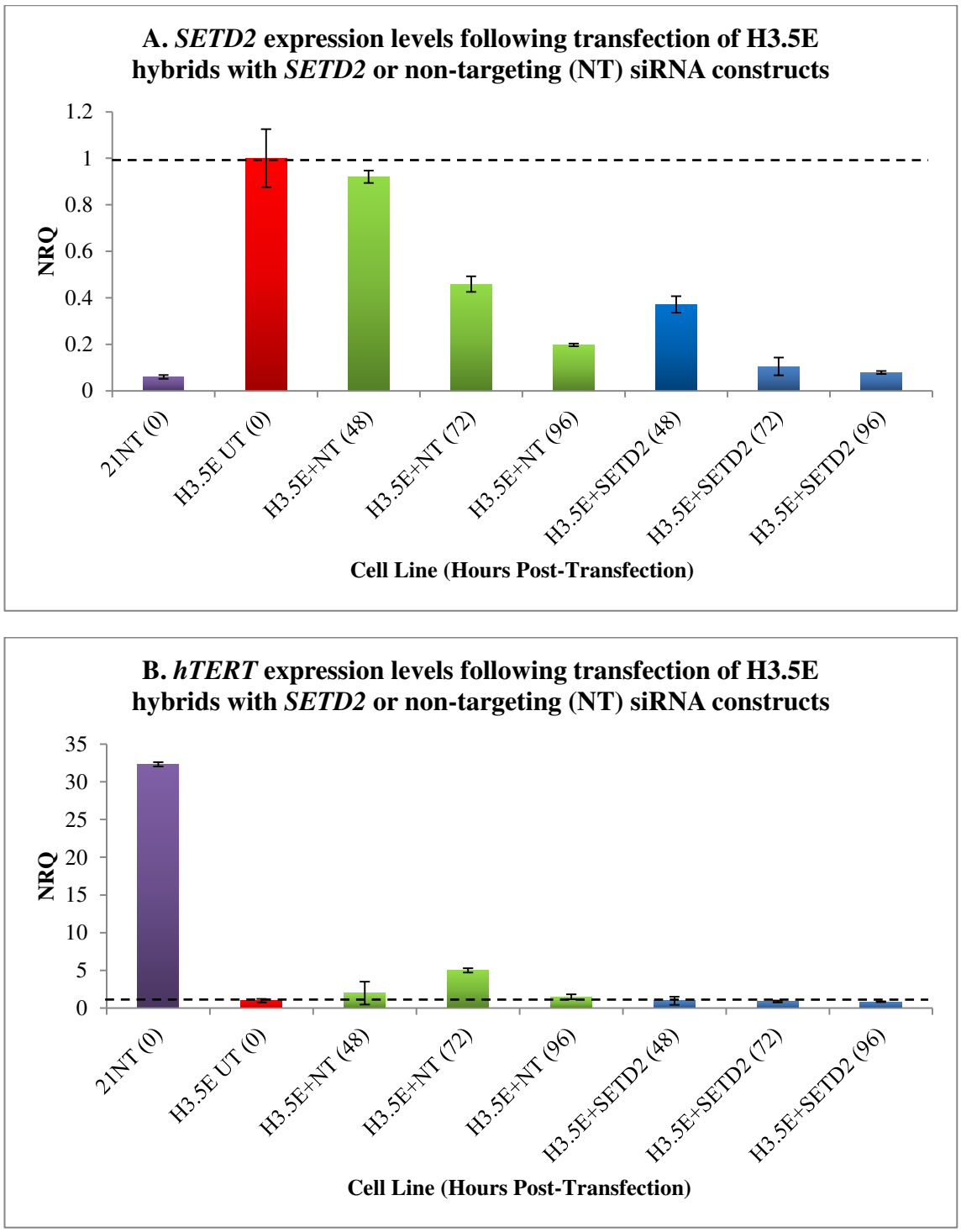


Figure 4.15 - *Normalized relative quantities of* ***(A)*** *SETD2 and* ***(B)*** *hTERT mRNA 48-96 hours post transfection of H3.5E hybrid clones with non-targeting (NT, green) or a mixture of four SETD2-siRNA construct molecules (blue), relative to untreated (UT) H3.5E hybrids (red) harvested on the day of transfection. Error bars represent the standard error of triplicate repeats for each sample.*

4.3.4 Analysis of a Two Stable 21NT-FLJ Transfection Clones

The results presented so far provide no evidence to show that *SETD2* may function as a *hTERT* transcriptional repressor. Therefore, the functional role of *FLJ* as a candidate repressor of *hTERT* was explored further. As demonstrated previously, breast cancer cell lines have been found to exhibit significantly lower levels of *FLJ* expression when compared with normal HMEC cell strains. Additionally, the majority of breast cancer cell lines employed during this study were found to have undergone allele loss that included the *FLJ* genomic locus. Similarly to *SETD2*, *FLJ* allele loss was observed within post-crisis H3.5L hybrids compared with pre-crisis H3.5E hybrid clones (Figure 4.14B). As shown in Figure 4.16, H3.5E hybrid clones were found to display a significant increase in *FLJ* expression levels relative to untreated 21NT cells. In addition *FLJ* expression levels were found to be substantially reduced within H3.5L hybrids compared with H3.5E hybrids (Figure 4.16). These results indicate that chromosome 3p loss within post-crisis H3.5L hybrids may also be associated with loss of *FLJ* function.

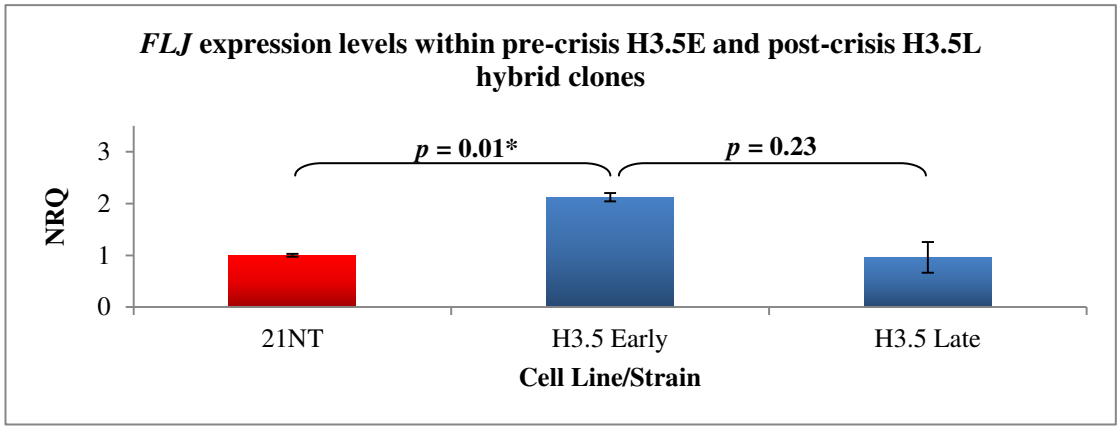


Figure 4.16 - *Normalized relative quantities (NRQ) of FLJ mRNA within pre-crisis H3.5E and post-crisis H3.5L hybrid clones relative to parental 21NT cells. NRQ values represent the mean of two independent qPCR runs for each sample and error bars represent the standard error of the mean. A student's t-test was used to compare log-transformed mean NRQ values of all samples.*

In order to explore a causal link between *FLJ* and *hTERT* gene regulation, the effect of forced *FLJ* overexpression on *hTERT* transcriptional activity within the 21NT cell was examined. After cloning the human *FLJ* open reading frame (ORF) into the pCMVNeo plasmid vector, my colleague Dr. T. Roberts transfected target pCMVNeo-*FLJ* and empty vector control pCMVNeo constructs into the 21NT cell line and cultured cells in the presence of neomycin to select for cells that had undergone stable integration of plasmid constructs. Individual 21NT-pCMVNeo and 21NT-pCMVNeo-*FLJ* clones were isolated and propagated as individual cell lines. Aliquots of two 21NT-pCMVNeo and two 21NT-pCMVNeo-*FLJ* stable clones were provided to me for downstream analysis.

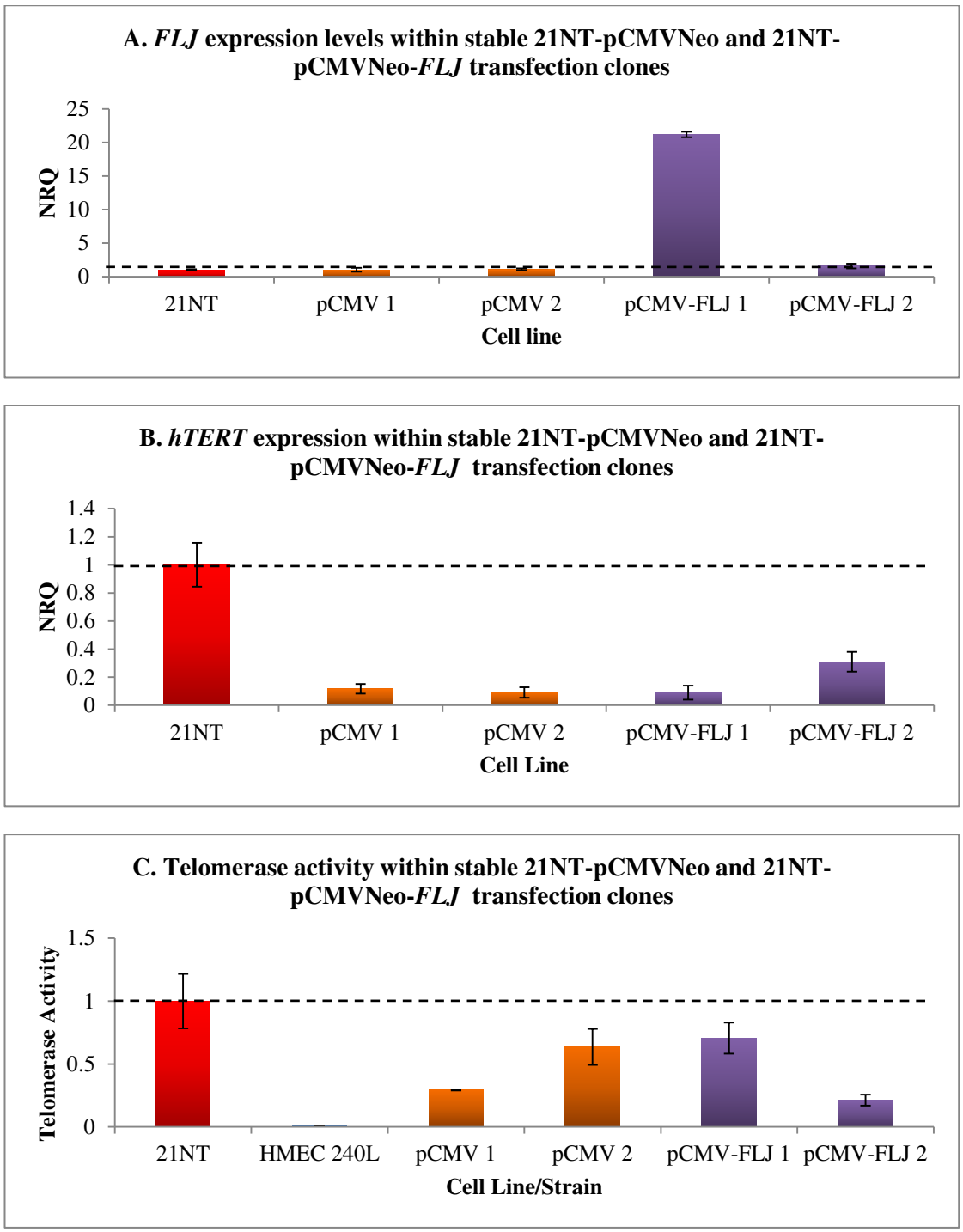


Figure 4.17 - *Normalized relative quantities of* ***(A)*** *FLJ and* ***(B)*** *hTERT mRNA within two stable 21NT-pCMVNeo (orange) and two stable 21NT-pCMVNeo-FLJ (purple) clones relative to untreated 21NT cells. Error bars represent the standard error of the mean NRQ values from triplicate repeats for each sample.* ***(C)*** *Telomerase activity within all stable clones relative to untreated 21NT cells. The HMEC 240L sample was included as a negative control. Error bars represent the standard deviation of triplicate repeats for each sample.*

As shown in Figure 4.17A, stable 21NT-pCMVNeo-*FLJ* clones 1 and 2 were found to exhibit 1.6-fold and 21-fold higher *FLJ* expression levels relative to untreated 21NT cells respectively. Both 21NT-pCMVNeo-*FLJ* stable clones were found to display a 90% and 70% reduction in *hTERT* expression levels, which may indicate that *FLJ* could function as a transcriptional repressor of *hTERT*. In further support of this, 21NT-pCMVNeo-*FLJ* clones demonstrated a reduction in telomerase activity, outside of the normal range of clonal variation exhibited by 21NT cells (Chapter 3.3.1, Figure 3.3C). However, despite observing no change in *FLJ* expression levels within stable empty vector control 21NT-pCMVNeo clones 1 and 2, *hTERT* expression levels were found to be reduced by around 90% (Figure 4.17B). Similarly, both control 21NT-pCMVNeo clones displayed a similar reduction in telomerase activity as stable 21NT-pCMVNeo-*FLJ* clones, relative to untreated 21NT cells (Figure 4.17C), which indicates that the pCMVNeo vector construct alone influences *hTERT* expression. As previously shown in Figure 4.9C, two out of five stable empty vector control 21NT-pCMVNeo clones isolated during an investigation of *SETD2* in *hTERT* transcriptional regulation, exhibited 80% lower *hTERT* expression levels relative to untreated 21NT cells. One stable 21NT-pCMVNeo clone also displayed 60% lower levels of telomerase activity compared with untreated 21NT cells (Figure 4.9C). These results demonstrate that the (empty) pCMVNeo vector consistently influences *hTERT* expression within the 21NT cell line. Therefore, further investigation is necessary to determine whether the observed reduction in *hTERT* expression levels and telomerase activity within 21NT cells, is linked to increased *FLJ* expression levels. However, it should be noted that neither 21NT-pCMVNeo or 21NT-pCMVNeo-*FLJ* stable clones exhibited a greater than 90% reduction in telomerase activity, which has been previously associated with growth arrest of 21NT-chromosome 3 hybrid clones in the study by Cuthbert *et al.,* (1999). Therefore, it is likely that *FLJ* may not function as a transcriptional regulator of *hTERT*.

4.4 SUMMARY

Telomerase repression within 21NT breast cancer cells containing an exogenous intact copy of normal chromosome 3, was found to be due to transcriptional repression of the *hTERT* gene (Ducrest *et al.,* 2001). The observed change in chromatin structure around intron 2 of the *hTERT* gene to a 'closed' state within 21NT-chromosome 3 hybrids, provided an important insight into the mechanism by which chromosome 3 may mediate *hTERT* transcriptional repression within breast cancer cells (Szutorisz *et al.,* 2003). Subsequent studies that aimed to narrow the search for the chromosome 3-encoded *hTERT* repressor sequence, identified a 490kb region mapping to the 3p21.3 region, encompassing two putative candidate genes; a known histone methyltransferase enzyme (*SETD2)* and an uncharacterized long non-coding (lnc) RNA sequence (*FLJ).*

Consistent with findings presented by Sarakbi *et al.,* (2009) and Newbold and Mokbel (2010), the results from my study demonstrate a putative tumour suppressor role of *SETD2* in breast cancer. *SETD2* gene expression levels were found to be significantly reduced within a panel of nine breast cancer cell lines relative to normal human mammary epithelial cell (HMEC) strains. By comparison, a greater proportion of breast cancer cell lines were found to exhibit lower levels of *FLJ* expression than *SETD2*, which provides indirect evidence of a putative novel lncRNA tumour suppressor gene in breast cancer. Similar levels of *SETD2* and *FLJ* expression were observed within both primary tumour-derived and metastatic deposit-derived breast cancer cell lines, indicating that a reduction in both candidate gene expression levels occurs at an early point during disease pathogenesis and prior to metastatic spread. Interestingly, despite observing allele loss at the *SETD2-FLJ* genomic locus within over half of breast cancer cell lines examined, those harbouring two alleles at this locus also exhibited substantially lower levels of *SETD2* and *FLJ* expression levels. Additionally, breast cancer cell lines with three alleles at this locus were also found to exhibit low *FLJ* expression levels. Therefore, copy number variation at the *SETD2-FLJ* locus does not account for all transcriptional deregulation of *SETD2* and *FLJ* within breast cancer cells.

Independent sequencing studies and cohort analyses have identified *SETD2* mutations within multiple cancer types, such as acute MLL-rearranged leukaemia (22%), high grade glioma (HGG, 15%), acute lymphoblastic leukaemia (ALL, 12%) and clear cell renal cell carcinoma (ccRCC, 11.6%) (Zhu *et al.,* 2014, Fontebasso *et al.,* 2013, Mar *et al.,* 2014, Hakimi *et al.,* 2013, respectively). Interestingly, *SETD2* mutations have been identified within a panel of ccRCC cell lines that had undergone copy number loss within the 3p21 locus (Duns *et al.,* 2010). These mutations were found to disrupt SETD2 protein function either through introduction of premature stop codons, aberrant splicing or through single missense mutations within specific protein domains. A recent screen of somatic cancer mutations in protein lysine

methyltransferase (PKMT) enzymes revealed that there are 339 mutations within the *SETD2* gene; a large proportion of these are frame-shift and nonsense mutations that are predicted to confer loss of protein function (Kudithipudi and Jeltsch., 2014). According to current mutation data within the Catalogue of Somatic Mutations in Cancer (COSMIC) database, 1.72% of breast tumour samples harbour *SETD2* mutations, all of which are derived from patients with invasive breast carcinoma (http://www.sanger.ac.uk/cosmic, Forbes *et al.,* 2015). According to the same database, *SETD2* mutations were not identified during a mutation screen of H5S78T, HCC1143, BT20 and BT474 breast cancer cell lines. The BT20 and H5S78T cell lines were found to harbour 2 and 3 alleles at the *SETD2* genomic locus within my study respectively. These observations together with the low frequency of *SETD2* mutations in breast cancer could suggest that other mechanisms of *SETD2* loss of function may exist within breast cancer cells.

In support of the hypothesis that *SETD2* may function as a regulator of *hTERT,* a single *hTERT*-repressed 21NT-chromosome 3 hybrid clone (H3.5E) was found to exhibit significantly higher levels of *SETD2* expression than parental 21NT cells. In addition, two alleles were observed at multiple loci within the *SETD2-FLJ* genomic region of H3.5E hybrid cells, indicating that the exogenous copy of chromosome 3 was not only associated with *hTERT* repression but also with increased levels of *SETD2* expression. This particular hybrid was reported to have entered crisis and a telomerase-positive hybrid cell colony was found to have emerged. Further characterization of the pre-crisis and post-crisis forms of this hybrid within my study, revealed an increase in *hTERT* expression levels and cytogenetic analysis demonstrated consistent loss of chromosome 3p material within a single copy of chromosome 3. These results are in agreement with findings presented by Cuthbert *et al.,* (1999), Ducrest *et al.,* (2001) and Szutorisz *et al.,* (2003) demonstrating that a transcriptional repressor of *hTERT* is present on the short arm of chromosome 3. Copy number variation analysis demonstrated allele loss at the *SETD2-FLJ* genomic locus and expression levels of *SETD2* were significantly reduced after the hybrid clone entered crisis. This provided further evidence that *SETD2* may function as a *hTERT* repressor sequence.

Stable integration of pCMVNeo-*SETD2* plasmid constructs into the 21NT cell line produced two stable clones overexpressing *SETD2* to a similar level observed within *hTERT*-repressed pre-crisis H3.5 clones. Despite observing a substantial reduction in *hTERT* expression levels within one clone, telomerase activity was not found to be repressed within either stable clone. In fact, one clone was found to display almost 4-fold higher levels of telomerase activity. Similarly, transient high-level overexpression of *SETD2* within 21NT cells, was consistently associated with increased levels of *hTERT* expression. In addition, siRNA-mediated knockdown of *SETD2* expression within pre-crisis H3.5E hybrid cells, was not associated with any observable change in *hTERT* expression levels. The results presented within my study provide evidence to show that *SETD2* does not function as a negative regulator of *hTERT* transcription

and is therefore unlikely to be responsible for telomerase repression in 3p monochromosomal hybrids.

In 2003, Smith *et al.,* provided evidence to show that the telomerase enzyme may possess telomere-stabilization independent effects on promoting cell growth. An increase in cellular proliferation was observed within post-stasis HMEC strains ectopically expressing *hTERT*, but not normal HMEC controls, when both were cultured in a low-mitogen environment. The reduced dependency on extracellular growth factors, was thought to be due to the observed telomerase-dependent upregulation in the expression of genes involved in mitogenic signalling pathways, such as the epidermal growth factor receptor (*EGFR*). Apart from facilitating cellular immortality, telomerase may play additional roles in tumorigenesis. In my study, transient high-level *SETD2* expression within 21NT cells was associated with increased *hTERT* expression levels. Similarly, a positive correlation between *SETD2* and telomerase activity was observed within breast cancer cell lines. Interestingly, the number of stable 21NT-pCMVNeo-*SETD2* clones that emerged following two weeks of neomycin selection was significantly higher than the number of stable empty vector control 21NT-pCMVNeo stable clones that emerged. This suggests that overexpression of *SETD2* may promote the growth of 21NT breast cancer cells. Taking into account the results presented by Smith *et al.,* (2003) and the observed relationship between *SETD2* and *hTERT* expression, it is possible that the observed increase in 21NT cell proliferation is due to increased *hTERT* expression levels. However, further investigation into a direct causal link between *SETD2* and *hTERT* expression levels and cell growth is required.

Transcriptome profiling of a panel of 42 primary ccRCC tumour samples, revealed an association between *SETD2* mutations and genome-wide aberrant RNA splicing defects affecting around 25% of all actively transcribed genes (Simon *et al.,* 2014). This was found to be due to changes in chromatin accessibility and nucleosome positioning, which was thought to lead to the formation of alternative transcription start sites (TSS) and intron retention. A large proportion of deregulated transcripts affected known tumour suppressor genes and those involved in the DNA damage response (DDR) pathway. This demonstrates the important function that *SETD2* plays in maintaining transcriptome stability within human cells, and provides an understanding of how loss of *SETD2* function may promote tumorigenesis. It has recently been shown that knockdown of *SETD2* within U2OS osteosarcoma cells, reduced histone H3 lysine 36 (H3K36) trimethylation marks and decreased the clonogenic survival of cells following treatment with DNA damage-inducing agents, compared with non-targeting controls (Pfister *et al.,* 2014). The H3K36 trimethylation activity of *SETD2* was found to be required for the recruitment of C-terminal binding protein interacting protein (CtlP) to sites of DNA double strand breaks (DSB) in order to facilitate repair and promote genomic stability in human cells. Expression of wild-type *SETD2* through stable integration of doxycycline-

inducible Tet operator-*SETD2* constructs into U2OS cells, was found to increase H3K36 trimethylation levels and rescue homologous recombination (HR) efficiency following knockdown of endogenous *SETD2*. In addition, overexpression of *SETD2* to around 100-fold higher levels within MCF7 breast cancer cells, was found to increase the expression levels of *p53* and specific downstream *p53* target genes involved in apoptosis and cell cycle control (Xie *et al.,* 2008). These studies demonstrate an important function of *SETD2* in maintaining genomic stability through activation of DNA damage response pathways and DSB repair. However, the effect of reconstituting wild-type *SETD2* expression on cancer cell growth was not reported within the Pfister *et al.,* (2014) and Xie *et al.,* (2008) studies. *SETD2* gene expression analysis within my study demonstrated that both primary tumour and metastatic deposit-derived breast cancer cell lines exhibit low levels of expression, indicating that a reduction in *SETD2* function may occur early in disease pathogenesis. Based on the function of *SETD2* to maintain genomic stability, loss of *SETD2* function within pre-neoplastic cells, may accelerate the acquisition of somatic cancer mutations that drive tumorigenesis. This has been observed within hereditary non-polyposis coli (HNPCC), where inactivating mutations in DNA mismatch repair (MMR) genes *hMLH1* or *hMSH2,* results in the accumulation of mutations in tumour suppressor and oncogenes leading to tumorigenesis (Lengauer *et al.,* 1998).

The results of my study described in this chapter contrast with findings by Sarakbi *et al.,* (2009) and Newbold and Mokbel (2010), demonstrating a putative tumour suppressor role of *SETD2* in breast cancer. Sarakbi *et al.,* (2009) compared *SETD2* expression levels within breast tumour samples derived from patients with different breast cancer subtypes, tumour grades, clinical outcomes and clinicopathological features such as ER status, with normal breast tissue samples. Overall, malignant breast tumour samples were found to exhibit significantly lower levels of *SETD2* mRNA than normal breast tumour samples. In addition, breast tumour samples derived from patients who developed local recurrence, metastases or died of breast cancer were found to exhibit significantly lower *SETD2* expression levels than patients who were free of disease for greater than 10 years, which supports the role of *SETD2* as a putative tumour suppressor gene. In contrast, my results are the first to demonstrate an association between increased *SETD2* expression levels and an increase in 21NT breast cancer cell growth. The 21NT cell line is derived from a nuclear grade 3, primary IDC breast tumour from a 36-year old female, which had spread to three lymph nodes and the lungs (Band *et al.,* 1990). This cell line has been shown to harbour a frame-shift mutation in *p53,* is *HER2*-positive and is negative for both ER and PgR receptors (Band *et al.,* 1990, Liu *et al.,* 1994). Using the Kaplan-Meier Plotter (KPM) program, which assesses the effect of 54,675 genes on the survival rates of over 4,000 breast cancer patients, the effect of *SETD2* expression on relapse-free survival (RFS) for breast cancer patients was examined (Gyorffy *et al.,* 2010). Consistent with the findings presented by Sarakbi *et al.,* (2009), breast cancer patients with low levels of *SETD2* expression

show a significant reduction in RFS compared with patients with high *SETD2* expression levels (Figure 4.18A). In complete contrast, high *SETD2* expression levels within breast tumours that display similar characteristics to the 21NT cell line including, ER-negative and *p53*-mutant or ER-negative, PgR-negative and HER2-positive breast tumours, are associated with a significantly reduced RFS (Figure 4.18B and C). Therefore, increased *SETD2* expression levels within a subset of breast tumours with certain molecular features is associated with reduced patient survival. The results presented within my study provide evidence to show that increased *SETD2* expression within 21NT cells is associated with increased clonogenic survival and *hTERT* expression, which may promote tumour cell growth and survival.

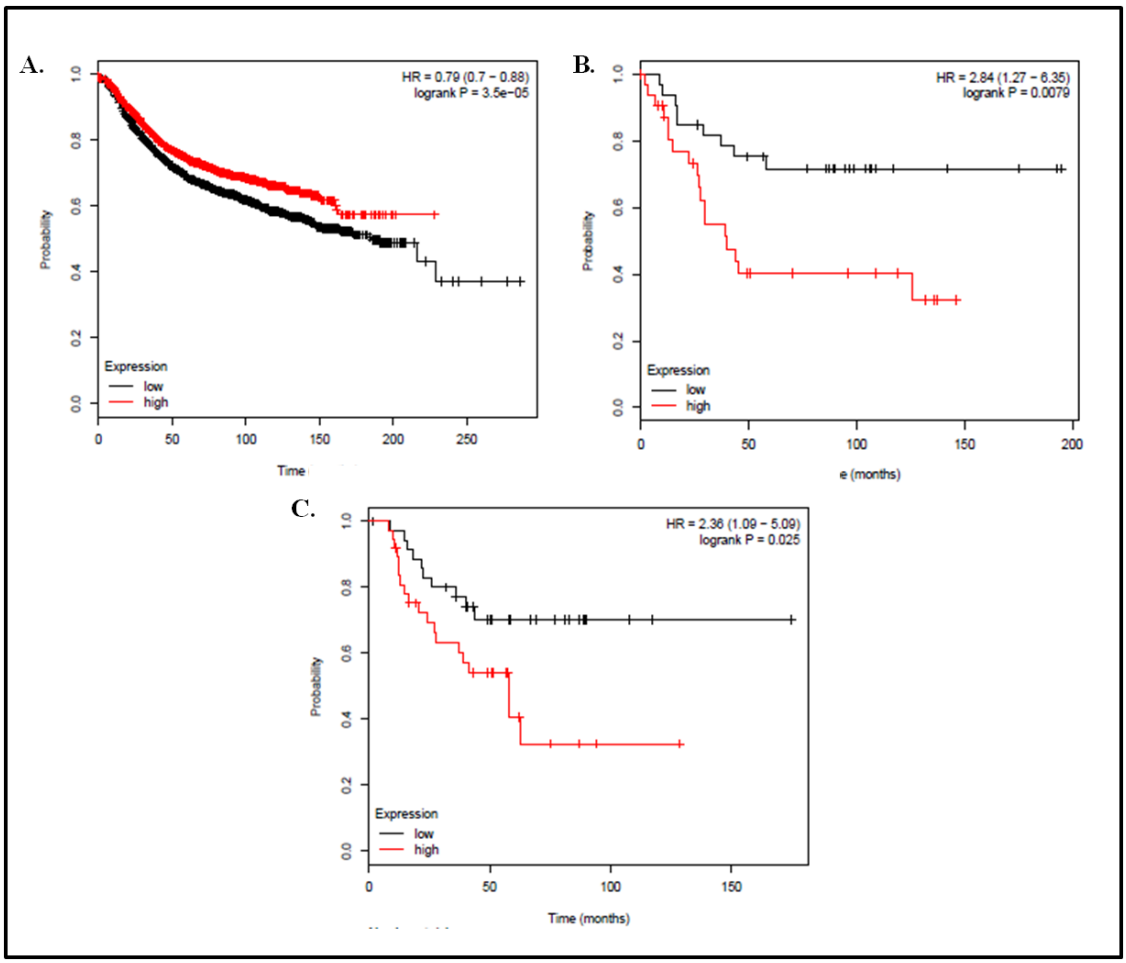


Figure 4.18 - *Kaplan-Meier relapse-free survival (RFS) analysis depending on SETD2 expression of* ***(A)*** *all breast cancer subtypes,* ***(B)*** *ER-negative breast cancers with p53 mutations and* ***(C)*** *ER-negative, PgR-negative and HER2-positive breast cancer patients. Data and plots obtained from Kaplan-Meier Plotter (Gyorffy et al., 2010, http://kmplot.com/analysis/index.php?p=background).*

Based on the observed effect of *SETD2* on *hTERT* transcriptional regulation, the putative function of *FLJ* to mediate transcriptional repression of *hTERT* was explored further. A significant increase in *FLJ* expression levels were observed within the *hTERT*-repressed pre-crisis H3.5E hybrid clone. Following the crisis event, this hybrid displayed substantially lower levels of *FLJ* expression and had lost one allele at the *FLJ* genomic locus. These findings supported the hypothesis that *FLJ* may play a functional role in *hTERT* repression. Interestingly, stable overexpression of *FLJ* within parental 21NT cells was associated with a greater than 70% reduction in *hTERT* expression levels relative to untreated 21NT cells. However, a stable 21-fold overexpression level of *FLJ* was only associated with a 30% reduction in telomerase activity compared with untreated controls. This suggests that *FLJ* may not have been responsible for the >90% reduction in telomerase activity observed within 21NT-chromosome 3 hybrids generated in the study by Cuthbert *et al.,* (1999). Importantly, stable 21NT-pCMVNeo empty vector control clones were found to exhibit a 90% reduction in *hTERT* expression and a 36-70% reduction in telomerase activity relative to untreated 21NT cells, indicating that stable integration of the pCMVNeo vector alone influenced *hTERT* expression. Therefore, it is unlikely that the observed reduction in *hTERT* expression levels and telomerase activity within stable 21NT-pCMNeo-*FLJ* clones is associated with *FLJ* overexpression. These findings therefore suggest that *FLJ* does not function as a negative regulator of *hTERT* transcription or telomerase activity within the 21NT breast cancer cell line.

Based on the identification of *SETD2* and *FLJ* within a chromosome 3-fragment previously found to cause telomerase repression within 21NT breast cancer cells, the aim of this study was to explore the functional role of these candidate genes in *hTERT* transcription regulation. Despite growing evidence of *SETD2* as an important tumour suppressor gene in multiple cancer types, the results presented here suggest that *SETD2* does not function as a negative transcriptional regulator of *hTERT*. In addition reconstitution of *SETD2* expression within 21NT cells was found to have an unanticipated effect on cell growth, which requires further investigation. The findings presented within this study also identify a potentially novel tumour suppressor lncRNA (*FLJ*) in breast cancer localized to the 3p21.3 region. However, as with *SETD2*, *FLJ* does not appear to mediate transcriptional repression of *hTERT* within 21NT cells and, therefore, any role its loss of function may have in cancer development or progression remains to be elucidated.

CHAPTER 5

5 EXPLORING THE ROLE OF TUMOUR SUPPRESSOR GENES WITHIN THE CHROMOSOME 3P21.1-P21.3 REGION AS CANDIDATE REGULATORS OF TELOMERASE IN BREAST CANCER

5.1 INTRODUCTION

As demonstrated by Cuthbert *et al.,* (1999), introduction by MMCT of a normal copy of human chromosome 3 into the 21NT breast cancer cell line is associated with strong telomerase repression and induction of cellular senescence pathways. Deletion-mapping of chromosome 3 within telomerase-positive segregant 21NT-chromosome 3 hybrids identified two regions that may harbour the putative telomerase repressor sequence, namely 3p21.3-p22 and 3p12-p21.1. Subsequent studies showed that the normal chromosome 3 copy mediates telomerase repression within 21NT cells through chromatin remodelling and transcriptional repression of the *hTERT* promoter (Ducrest *et al.,* 2001, Szutorisz *et al.,* 2003). Loss of heterozygosity (LOH) of chromosome 3p has been found to occur within 30-87% of primary breast tumour samples (Ali *et al.,* 1989, Sato *et al.,* 1991, Chen *et al.,* 1994, Matsumoto *et al.,* 1997, Martinez *et al.,* 2001, Maitra *et al.,* 2001, De Oliveira *et al.,* 2012). Microsatellite and polymorphic marker mapping of chromosome 3p within these breast tumour samples revealed allele loss of multiple regions of chromosome 3p including 3p24-26, 3p25, 3p22-24, 3p21.3, 3p13-14.2 and 3p12. However, some studies have reported that the 3p21-p22 region is the most frequent site of LOH in breast cancer (Maitra *et al.,* 2001, Martinez *et al.,* 2001). In addition, around 36% of pre-neoplastic breast lesions have been found to undergo LOH within 3p21.3, which suggests that loss of this region is a frequent and early event in breast cancer development.

The findings presented within my study so far provide evidence to show that the chromosome 3p candidate telomerase repressor genes *SETD2* and *FLJ* may not function as transcriptional regulators of *hTERT* (Chapter 4). These candidate genes are therefore unlikely to be responsible for the telomerase repression observed within 21NT-chromosome 3 hybrids in the Cuthbert *et al.,* (1999) study. A review of relevant literature identified a novel set of candidate telomerase repressor genes that (i) are located close to or within the 3p21.3-p22 region, (ii) have been previously implicated in breast cancer and (iii) have been shown to play a functional role in the epigenetic regulation of target gene transcription through chromatin remodelling. As a result, four candidate genes *BAP1, RASSF1A, PBRM1* and *PARP-3* were selected to investigate their putative role in *hTERT* transcription regulation.

5.1.1 BAP1

The BRCA1 associated protein-1 (BAP1) is a nuclear-localized, ubiquitin hydrolase enzyme that was originally identified during a yeast two-hybrid screen for Breast/Ovarian Cancer Susceptibility Gene (BRCA1)-interacting proteins (Jensen *et al.,* 1998). In humans, *BAP1* is localized to the 3p21.1 region, approximately 52.4Mb from the telomere of chromosome 3 (National Centre for Biotechnology Information, NCBI). *BAP1* encodes a 729 amino acid protein, containing an amino-terminal ubiquitin carboxy-terminal hydrolase (UCH) domain, BRCA1, BRC1-associated RING domain 1 (BARD-1), host cell factor-1 (HCF-1) and Yin Yang 1 (YY1) protein binding domains and nuclear localization signals (Jensen *et al.,* 1998, Machida *et al.,* 2009, Yu *et al.,* 2010, reviewed by Carbone *et al.,* 2013). *BAP1* is expressed ubiquitously within multiple adult human tissues including normal breast tissue, with higher transcript levels observed within the testis, ovaries and placenta (Jensen *et al.,* 1998).

According to a recent review of the TGCA (The Cancer Genome Atlas Network) and MSKCC (Memorial Sloan Kettering Cancer Centre) large-scale whole-exome sequencing studies, 6-12% of clear cell renal cell carcinoma (ccRCC) patients have been found to harbour *BAP1* mutations, which are associated with poor cancer-specific survival (Hakimi *et al.,* 2013). In addition, mutations within the UCH domain of *BAP1* have been identified within lung cancers (Jensen *et al.,* 1998). It has recently been shown that around 70% of germline *BAP1* mutation carriers develop malignancies at an early age, with 25% of these developing two or more types of malignancy (Carbone *et al.,* 2013). The most common malignancies associated with germline *BAP1* mutations are uveal melanoma (UVM) and mesothelioma, which are thought to characterize a novel germline *BAP1* mutation-associated cancer syndrome. However, the incidence of other cancer types such as renal, lung, ovarian and breast cancers have also been reported within germline mutation carriers, which indicates that *BAP1* may play a crucial role in preventing tumorigenesis within multiple tissues. *BAP1* is located within a common site of loss of heterozygosity (LOH) within mesotheliomas, metastasizing uveal melanomas, breast, lung and renal carcinomas (reviewed by Angeloni., 2007, Carbone *et al.,* 2013). Chromosome 3p losses have been observed within pre-neoplastic lesions of the breast and lung, indicating that loss of *BAP1* and other tumour suppressor genes within this region at an early point may facilitate cancer development (Maitra *et al.,* 2001, Hung *et al.,* 1995). In support of this, Stephens *et al.,* (2012) identified *BAP1* mutations as likely drivers of breast tumorigenesis during a mutation screen of 21,416 protein-coding genes within 100 breast cancer samples. By reconstructing the karyotypic evolution of the HCC1187 triple-negative ductal-breast carcinoma cell line, Newman *et al.,* (2013) also found that a mutation of *BAP1* had likely occurred at an early point during its evolution. These findings demonstrate that loss of *BAP1* function may occur at an early point during the development of multiple cancer types, indicating that this gene may function as a critical tumour suppressor.

In 1998, Jensen *et al.,* observed a significant reduction in the number of cell colonies that emerged following co-transfection of MCF7 breast cancer cells with *BAP1* and *BRCA1* compared with the number of colonies that emerged following transfection of *BRCA1* alone or empty vector controls. Interestingly, Xiong *et al.,* (2003) demonstrated that overexpression of wild-type *BRCA1* was associated with repression of telomerase activity and progressive telomere shortening within the DU-145 prostate and T47D and HCC1937 breast cancer cell lines. This was found to be due to repression of the *hTERT* promoter activity, which was dependent on the amino terminal of BRCA1. Despite observing no change in *c-MYC* expression levels, BRCA1 overexpression was found to repress *hTERT* transcription, in part, through inhibition of c-MYC transcriptional activity (Xiong *et al.,* 2003). These findings, together with the known interaction between BAP1 and the amino terminal-RING finger domain of BRCA1 (Jensen *et al.,* 1998) and the location of *BAP1* within a chromosome 3p region that has been implicated in telomerase regulation in breast cancer (Cuthbert *et al.,* 1999), suggests that there may be a putative link between BAP1 and *hTERT* transcriptional regulation.

Jensen *et al.,* (1998) found that transfection of *BAP1* alone was also associated with growth suppression of MCF7 cells, which indicates that aside from enhancing *BRCA1*-mediated growth suppression, *BAP1* may exert other *BRCA1*-independent tumour suppressor functions in breast cancer. In support of this, Ventii *et al.,* (2008) observed a 60% reduction in the number of cell colonies that emerged following overexpression of *BAP1* within the *BRCA1*-deficient HCC1937 breast cancer cell line. Lentivirus-mediated expression of *BAP1* within the NCI-H226 non-small cell lung cancer (NSCLC) cell line, known to harbour a *BAP1* mutation, was found to suppress cell growth *in vitro* and caused a significant reduction in tumour growth rate and overall volume in nude mice compared to negative controls. The tumour suppressor function of *BAP1* was found to be dependent on the presence of functional deubiquitinating UCH and nuclear localization signal (NLS) domains, as transfection of recombinant *BAP1* cDNA harboring mutations in either region did not confer a significant reduction in the growth of NCI-H226 cells *in vitro* or *in vivo.*

Independent studies have shown that *BAP1* is a chromatin-bound protein that forms complexes with transcription factors that are known to regulate the expression of target genes through chromatin remodelling including host cell factor-1 (HCF-1), additional sex combs-like 1 and 2 (ASX1 and ASX2), histone acetyltransferase 1 (HAT1) and the histone H3 lysine 4 demethylase KDM1B (Machida *et al.,* 2009, Yu *et al.,* 2010). Sheuermann *et al.,* (2010) confirmed that BAP1 interacts with ASLX1 in human cells to form the Polycomb repressive deubiquitinase (PR-DUB) complex, which functions to deubiquitinate histone H2A. The Drosophila PR-DUB complex was found to exhibit the same function and was shown to bind to polycomb group response elements (PRE) within target polycomb group (PcG) gene promoters. Inactivation of the BAP1 orthologue *calypso* caused de-repression of PcG target HOX genes in

Drosophila, indicating that the de-ubiquitinating activity of PR-DUB is essential for mediating repression of target gene expression (Scheuermann *et al.,* 2010). It is therefore possible that BAP1 may play an important role in repressing the expression of target genes in human cells through chromatin remodelling.

The HCF-1 binding motif (HBM) of *BAP1* was found to bind to and de-ubiquitinate lysine residues within the Kelch domain of HCF-1 (Machida *et al.,* 2009). Over-expression of the C91S *BAP1* mutant, deficient in ubiquitin hydrolase activity, within MCF-10A and MCF12A breast epithelial cell lines was found to inhibit cell growth, whereas wild-type *BAP1* overexpression did not. This was thought to be due to the sequestration of BAP1-binding sites from endogenous wild-type BAP1 by the C91S mutant. Interestingly, transfection of C91S mutants containing a mutated HBM domain were not able to suppress MCF-10A cell growth, which indicates that the ability of BAP1 to bind to and de-ubiquitinate HCF-1 is essential for cell growth regulation in breast epithelial cells. HCF-1 has been found to interact with a variety of transcription factors such as E2F and Sp1 (Gunther *et al.,* 2000, Knez *et al.,* 2006) and chromatin remodelling enzymes including histone acetyltransferase (HAT) and methyltransferase (HMT) enzymes (Tyagi *et al.,* 2007, Wysocka *et al.,* 2003). It is thought that HCF-1 functions to activate or represses the expression of a wide variety of target genes involved in cell cycle progression by coordinating the recruitment of appropriate chromatin-modifying complexes to gene promoters (Wysocka *et al.,* 2003). RNA interference-mediated depletion of BAP1 within the U2OS osteosarcoma cell line was associated with an increase and decrease in the expression levels of genes involved in cell cycle control, DNA replication and repair, metabolism and survival, indicating that *BAP1* may possess both activating and repressing functions (Yu *et al.,* 2010). Of particular interest, several E2F target genes were also found to be deregulated as a result of *BAP1* knockdown. It was recently shown that BAP1 directly interacts with E2F-1 and E2F-4 transcription factors and it has been proposed that BAP1 may function to regulate E2F target gene expression through interaction with HCF-1 and E2F (Eletr and Wilkinson., 2012). Ectopic expression of the E2F-1 transcription factor has been found to repress the transcription of *hTERT* within HeLa and U2OS cancer cell lines through direct binding to Sp1 sites within the *hTERT* promoter (Won *et al.,* 2002).

Based on the increased incidence of malignancy within germline *BAP1* mutation carriers together with the observed growth suppression of breast and lung cancer cells upon overexpression of wild-type *BAP1 in vitro,* there is strong evidence to suggest that *BAP1* plays a critical role in preventing tumorigenesis (Jensen *et al.,* 1998, Ventii *et al.,* 2008, reviewed by Carbone *et al.,* 2013). *BAP1* has been found to interact with multiple proteins that regulate the transcription of target genes through chromatin remodelling (Machida *et al.,* 2009, Yu *et al.,* 2010). It is thought that *BAP1* influences a wide range of cellular processes including cell cycle regulation, DNA damage repair and survival (Yu *et al.,* 2010). The ubiquitin carboxy-terminal

hydrolase (UCH) domain and nuclear localization signals (NLS) have been found to be essential for mediating the tumour suppressive function of *BAP1 (*Ventii *et al.,* 2008)*,* however the precise mechanism by which *BAP1* functions to suppress cancer cell growth is unknown. Taking into account the observed interaction between BAP1 and a known regulator of *hTERT,* together with the role of BAP1 as a transcriptional regulator and its location within a common region of loss associated with telomerase regulation (Cuthbert *et al.,* 1999), an aim of this study was to explore a functional link between *BAP1* and *hTERT* regulation in breast cancer cells.

5.1.2 RASSF1A

Ras association domain family 1 (RASSF1) is a member of a Ras-association domain containing family of proteins, which have been shown to mediate cancer cell growth suppression through induction of pro-apoptotic signalling pathways (reviewed by Donninger *et al.,* 2007). The *RASSF1* gene was originally identified by Damman *et al.,* (2000) and is located within a common 120kb homozygous region of deletion within chromosome 3p21.3 in lung and breast cancer cells (Sekido *et al.,* 1998). According to current records, the *RASSF1* genomic locus encodes ten transcript variants, five of which are protein coding (Ensembl, Flicek *et al.,* 2014), which are produced by alternative splicing and promoter usage (Damman *et al.,* 2000, Burbee *et al.,* 2001). The *RASSF1A* transcript isoform is 1.8kbp in length and encodes a 340 amino acid protein containing an N-terminal diacylglycerol (DAG) binding domain, a C-terminal Ras-association domain and Sav/RASSf/Hpo (SARAH) protein interacting domain (Damman *et al.,* 2000, Burbee *et al.,* 2001, Donninger *et al.,* 2007).

RASSF1A transcripts have been detected within a variety of normal human tissues and nonmalignant epithelial cell cultures, but are undetectable within 100% of SCLC cell lines, 65% of NSCLC cell lines, 62-65% of breast cancer cell lines and 49% of primary breast tumours (Damman *et al.,* 2000, Damman *et al.,* 2001, Burbee *et al.,* 2001). Loss of *RASSF1A* expression within these cancer types was found to be associated with CpG island hypermethylation within the *RASSF1A* promoter region. Subsequent studies showed that the *RASSF1A* promoter is frequently hypermethylated in over 30 different cancer types including ccRCC, hepatocellular, cervical, head and neck and testicular (reviewed by Agathanggelou *et al.,* 2005 and Donninger *et al* 2007). Pan *et al.,* (2005) found that 74% of tissue samples derived from primary nasopharyngeal carcinomas (NPC) harbored one or more *RASSF1A* mutations, the majority of which were missense. The same proportion of samples also showed complete *RASSF1A* promoter methylation, indicating that mechanisms of *RASSF1A* inactivation in human cancer involve copy number loss, epigenetic silencing and mutation. It is thought that *RASSF1A* is one of the most frequently inactivated genes in cancer, which demonstrates its importance as a tumour suppressor gene (Agathanggelou *et al.,* 2005, Donninger *et al.,* 2007). Independent

studies have shown that *RASSF1A* promoter hypermethylation in breast cancer patient tumour samples is associated with more progressive disease and poor disease-free survival, indicating that loss of *RASSF1A* may be a useful prognostic marker for breast cancer patients (Muller *et al.,* 2003, Martins *et al.,* 2011, Hagrass *et al.,* 2013, Xu *et al.,* 2012).

Loss of *RASSF1A* is thought to occur as an early event during breast tumorigenesis (Strunnikova *et al.,* 2005, Dumont *et al.,* 2009). Silencing of *RASSF1A* transcription was found to occur within proliferating human mammary epithelial cells (HMEC) with increasing passages due to elevated histone H3 deacetylation and lysine 9 trimethylation. HMECs that had overcome the stress-induced senescence barrier by silencing of $p16^{INK4A}$, were then found to undergo DNA methylation at the *RASSF1A* promoter (Strunnikova *et al.,* 2005). Similarly, variant HMEC cell strains (vHMEC), which are derived from a rare subpopulation of pre-malignant and $p16^{INK4A}$-silenced cells obtained from disease-free women, transduced with the Ha-RasV12 oncogene were found to exhibit characteristic features of malignancy including chromosomal instability, anchorage independent growth and increased levels of telomerase activity and *RASSF1A* promoter methylation (Dumont *et al.,* 2009). However, these cells were not found to form tumours upon injection into mammary glands of immunocompromised mice, and were therefore considered to be non-tumorigenic. Increased levels of *RASSF1A* promoter methylation were also observed within other immortal, non-tumorigenic breast cancer cell lines and pre-malignant breast lesions (Dumont *et al.,* 2009). These findings indicate that DNA methylation of the *RASSF1A* promoter region occurs at an early point during breast cancer development and coincides with telomerase re-activation and cellular immortalization; an essential pre-requisite for clonal evolution and malignant transformation (Newbold *et al.,* 1982, Counter *et al.,* 1998).

Forced overexpression of *RASSF1A* in nasopharyngeal, NSCLC, lung, prostate, renal and breast carcinoma cell lines is associated with a substantial reduction in clonogenic survival, malignant potential and tumorigenicity *in vitro* and *in vivo,* which demonstrates that *RASSF1A* is an important tumour suppressor gene in multiple cancer types (Damman *et al.,* 2000, Burbee *et al.,* 2001, Chow *et al.,* 2004, Dreijerink *et al.,* 2001, Kuzmin *et al.,* 2002, Thaler *et al.,* 2012). This is supported by the observation that *Rassf1a* knockout mice have been found to exhibit an increased propensity for spontaneous tumour development within the lungs, gastrointestinal tract, breast and immune system compared to heterozygous or wild-type mice (Tommasi *et al.,* 2005, van der Weyden *et al.,* 2005).

RASSF1A has been shown to play a functional role in maintaining genomic stability through interacting with and stabilizing microtubules to facilitate spindle assembly and chromosome attachment during mitosis (Liu *et al.,* 2003, Vos *et al.,* 2004, Song *et al.,* 2005, Donninger *et al.,* 2007). r*assf1a* knockout mouse embryonic fibroblasts treated with the tubulin-

depolymerizing agent nocodazole were found to lose microtubule structure, whereas treated mouse fibroblasts overexpressing *rassf1a* exhibited largely intact microtubule structures (Liu *et al.,* 2003). The Ras-association domain of RASSF1A was found to be essential for mediating this effect. However, van der Weyden *et al.,* (2005) demonstrated that *rassf1a* knockout mouse fibroblasts did not exhibit significant changes in cell cycle control or genomic stability compared to wild-type cells, but were instead found to be defective in pro-apoptotic signalling pathways (Baksh *et al.,* 2005). Overexpression of *RASSF1A* within the MCF-7 breast cancer cell line was found to induce apoptosis, whereas knockdown of *RASSF1A* within the U2OS osteosarcoma cell line was found to inhibit death receptor induced apoptosis (Baksh *et al.,* 2005). Both Baksh *et al.,* (2005) and Foley *et al.,* (2008) showed that recruitment of RASSF1A and MOAP-1 (also known as MAP-1) to death receptors and subsequent activation of Bax, was essential for mediating death receptor-induced apoptosis.

Aside from playing a functional role in microtubule stabilization and pro-apoptotic signalling pathways, RASSF1A has also been found to modulate cell cycle progression (Shivakumar *et al.,* 2002, Fenton *et al.,* 2004, Thaler *et al.,* 2009). RASSF1A has been found to interact with $p120^{E4F}$; a transcription factor that mediates cell cycle inhibition through interaction with pRb, transcriptional repression of cyclin A and stabilization of cyclin-dependent kinase inhibitors (CDKI) $p21^{Cip1/Waf1}$ and $p27^{kip1}$ (Fenton *et al.,* 2004, Fajas *et al.,* 2000, Fajas *et al.,* 2001, Fernandes *et al.,* 1998). Shivakumar *et al.,* (2002) found that *RASSF1A*-mediated growth inhibition of H1299 NSCLC cells in G1-phase of the cell cycle was associated with the inhibition of cyclin D1 protein accumulation and activation of pRb. Similarly, doxycycline-inducible *RASSF1A* expression within the A549 lung carcinoma cell line was associated with cell growth inhibition in G1 phase and cellular senescence, which was found to be due to *p53*-independent activation of the cyclin-dependent kinase inhibitor *p21* (Thaler *et al.,* 2009). Both studies showed that expression of the human papillomavirus (HPV) E7 oncoprotein within H1299 and A549 lung cancer cell lines expressing exogenous *RASSF1A,* inhibited *RASSF1A*-mediated cell cycle arrest. The E7 viral oncoprotein is known to disrupt the interaction between pRb pocket proteins and E2F transcription factors, and has also been found to disrupt *p53*-mediated repression of *hTERT* by *p21* (Farthing and Vousden., 1994, Shats *et al.,* 2004). Interestingly, overexpression of *RASSF1A* within MCF-7 breast cancer cells was associated with upregulation of $p21^{Cip1/Waf1}$ and induction of senescence pathways.

p21 is part of the Cip/Kip family of cyclin dependent kinase (Cdk) inhibitors and targets cyclin E-Cdk2, cyclin D1-Cdk4 and cyclin D-Cdk6 complexes to regulate cell cycle progression through the G1 phase (reviewed by Shay and Roberts., 1995). Similar to the effect of *RASSF1A* overexpression on cell cycle inhibition within lung cancer cell lines (Shivakumar *et al.,* 2002, Thaler *et al.,* 2009), Niculescu *et al.,* (1998) found that induction of *p21* expression is associated with increased levels of active hypophosphorylated pRb protein levels and cell cycle arrest in

G1 phase. Hypophosphorylated pRb and associated pocket proteins p107 and p130, are known to mediate cell cycle inhibition through interaction with E2F transcription factors and repression of E2F-target gene expression (reviewed by Weinberg 1995, Hurford *et al.,* 1997). Lai *et al.,* (2001) found that pRb pocket protein-mediated transcriptional repression of E2F-target genes involves recruitment of histone deacetylases (HDAC) to mediate chromatin remodelling at gene promoters. Interestingly, interaction of E2F with pRb pocket proteins and recruitment of HDAC enzymes was found to be essential for *p53*-mediated transcriptional repression of *hTERT* expression through *p21* in breast cancer cells (Shats *et al.,* 2004). In further support of this, Won *et al.,* (2004) found that the dynamic assembly and disassembly of E2F-pRb pocket protein-HDAC complex members at the *hTERT* promoter during different phases of the cell cycle within normal human fibroblasts, is associated with changes in *hTERT* expression levels. Loss of E2F-pRb pocket protein-HDAC complex binding and *hTERT* de-repression was observed during S-phase of the cell cycle within IMR90 normal human fibroblasts. This is consistent with findings presented by Masutomi *et al.,* (2003), demonstrating that *hTERT* expression levels and telomerase activity are elevated during S-phase of the cell cycle within normal human BJ, WI-38 and TIF-3 fibroblasts. Disruption of pRb-pocket protein complex members by forced expression of the E7 viral oncoprotein led to de-repression of *hTERT* and telomerase activity (Won *et al.,* 2004). These findings provide evidence of a functional link between cell cycle and *hTERT* regulation in normal human somatic cells through direct binding of E2F-pRb pocket proteins and recruitment of HDAC enzymes to the *hTERT* promoter.

Shivakumar *et al.,* 2002 and Thaler *et al.,* (2009) demonstrated that *RASSF1A* mediates cell cycle arrest and senescence of lung cancer cell lines through activation of pRb-dependent pathways. Taking into account what is currently known about pRb-mediated *hTERT* transcriptional regulation within normal human somatic cells, together with the relative timing of *RASSF1A* promoter methylation and telomerase re-activation during the early stages of breast tumorigenesis, the putative role of *RASSF1A* in *hTERT* transcriptional regulation within breast cancer cells was investigated within this study. It is important to note that overexpression of *RASSF1A* within the RCC23 renal cell carcinoma cell line was not found to be associated with repression of *hTERT* promoter activity (Tanaka *et al.,* 2005). Similarly to MMCT-mediated introduction of normal human chromosome 3 into the 21NT cell line by Cuthbert *et al* (1999), activation of cellular senescence pathways and growth arrest of RCC23-chromosome 3 hybrids was associated with *hTERT* transcriptional repression and loss of telomerase activity (Ohmura *et al.,* 1998). However, whole cell fusion of 21NT and RCC23 cells was found to produce telomerase-repressed hybrids, suggesting that distinct chromosome 3-encoded telomerase repressor genes may function to inhibit *hTERT* expression within breast and renal cancer cells (Tanaka *et al.,* 2005). Therefore, these findings do not exclude the possibility that *RASSF1A* may function as a repressor of *hTERT* in breast cancer cells.

5.1.3 PBRM1/BAF180

The polybromo-1 (*Pb1/PBRM1)* gene, also known as BRG1-Associated Factor (BAF) 180, is located within chromosome 3p21 and encodes a 1689 amino acid protein sequence containing six bromodomains (BD), two protein-protein interaction bromoadjacent homology domains (BAH) and a DNA-binding high mobility group (HMG) domain (Horikawa and Barrett, 2002). PBRM1 was originally identified during a screen of BRG1-associated protein factors within a variety of mammalian cell lines and forms part of the SWI/SNF (switch/sucrose non-fermentable) chromatin remodelling complex B, also known as PBAF (Wang *et al.,* 1996). Mammalian SWI/SNF complexes contain either BRM or BRG1 ATPase enzymes together with around 8-10 BAF protein subunits, which function to actively reposition nucleosomes to regulate target gene expression, chromosome organization, DNA repair, replication and recombination (Wang *et al.,* 1996, Reisman *et al.,* 2009, Euskirchen *et al.,* 2011). The presence of PBRM1 and BAF200 within the BRG1-containing PBAF complex distinguishes it from BRM-BAF and BRG1-BAF complexes which contain BAF250A (*ARID1A*) or BAF250B (*ARID1B*) (Nie *et al.,* 2000, Lemon *et al.,* 2001).

Protein bromodomains (BD) typically bind acetyl-lysine residues within histone tails and have been identified within yeast chromatin remodelling complexes (Horn and Peterson, 2001). The six BDs of PBRM1 are thought to be responsible for targeting the PBAF complex to specific acetylated histone lysine residues to facilitate chromatin remodelling. However, studies have reported conflicting results for the specific histone acetyl-lysine binding sites of individual PBRM1-BDs (Chandrasekaran and Thompson, 2007, Charlop-Powers *et al.,* 2010, reviewed by Brownlee *et al.,* 2012). Using fluorescence anisotropy, Chandrasekaran and Thompson (2007) demonstrated that PBRM1-BDs bind preferentially to histone H3 lysine residues including H3K4Ac, H3K23Ac, H3K9Ac and H3K14Ac. In contrast, Charlop-Powers *et al.,* (2010) found that each BD of PBRM1 bound to a different set of histone-acetyl lysine residues on histones H2, H3 and H4 including H3K36Ac, H2BK115Ac, H3K115Ac, H4K12Ac. These conflicting findings may be due to differences in the experimental methods employed, therefore the precise acetyl-lysine residues targeted by PBRM1-BDs remain unclear (Brownlee *et al.,* 2012).

The yeast RSC (Remodels the Structure of Chromatin) complex is homologous to the human PBAF complex and has been found to play an essential role in mediating cell cycle progression during mitosis (Cao *et al.,* 1997, Xue *et al.,* 2000). Human PBAF has been found to be associated with chromosome kinetochores and spindle poles during prometaphase of the cell cycle, which suggests that PBAF may also be required for cell cycle progression (Xue *et al.,* 2000). PBRM1 has been found to interact with actin filaments and is therefore thought to be responsible for mediating actin-dependent chromosome positioning within the nucleus (Bettinger *et al.,* 2004, reviewed by Thompson, 2009). Unlike other SWI/SNF complexes, the

PBAF complex has also been found to be required for ligand-dependent transcriptional activation by nuclear hormone-receptors, Sp1 and SREBP-1a, which demonstrates that the chromatin remodelling PBAF complex exhibits distinct functional roles in transcriptional regulation of specific target genes (Lemon *et al.,* 2001).

Recent reviews of large-scale exome sequencing studies have found that SWI/SNF complex members are mutated in a high frequency (19%) of all human malignancies including ovarian (75%), hepatocellular (33-40%), gastric (32-36%), head and neck (11%) and breast cancer (9-11%) (Kadoch *et al.,* 2013, Shain and Pollack, 2013). These studies have shown that the SWI/SNF complex is the most commonly mutated chromatin remodelling complex in human cancer and have found that individual protein subunits appear to be mutated more frequently in certain cancer types. This suggests that SWI/SNF complexes may exert tissue-specific tumour suppressor functions. According to current COSMIC database records, *PBRM1* mutations have been detected within over sixteen different human tumour types and cancer cell lines, including kidney, biliary tract, breast and oesophageal, (www.cancer.sanger.ac.uk, Forbes *et al.,* 2014). Exome sequencing of 257 renal cell carcinoma (RCC) tumour samples revealed that *PBRM1* is the second most commonly mutated gene in RCC, with 41% of ccRCC samples harboring truncating mutations of *PBRM1* (Varela *et al.,* 2011). Consistent with these findings, around 70% of ccRCC tissue samples have been found to exhibit undetectable levels of *PBRM1* expression, which demonstrates that other mechanisms of functional inactivation may be present within cancer cells (Pawlowski *et al.,* 2012). The same study also demonstrated a significant correlation between loss of *PBRM1* expression and advanced tumour stage and poor patient survival compared with *PBRM1*-positive ccRCC patients. These findings demonstrate that loss of *PBRM1* may play an important role in RCC disease pathogenesis. Xia *et al.,* (2008) identified four truncating mutations within the bromodomains (BD) of *PBRM1* in multiple breast cancer cell lines. The presence of *PBRM1* mutations was correlated with loss of wild-type *PBRM1* expression. In addition 48.1% of breast tumour samples were found to have undergone LOH at the *PBRM1* genomic locus, which demonstrates that *PBRM1* may also function as an important tumour suppressor gene in breast cancer (Xia *et al.,* 2008). LOH of discreet regions of chromosome 3p is a frequent event in renal, breast and lung cancers (Zarbarovsky *et al.,* 2002, Senchenko *et al.,* 2004). Interestingly, despite the identification of *PBRM1* mutations within renal and breast cancer cell lines (Varela *et al.,* 2011, Xia *et al.,* 2008), Sekine *et al.,* (2005) found that NSCLC and SCLC cell lines with chromosome 3p LOH, do not exhibit loss of *PBRM1* mRNA or protein levels. These findings indicate that *PBRM1* may function as a tissue-specific tumour suppressor gene.

Forced overexpression of *PBRM1* within the *PBRM1*-deficient HCC1143 breast cancer cell line was associated with a significant increase in the number of cells in G1 phase-cell cycle arrest compared with empty vector controls (Xia *et al.,* 2008). This was found to be partly due

to activation of the cyclin-dependent kinase inhibitor *p21*. A dosage-dependent increase in *p21* expression levels was observed following overexpression of *PBRM1* within *p53*-deficient HCC1143 and SUM1315 breast cancer cell lines, which indicates that *PBRM1* mediates *p53*-independent induction of *p21* expression. Endogenous *PBRM1* was found to bind directly to the *p21* promoter within MDA-MB-468 breast cancer cells, which express wild-type *PBRM1* mRNA. The same study also found that knockdown of *PBRM1* within normal human breast epithelial MCF-10A cells treated with γ-irradiation or transforming growth factor β (TGF-β) abrogated induction of *p21* expression and cell cycle arrest in G1 phase. *p53*-mediated transcriptional activation of *p21* is essential for sustained cell growth arrest following γ-irradiation-induced DNA damage (Bunz *et al.,* 1998). On the other hand, members of the SMAD family of transcription factors have been found to be essential for TGF-β-mediated induction of *p21* expression and cell growth arrest, independently of *p53* (Datto *et al.,* 1995, Pardali *et al.,* 2000). Therefore, the findings presented by Xia *et al.,*(2008) demonstrate that *PBRM1* may function as a critical tumour suppressor gene in normal mammary epithelial cells by mediating *p21* transcriptional activation and cell cycle arrest in response to DNA damage and extracellular growth factor-induced signalling pathways.

Inactivation of *p21* within normal human fibroblasts has been associated with an increase cellular lifespan and bypass of cellular replicative senescence pathways (Brown *et al.,* 1997). *p21*-deficient normal human fibroblasts were found to undergo continued telomere erosion until cells entered crisis, which demonstrates that *p21* plays an important role in activating replicative senescence pathways in response to critical telomere shortening. Interestingly, knockdown of *PBRM1* within normal human BJ fibroblasts has been associated with decreased basal and *p53*-induced levels of *p21* expression, increased cellular proliferation and delayed induction of replicative senescence pathways (Burrows *et al.,* 2010). Similarly, knockdown of another PBAF complex member *BRD7* within normal human fibroblasts was also associated with decreased basal and *p53*-induced *p21* expression levels, delayed replicative senescence and resistance to oncogene-induced senescence (OIS) pathways (Burrows *et al.,* 2010). These findings suggest that the PBAF complex may be involved in mediating cellular senescence pathways through transcriptional regulation of *p21*. In support of this, the histone acetyl-lysine binding bromodomain (BD) of BRD7 was found to be necessary for induction of *p21* expression, which suggests that PBAF-mediated chromatin remodelling at the *p21* promoter may be essential for transcriptional activation. Interestingly, gene expression profiling of normal human fibroblasts following knockdown of *PBRM1* and treatment with the MDM2 inhibitor nutlin-3a showed that, in addition to *p21*, *PBRM1* is involved in the transcriptional regulation of a specific subset of *p53*-target genes including *MDM2* and *DcR1* (Burrows *et al.,* 2010). Together, the findings presented by Xia *et al.,* (2008) and Burrows *et al.,* (2010) demonstrate that *PBRM1* mediates *p53*-dependent and independent activation of *p21* expression in normal

human somatic cells and cancer cells and plays an important functional role in the induction of two critical tumour suppressor pathways; cell growth arrest and replicative senescence. However, knockdown of *p21* within HCC1143 breast cancer cells overexpressing *PBRM1* was only associated with a partial rescue of G1-phase cell cycle arrest (Xia *et al.,* 2008). In addition, Burrows *et al.,* (2010) demonstrated that aside from *p21, PBRM1* regulates the transcription of multiple *p53* target genes indicating that *PBRM1* may exhibit other critical tumour suppressor functions in human cells.

Recently, PBRM1 has been found to play important roles in maintaining genomic stability in human cells through mediating centromeric sister chromatid cohesion, DNA damage-induced transcriptional repression and non-homologous end joining (NHEJ) repair of DNA breaks (Brownlee *et al.,* 2014, Karkarougkas *et al.,* 2014). Targeted inactivation of cohesin complex proteins involved in mediating sister-chromatid cohesion during the cell cycle, has been linked to chromosomal instability and aneuploidy, which is thought to lead to tumorigenesis (Remeseiro *et al.,* 2012, Solomon *et al.,* 2011). Stable sh-RNA-mediated depletion of *PBRM1* within the U2OS osteosarcoma cell line was associated with a reduction in sister chromatid cohesion, increased chromosome miss-segregation, and defective recombination-based DNA damage repair (Brownlee *et al.,* 2014). Transfection of two *PBRM1* cDNA constructs harboring ccRCC-associated mutations within bromodomains 2 or 4 (BD2-T232 and BD4-M538) (Varela *et al.,* 2011), were unable to rescue sister-chromatid cohesion defects within shRNA-*PBRM1* U2OS cells. These findings demonstrate an important role for *PBRM1* in maintaining genome stability and preventing tumorigenesis through cohesion-dependent functions such as sister chromatid exchange during mitosis and DNA damage repair. In a later study, the same group also demonstrated that *PBRM1* and PBAF complex-mediated transcriptional repression of actively transcribed genes flanking euchromatic DNA double strand breaks, is important for efficient repair (Karkarougkas *et al.,* 2014). In addition, increased levels of γ-H2AX foci were observed at early time points following irradiation of *PBRM1, BRG1* or *PBAF*-depleted U2OS cells compared with controls, which indicates that the PBAF chromatin remodelling complex functions to promote repair at an early point following DNA damage. Similarly to Brownlee *et al.,* (2014), transfection of cancer-associated *PBRM1* mutants T232 and M538 within *PBRM1*-deficient cells were unable to rescue transcriptional repression or DNA repair defects. Therefore, aside from mediating cohesion-dependent functions, *PBRM1* promotes genomic stability by facilitating the efficient repair of DNA double strand breaks.

PBRM1 has been implicated in multiple tumour suppressor pathways including cell cycle arrest, replicative senescence, DNA damage repair and genome stability, which demonstrates that *PBRM1* plays an important role in preventing tumorigenesis. As discussed in Chapter 5.1.2, induction of *p21* expression has been found to be an essential for *p53*-mediated transcriptional repression of *hTERT* in breast cancer cells by E2F-pRB pocket protein-HDAC

complexes (Shats *et al.,* 2004). As demonstrated by Burrows *et al.,* (2010), *PBRM1* functions as an important mediator of replicative senescence pathways through *p53*-dependent activation of *p21* expression. In addition, *PBRM1* was found to be important for *p21* transcriptional activation and cell cycle arrest of breast cancer cells in response to TGF-β treatment (Xia *et al.,* 2008). It is thought that *PBRM1* may cooperate with SMAD family protein members to activate *p21* expression and mediate TGF-β-induced cell cycle arrest. Interestingly, TGF-β has been found to repress *hTERT* promoter activity and telomerase activity within breast cancer cells (Yang *et al.,* 2001). TGF-β may mediate *hTERT* transcriptional repression *in cis* through direct binding of SMAD3 to the *hTERT* promoter or *in trans* by SMAD3-mediated repression of the *hTERT* transcriptional activator *c-Myc* (Li *et al.,* 2006). Taking these findings into account, the functional role of *PBRM1* in *hTERT* expression regulation was investigated within this study.

5.1.4 PARP-3

Human PARP-3 belongs to a family of 17 poly(ADP-ribose) polymerase enzymes, which function to synthesize ADP-ribose polymers from Nicotinamide adenine dinucleotide (NAD) (reviewed by Aredia and Scovassi., 2014). PARP enzymes mediate the transfer ADP-ribose polymers to specific amino acid residues of nuclear proteins to facilitate the assembly of functional protein complexes that regulate apoptosis, DNA replication, repair and gene transcription. *PARP-3* (*ARTD3)* was originally discovered by Johnsson *et al.,* (1999) during a search of the expressed sequence tag (EST) library for PARP-1 sequence-related proteins in humans. *PARP-3* is located on chromosome 3p21.2 and encodes two major transcript isoforms, one of which contains an additional seven amino acids at the N-terminal (Augustin *et al.,* 2003, Rouleau *et al.,* 2007). Transcript variants 1 (*PARP-3*$_{short}$) and 2 (*PARP-3*$_{long}$) encode 60.1kDa and 61kDa proteins respectively and belong to the DNA-dependent sub-family of PARP enzymes, which catalyze mono(ADP-ribosyl)ation of target proteins (Rouleau *et al.,* 2007, Gibson and Kraus., 2012). Structurally, PARP-3 proteins contain an N-terminal conserved WGR nucleic acid binding domain and a C-terminal mono(ADP-ribose) polymerase catalytic domain (Johansson., 1999, Augustin *et al.,* 2003, Lehtiö *et al.,* 2009).

Green-fluorescent protein-tagged PARP-3 expression within the human HeLa and SV40-transformed African green monkey COS-7 kidney cells, revealed that both PARP-3 isoforms localize predominantly to the nucleus (Rouleau *et al.,* 2007). The same study also found that PARP-3 specifically interacts with components of the Polycomb group (group) complex including EZH2, Suz12, YY1, HDAC1 and HDAC2 and co-localize with many of these proteins at PcG bodies *in vitro*. This suggests that PARP-3 may be involved in transcriptional regulation of target genes through chromatin remodelling. Mass spectrometry analysis of co-immunoprecipitated proteins revealed that PARP-3 also interacts with multiple

components of DNA-damage repair pathways such as DNA-dependent protein kinase (DNA-PKcs), Ku70, Ku80, PARP-1 and DNA ligases (Rouleau *et al.,* 2007). The two major pathways that are involved in DNA DSB repair are homologous recombination (HR) and NHEJ (reviewed by Chapman *et al.,* 2012). The DNA PKcs/Ku70/Ku80 complex interacts with DNA ligase IV to mediate DNA double strand break repair through non-homologous end-joining (NHEJ) pathways (Spagnolo *et al.,* 2006). The Ku70-Ku80 heterodimer has been found to inhibit 5'-DNA end resection and promote activation of the NHEJ repair pathway. Recently, Beck *et al.,* (2014) demonstrated PARP-3 depletion was associated with a lack of Ku80 recruitment to sites of DSB and increased DNA end resection, which demonstrates that PARP-3 plays in important role in activating NHEJ-mediated DSB repair. Consistent with these findings, Boehler *et al.,* (2010) demonstrated that PARP-3 accumulates at micro-irradiation-induced DNA damage sites within mammalian cells. ShRNA-mediated depletion of PARP-3 within normal human fetal lung MRC-5 fibroblast cells was not only associated with a higher frequency of spontaneous DNA strand breaks but also a significant delay in γ-H2AX foci clearance following X-irradiation treatment compared to non-targeting controls (Boehler *et al.,* 2010). In the same year Rulten *et al.,* (2010) found that PARP-3 is responsible for targeting the aprataxin and PNKP like factor (APLF) to sites of DNA breaks through histone H1 ribosylation, which facilitates DNA ligation during NHEJ.

Aside from playing an important functional role in mediating DNA damage repair, PARP-3 has also been implicated in regulating cell cycle progression (Augustin *et al.,* 2003, Boehler *et al.,* 2010). Overexpression of PARP-3 within HeLa cells was associated with an increase in the proportion of cells arrested in G1/S phase following treatment with DNA-damage inducing agents, compared with empty vector controls which were arrested in G2/M (Augustin *et al.,* 2003). PARP-3 has been found to associate with centrosomes and interact with the Nuclear Mitotic Apparatus (NuMA) protein and Tankyrase 1, which are involved in regulating microtubule dynamics during mitosis (Augustin *et al.,* 2003, Boehlin *et al.,* 2010, Radulescu and Cleveland., 2010). Knockdown of PARP-3 within normal lung MRC-5 fibroblasts was associated with cell cycle arrest in metaphase and defects in spindle assembly, characterized by splayed microtubules and the presence of aberrant mitotic figures (Boehlin *et al.,* 2010). In the presence of DNA, PARP-3 was found to cause direct ADP-ribosylation and stimulate Tankyrase 1-mediated ADP-ribosylation of NuMA. Therefore, it is thought that PARP-3 plays a functional role in cell cycle progression through mediating correct microtubule assembly during mitosis.

Interestingly, knockdown of PARP-3 within normal human lung fibroblasts has also been associated with increased sister telomere fusions and loss, demonstrating that PARP-3 may also function to maintain telomere stability (Boehlin *et al.,* 2010). In 2007, Frias *et al.,* investigated the relationship between telomerase activity and the expression levels of genes

involved in DNA damage repair pathways in non-small cell lung cancer (NSCLC) and found that tumour samples exhibiting high levels of telomerase activity also displayed significantly reduced levels of *PARP-3* mRNA. Recently, transient overexpression of PARP-3 within the A549 lung adenocarcinoma cell line was associated with a significant reduction in telomerase activity 48 and 96 hours following transfection compared with empty vector controls. Moreover, shRNA-mediated depletion of *PARP-3* within the human Saos-2 osteosarcoma cell line to around 40% of controls, was associated with a greater than two-fold increase in telomerase activity levels. This provides evidence to show that *PARP-3* may function as a telomerase repressor gene in cancer. Taking these findings into account, together with the observation that *PARP-3* resides within a common region associated with telomerase regulation in breast cancer (Cuthbert *et al.,* 1999), the putative role of *PARP-3* in telomerase regulation within breast cancer cells was investigated within this study.

5.2 MATERIALS AND METHODS

5.2.1 GENE COPY NUMBER VARIATION ANALYSIS (CNV)

Nine breast cancer cell lines, 21NT, 21MT, MCF-7, H5S78T, HCC1143, BT474, BT20, MTSV and GI101, were cultured under normal growth conditions (Chapter 2.1.2.1) until subconfluent before they were harvested, pelleted and stored at -80^{o}C. Normal pre-stasis HMEC strains 184 and 240L and the post-stasis HMEC LONZA cell strain were cultured under normal growth conditions by my colleague Dr. H. Yasaei and were provided to me upon request as cell pellets derived from cells growing in log-phase. Genomic DNA (gDNA) was extracted from cell lines and HMEC cell strains as outlined in Chapter 2.2. Sample DNA extracts were diluted to 5ng/µl using nuclease-free water. Gene copy number variation (CNV) analysis of *BAP1, PARP-3* and *PBRM1* within breast cancer cell lines and normal HMEC strains was carried out as described in Chapter 2.5. Details of pre-designed CNV primer sets (ABI, US) for each gene are provided in Chapter 2.5, Table 2.6. The normal HMEC 184 cell strain was used to calibrate gene copy number within all samples.

5.2.2 GENE EXPRESSION ANALYSIS

All breast cancer cell lines, normal HMEC strains and stable 21NT transfection clones were subjected to the same procedure of RNA extraction, DNAse I treatment and cDNA synthesis as described in Chapter 2.3. Using the appropriate primer sequences and thermal cycling parameters described in Table 2.3 and Table 2.4 (Chapter 2.3.4), *BAP1, PBRM1, RASSF1A, PARP-3* and *hTERT* expression levels were examined using the same methods outlined in Chapter 2.3.4. As demonstrated in Chapter 4.2.1.1, GeNorm™ (Primer Design) analysis of normal HMEC strains and breast cancer cell lines demonstrated that the most

suitable endogenous control genes for examining target gene expression levels within this sample set were *YWHAZ* and *TOP1*. Therefore, target gene expression levels within normal HMEC strains and breast cancer cell lines, were normalized to *YWHAZ* and *TOP1* expression. Target gene and *hTERT* expression levels within 21NT transfection clones were normalized to the *GAPDH* endogenous control. The qbase^{PLUS} (Biogazelle) data analysis software program was used to calculate normalized relative quantities (NRQ) of target gene mRNA for all samples as described in Chapter 2.3.5.

5.2.3 TRANSFECTION OF TARGET GENES INTO THE 21NT BREAST CANCER CELL LINE

Overexpression of target genes *BAP1, RASSF1A* and *PBRM1* within the 21NT breast cancer cell line was achieved by transfection of mammalian expression vector constructs containing target gene cDNA clones and associated empty vector controls (Table 5.1).

Table 5.1 - Details of mammalian expression vectors used to overexpress *BAP1, RASSF1A* and *PBRM1* within 21NT breast cancer cells

Target gene		Plasmid Vectors	Accession Number	Vector Size (bp)	Source	Selectable Marker
BAP1	**Control**	pCMV6Neo	-	5838	OriGene, PCMV6NEO	Neomycin
	Target	pCMV6AC-*BAP1*	NM_004656	8442	OriGene, SC320669	
RASSF1A	**Control**	pcDNA3.1/V5-His-TOPO/*LacZ*	-	8592	Invitrogen, US	Neomycin
	Target	pcDNA3.1(+)-*RASSF1A*	NM_007182.4	7273	Professor Reinhard Damman[1], (Damman *et al.,* 2000)	
PBRM1	**Control**	pBABE-puro	-	5169	Morgenstern and Land[2]., 1990, Addgene #: 1764	Puromycin
	Target	pBABE-puro-*BAF180*	NM_018313.4	10,130	Professor Ramon Parsons[3], Xia *et al.,* (2008), Addgene #: 41078	

[1]Intitute of Genetics, Justus-Liebig University Giessen, Germany, [2]London Research Institute, Lincoln's Inn Fields, London, UK, [3]Institute for Cancer Genetics, Columbia University, New York, USA.

The pCMV6AC-*BAP1* and pcDNA3.1(+)-*RASSF1A* (the latter was a kind gift from Professor Reinhard Damman, Justus-Liebig University) plasmid constructs were received in the form of dried DNA samples, which were reconstituted in nuclease-free water (Ambion, Thermo Fisher Scientific, US) and transformed into OneShot® TOP10 Chemically Competent E.coli (Invitrogen, US) as described in Chapter 2.6.1. The pCMV6Neo and pcDNA3.1/V5-His-TOPO/*LacZ* empty vector plasmids were transformed into the same chemically-competent

E.coli cells by my colleague Dr. T. Roberts (Brunel University). These plasmids were provided to me upon request as frozen glycerol stock solutions (85% LB-Broth and 15% glycerol). The pBABE-puro and pBABE-puro-*BAF180* plasmids were received as bacterial stabs of transformed E.coli. Using aseptic techniques, sterile pipette tips were used to recover the bacterial cells, which were then placed into Falcon tubes containing 5ml of pre-sterilized 2.5% LB-broth (high salt, Sigma, US) and 100µg/ml ampicillin. Bacterial cell suspensions were incubated overnight in an orbital shaker at 37°C and 200rpm. The following day, cultures were either stored at 4ºC for a maximum of five days or prepared for long-term storage. Glycerol stock solutions of all transformed E.coli cultures were created as described in Chapter 2.6.2. To obtain sufficient quantities of plasmid DNA for transfection purposes, bacterial cultures were propagated using the methods described in Chapter 2.6.3. Extraction and purification of plasmid DNA was achieved using the QIAfilter Midi Plasmid Purification Kit (Qiagen, NL) according to manufacturer's guidelines (Chapter 2.6.4).

5.2.3.1 Restriction Digest of Target Gene Plasmid Vectors

In order to confirm the size of *BAP1, RASSF1A* and *PBRM1/BAF180* target gene cDNA sequences within each of the plasmid vector constructs described in Table 5.1, plasmids were subject to restriction endonuclease digestion. For each target gene plasmid construct, restriction enzymes used to clone the cDNA into the vector backbone were used to release the cDNA insert sequence with reaction components described in Table 5.2. Restriction digest reaction mixtures were incubated at 37ºC for one hour before being transferred to wet-ice to inhibit enzyme activity. 250ng of plasmid DNA was added to wells of a 0.75% agarose gel made with 1X Tris-Borate-EDTA buffer (Sigma-Aldrich, US) and 0.5µg/ml ethidium bromide (EtBr). To estimate the size of DNA fragments, the TrackIt™ 1Kb Plus DNA Ladder (Invitrogen, US) was also loaded into a single well of each gel. After submerging in 1X TBE buffer, gels were run at 60-70V for approximately 1 hour 30 minutes and resolved DNA fragments were visualized using an Alphaimager under UV light.

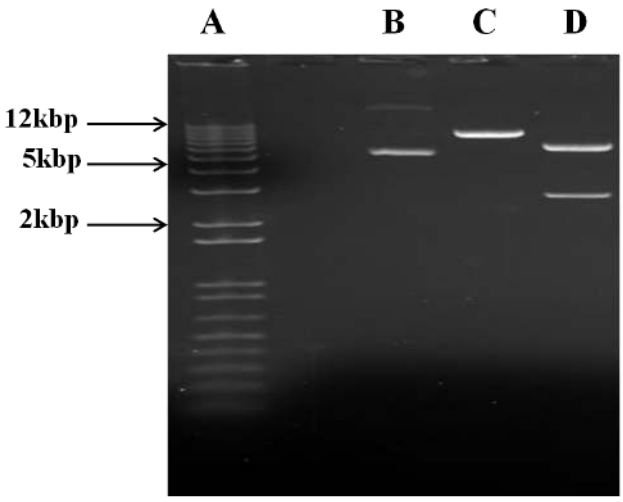
Table 5.2 - Restriction enzyme digest reaction conditions used to release target gene cDNA from plasmid vector backbones

Plasmid Vector	Cloning Sites (5'-3')	Vector Backbone (bp)	cDNA Insert Sequence (bp)	Restriction Digest Reaction Components*
pCMV6AC-*BAP1*	EcoRI-XhoI	5802	2640	1µg plasmid DNA, 1µl EcoRI-HF, 1µl XhoI-HF, 5µl NEB CutSmart® Buffer, nuclease-free water. Total Volume: 50µl
pcDNA3.1(+)-*RASSF1A*	HindIII-EcoRI	5428	1845	1µg plasmid DNA, 1µl HindIII-HF, 1µl EcoRI-HF, 5µl NEB CutSmart® Buffer, nuclease-free water. Total volume: 50µl
pBABE-puro-*BAF180*	EcoRI-EcoRI	5169	4961	1µg plasmid DNA, 1µl EcoRI-HF, 1µl NotI, 5µl NEBuffer EcoRI, nuclease-free water. Total volume: 50µl

** Restriction enzymes (20U/µl), NEB CutSmart® Buffer (10X) and NEBuffer EcoRI (10X) were purchased from New England Biolabs (NEB).*

As shown is Figure 5.1A and B, pCMV6AC-*BAP1* and pcDNA3.1(+)-*RASSF1A* plasmid constructs were found to contain the expected target gene cDNA sequence lengths of 2.6kbp and 1.8kbp respectively. The pBABE-puro plasmid and *BAF180* cDNA sequence are very similar in size (Table 5.2). Therefore in order to distinguish DNA fragments following restriction digest of pBABE-puro-*BAF180* with EcoRI (Figure 5.1Cii, Lane C), the NEBcutter software program (Posfai *et al.,* 2005) was used to identify a different restriction enzyme that would cleave a single site within the vector backbone. As shown in Figure 5.1C, a single NotI restriction digest site was identified and used to confirm the *BAF180* cDNA insert length of around 5kbp (Figure 5.1Cii, Lane D).

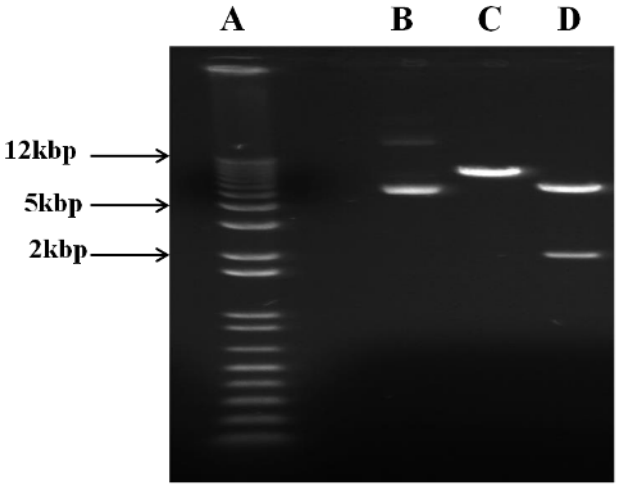
A. pCMV6AC-*BAP1* Plasmid Vector Digest



Key:
A – 1Kbp DNA Ladder
B – pCMV6AC-*BAP1* Circular
C – pCMV6AC-*BAP1* + XhoI
D – pCMV6AC-*BAP1* + XhoI and EcoRI

pCMV6AC = 5802bp
***BAP1* cDNA** = 2640bp

B. pcDNA3.1(+)-*RASSF1A* Plasmid Vector Digest



Key:
A – 1Kbp DNA Ladder
B – pcDNA3.1(+)-*RASSF1A* Circular
C – pcDNA3.1(+)-*RASSF1A* + HindIII
D – pcDNA3.1(+)-*RASSF1A* + HindIII and EcoRI

pcDNA3.1 (+) = 5428bp
***RASSF1A* cDNA** = 1845bp

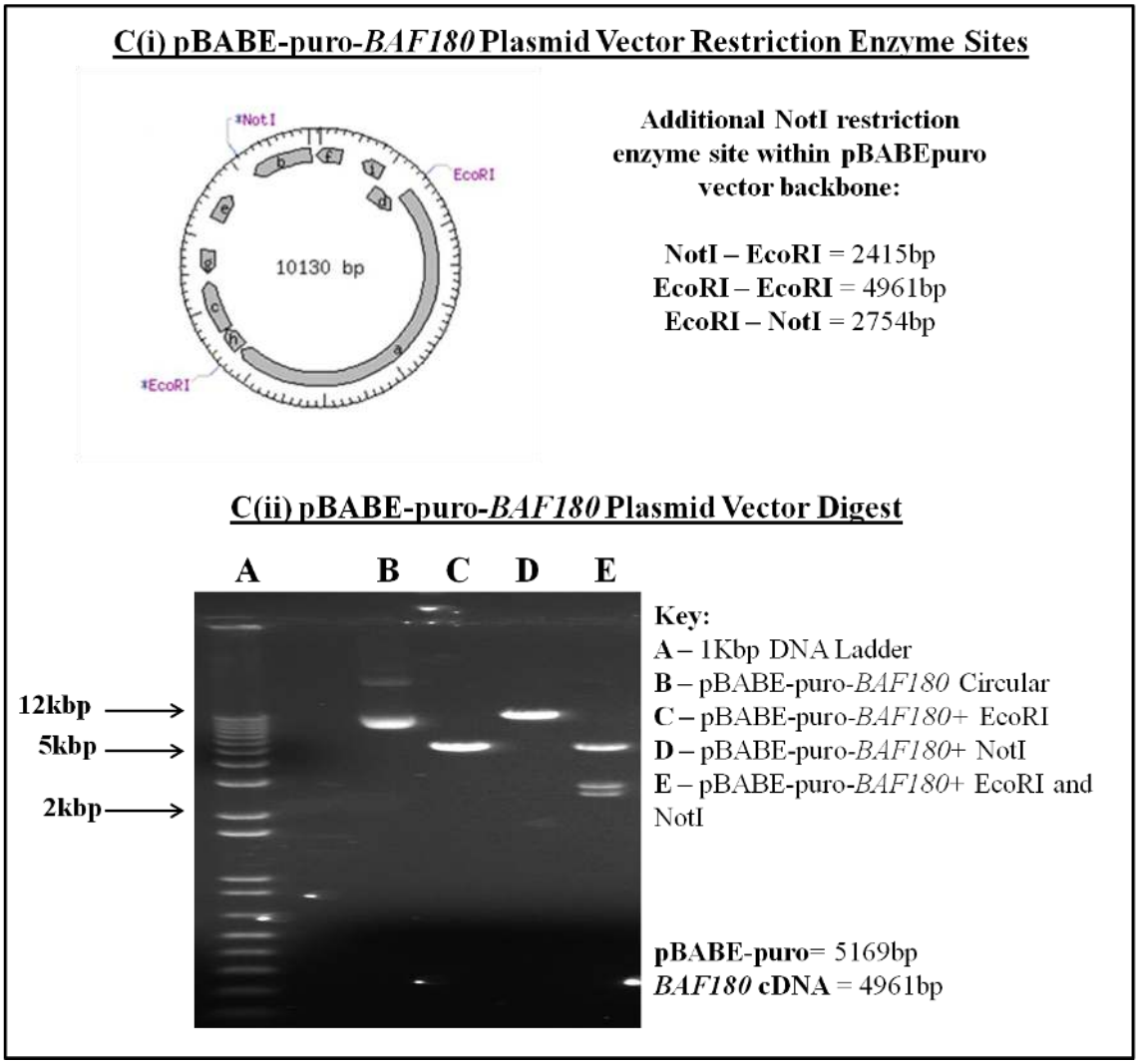


Figure 5.1 - *Gel electrophoresis of DNA fragments following restriction digest of* ***(A)*** *pCMV6AC-BAP1 with XhoI and EcoRI,* ***(B)*** *pcDNA3.1(+)-RASSF1A with HindIII and EcoRI.* ***(C)*** ***(i)*** *Relative positions of NotI and EcoRI restriction sites determined using the NEBcutter software program and expected fragment lengths (Posfai et al., 2005).* ***(ii)*** *Gel electrophoresis of DNA fragments following digestion of pBABEpuro-BAF180 with EcoRI and NotI.*

5.2.3.2 Transfection of Plasmid Vector Constructs into the 21NT Cell Line

Overexpression of *BAP1, RASSF1A* and *PBRM1(BAF180)* within the 21NT breast cancer cell line was achieved by transfection of pCMV6AC-*BAP1,* pcDNA3.1(+)-*RASSF1A,* pBABE-puro-*BAF180* and associated empty vector plasmids (Table 5.1) using the *Trans*IT®-BrCa Transfection Reagent (Mirus) as described in Chapter 2.7.1.

5.2.3.3 Selection and Propagation of Stable Transfection Clones

In order to examine the long-term effect of forced *BAP1, RASSF1A* and *PBRM1* target gene expression within the 21NT cell line, positive selection of 21NT clones that had undergone stable integration of target gene vector constructs was carried out. 48 hours post-transfection with target gene and empty vector control plasmids, P60 dishes of treated 21NT cells were

passaged at a ratio of 1:8 into four P100 dishes using methods described in Chapter 2.1.3. Cells were maintained in complete medium for two weeks supplemented with either 400µg/ml neomycin or 1µg/ml puromycin depending on the selectable marker present within the plasmid vector (Table 5.1). During this time, dishes were monitored regularly for the emergence of stable clones. At least five stable 21NT-target gene and five stable 21NT-empty vector control clones were isolated at random and propagated as individual cell lines as described in Chapter 2.1.5. Once stable clones had reached sub-confluence on P35 dishes (passage 1), cells were harvested, counted (Chapter 2.1.6) and aliquots of $5x10^5$ cells were pelleted and immediately stored at -80ºC for downstream analysis of telomerase activity. The remaining cells were re-plated on a P60 dish and were maintained in culture for cell growth analysis or propagated until three sub-confluent P100 dishes were obtained. At this point, one dish was harvested for cryopreservation (Chapter 2.1.4) and the remaining dishes were harvested, pelleted and stored at -80ºC for downstream analysis.

5.2.3.4 Cell Growth Analysis

As described above, individual stable 21NT transfection clones that had reached sub-confluence on P35 dishes (passage 1) were counted and an aliquot of $5x10^5$ cells were taken for downstream analysis of telomerase activity. The remaining cells were re-plated onto P60 dishes and were maintained in under normal growth conditions in the presence of appropriate selection antibiotics. At 70-90% confluence, cell number was ascertained (Chapter 2.1.6) and $5x10^5$ cells were re-established on P100 dishes containing complete culture media. This procedure of serial sub-cultivation was repeated a maximum number of five times before clones were cryopreserved (Chapter 2.1.4). Cell growth rate was assessed by calculating the total number of population doublings (PD) that had occurred between seeding $5x10^5$ cells and harvesting at 70-90% confluence (Chapter 2.8.1). The same procedure was carried out for individual stable 21NT-empty vector transfection clones and untreated 2NT cells.

5.2.3.5 Colony Forming Unit (CFU) Assay

In order to determine the effect of stable *BAP1, RASSF1A* and *PBRM1* expression on the clonogenic survival of 21NT cells, a colony forming unit (CFU) assay was carried out for each set of target genes and associated empty vector control plasmids (Table 5.1) as described in Chapter 2.7.3.

5.2.3.6 Clone Size Estimation

To examine the effect of stable *BAP1, RASSF1A* and *PBRM1* target gene expression on the growth rate of 21NT cells, the diameter of stable 21NT-target gene and associated 21NT-empty vector control clones (Table 5.1) that emerged following two weeks of antibiotic selection was examined. Fixed and Methyl Blue-stained stable clones from the CFU assay were examined under a Zeiss Axioskop microscope (brightfield illumination). Photomicrographs of

10-18 individual 21NT-target gene and associated 21NT-empty vector control clones were taken at 2.5x magnification. As shown in Figure 5.2, a 10mm reticule was used to measure the diameter of each stable 21NT transfection clone. The clone diameter of all individual stable 21NT-target gene and 21NT-empty vector control clones was recorded and the mean diameter (mm) ± SE of all stable clones for each transfected plasmid was calculated. A student's t-test was used to compare the mean clone diameter of each set of stable 21NT-target gene and 21NT-empty vector control clones.

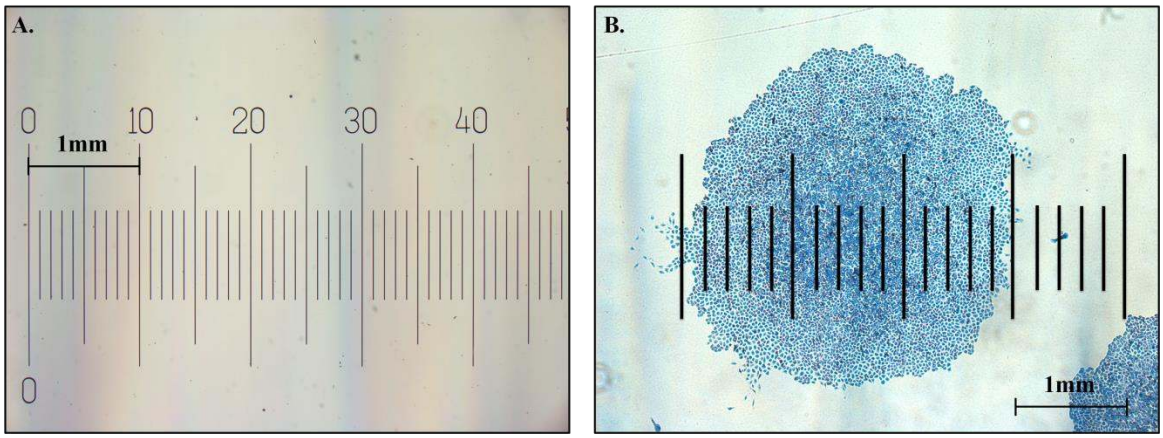


Figure 5.2 - *Estimation of stable clone diameter. Representative photomicrograph images at 2.5x magnification of* ***(A)*** *10mm reticule,* ***(B)*** *a single stable 21NT-pBABEpuro-PBRM1 clone fixed and stained with Methyl Blue.*

5.2.4 TELOMERASE ACTIVITY

To determine the effect of stable *BAP1, RASSF1A* and *PBRM1* target gene overexpression on telomerase activity within 21NT cells, the level of telomerase activity was examined within stable 21NT-target gene and associated 21NT-empty vector control clones. Protein was extracted from aliquots of $2.5x10^5$ cells obtained from each stable clone at passage 1 and quantified using the same methods described in Chapter 2.4.1. The Telomere Repeat Amplification Protocol (TRAP) assay was used to determine telomerase activity within all samples as described in Chapter 2.4.2. For each target gene examined, five stable 21NT-target gene clones and three stable 21NT-empty vector control clones were included in each assay. Telomerase activity was quantified as described in Chapter 2.4.3 and expressed as the mean ± SE of triplicate repeats for each sample relative to untreated wild-type 21NT controls.

5.3 RESULTS AND DISCUSSION

5.3.1 CANDIDATE GENE COPY NUMBER VARIATION AND EXPRESSION ANALYSIS WITHIN NORMAL HMEC STRAINS AND BREAST CANCER CELL LINES

The initial aim of this study was to investigate a putative tumour suppressive function of the selected candidate genes *BAP1, RASSF1A, PBRM1* and *PARP-3* in breast cancer cells. To do this, candidate gene copy number variation (CNV) and expression levels were examined within the same panel of nine breast cancer cell lines and three HMEC strains employed within Chapter 4.3.1. Over half of breast cancer cell lines were found to have undergone allele loss within the *SETD2-FLJ* genomic locus. As shown in Figure 5.1, all four selected candidate genes are located within a 2.3Mb region within 3p21.1-21.3. Taking into account the high frequency of allele loss within the 3p21.3 region in breast cancer (Maitra *et al.,* 2001, Martinez *et al.,* 2001), CNV analysis of selected candidate genes within breast cancer cell lines was carried out. According to the Database of Genomic Variants (DGV, MacDonald *et al.,* 2013), copy number loss of *RASSF1A* has been observed within the genome of healthy individuals. The phenotypic consequence of this structural variation is unknown. However, according to the Database of Genomic Variation and Phenotype in Humans using Ensembl Resources (DECIPHER), loss/deletion of sequences involving the 3p21.31 region (including *RASSF1*) is associated with particular characteristics such as delayed speech and intellectual disability (Firth *et al.,* 2009). Therefore, CNV analysis of the *RASSF1A* genomic locus within breast cancer cell lines could not be reliably determined within this study because matched normal tissue samples were not used to calibrate gene copy number alterations within breast cancer cell lines.

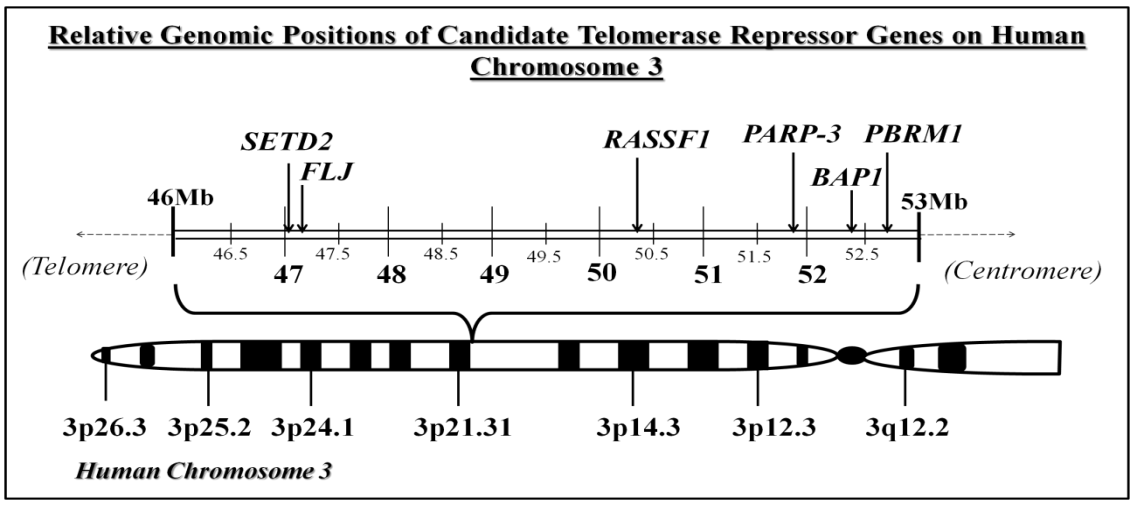


Figure 5.3 - *Graphical representation of the approximate positions of selected candidate genes RASSF1A, PARP-3, BAP1, PBRM1 within the 3p21.1-p21.3 region of human chromosome 3 and their locations relative to the SETD2-FLJ genomic locus. Genomic coordinates of all candidate genes were obtained from the National Centre of Biotechnology Information (NCBI) database.*

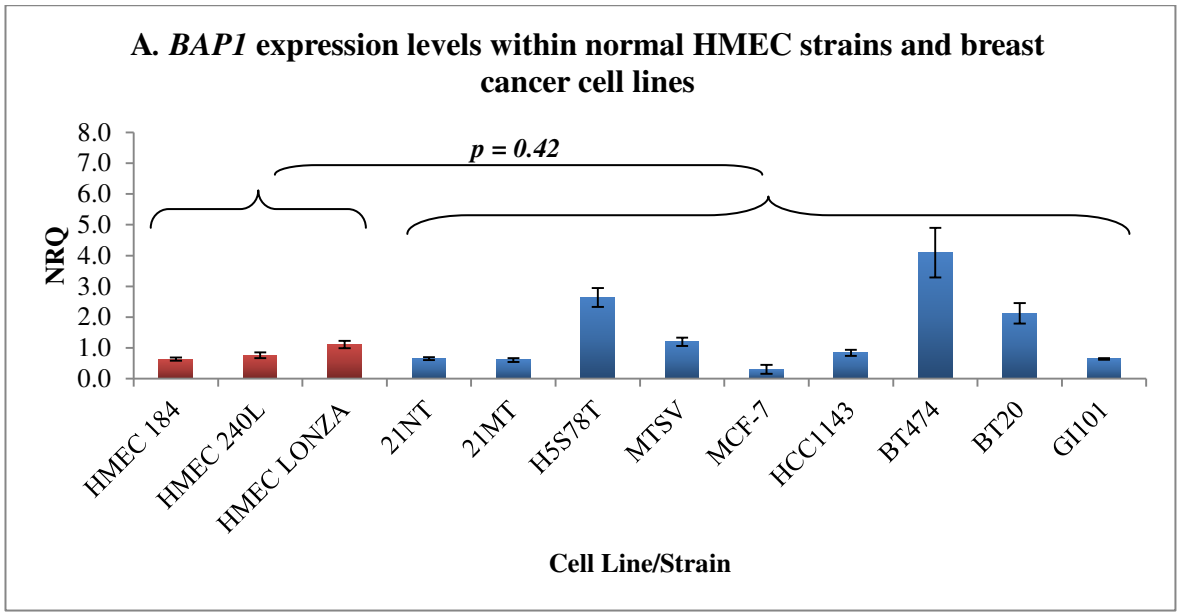
Table 5.3 - Candidate gene copy number (CN) variation analysis within normal HMEC strains and a panel of nine breast cancer cell lines

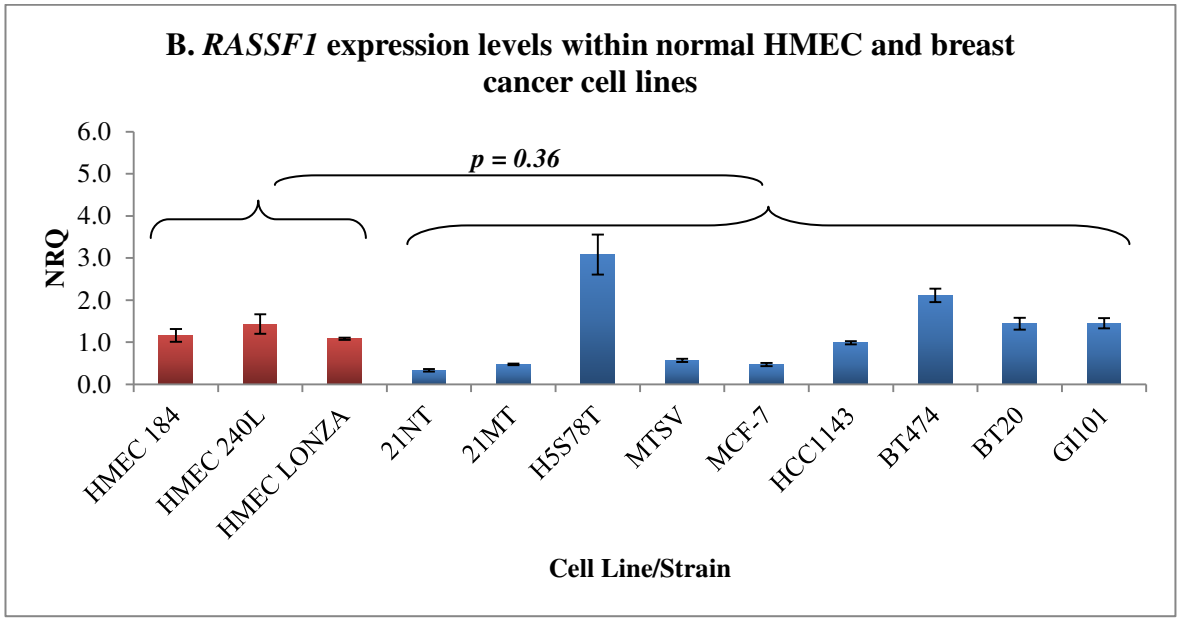
Candidate Gene		Normal HMEC Strains			Breast Cancer Cell Lines								
Gene	Distance from Telomere (Mb)	240L	LONZA	184	BT20	MCF7	H5S78T	MTSV	21NT	21MT	HCC1143	BT474	Gi101
SETD2	47.1	+/+	+/+	+/+	+/+	+/+	+/+/+	+/+/+	+/-	+/-	+/-	+/-	+/-
FLJ	47.2	+/+	+/+	+/+	+/+	+/+	+/+/+	+/+/+	+/-	+/-	+/-	+/-	+/-
PARP3	51.9	+/+	+/+	+/+	+/+	+/+	+/+/+	+/+/+	+/-	+/-	+/-	+/-	+/-
BAP1	52.4	+/+	+/+	+/+	+/+	+/+	+/+/+	+/+/+	+/-	+/-	+/-	+/-	+/-
PBRM1	52.6	+/+	+/+	+/+	+/+	+/+	+/+/+	+/+	+/-	+/-	-/-	+/-	+/-

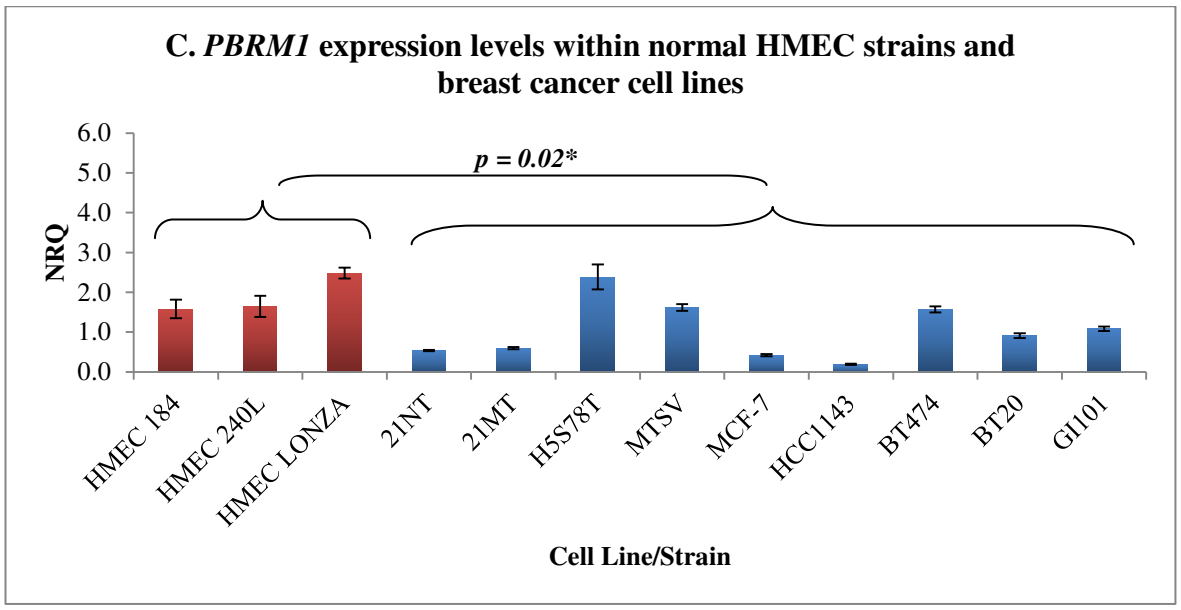
-/-	+/-	+/+	+/+/+
CN = 0	CN = 1	CN = 2	CN = 3

As shown in Table 5.3, breast cancer cell lines that initially demonstrated allele loss within the *SETD2-FLJ* genomic locus, were also found to have undergone copy number loss at *PARP-3, BAP1* and *PBRM1* loci. Based on the relative positions of each gene examined (Figure 5.3), it is likely that the *RASSF1A* locus may have also undergone copy number loss within the same breast cancer cell lines. This suggests that a minimal 5.5Mb region within chromosome 3p21.1-3p21.3, which encompasses five known tumour suppressor genes, was found to have undergone copy number loss within over half of breast cancer cell lines examined. Interestingly, the SV40-immortalized breast cell line MTSV and the HCC1143 breast cancer cell line were found to have undergone distinct copy number alterations within the *PBRM1* locus compared with other loci examined. The HCC1143 breast cancer cell line has been found to harbour an intragenic homozygous deletion of *PBRM1* involving exons 12-22 (Xia *et al.,* 2008). The CNV primer set used to detect copy number alterations of *PBRM1* within breast cancer lines employed in my study, were designed to detect a region within intron 14. These findings are therefore consistent with those presented by Xia *et al.,* (2008) and may also indicate a particularly important tumour suppressive role of *PBRM1* in breast cancer. In addition, copy number loss at all genomic loci was observed within both 21NT and 21MT cell lines, which are derived from primary breast tumour and metastatic deposits of the same patient respectively. This suggests that allele loss within the chromosome 3p21.1-p21.3 region may occur at an early point during breast tumorigenesis and prior to metastatic spread. The results presented here are consistent with previous studies demonstrating that chromosome 3p loss is a frequent and early

event in breast cancer (Ali *et al.,* 1989, Sato *et al.,* 1991, Chen *et al.,* 1994, Matsumoto *et al.,* 1997, Martinez *et al.,* 2001, Maitra *et al.,* 2001 and De Oliveira *et al.,* 2012). In addition, the results presented in Table 5.3, provide additional supporting evidence that *RASSF1A, PARP-3, BAP1* and *PBRM1* may function as important tumour suppressor genes in breast cancer. In order to explore the functional status of these candidate telomerase repressor genes further, the expression levels of *BAP1, RASSF1A, PBRM1* and *PARP-3* were examined within the same panel of breast cancer cell lines and compared with normal HMEC strains.







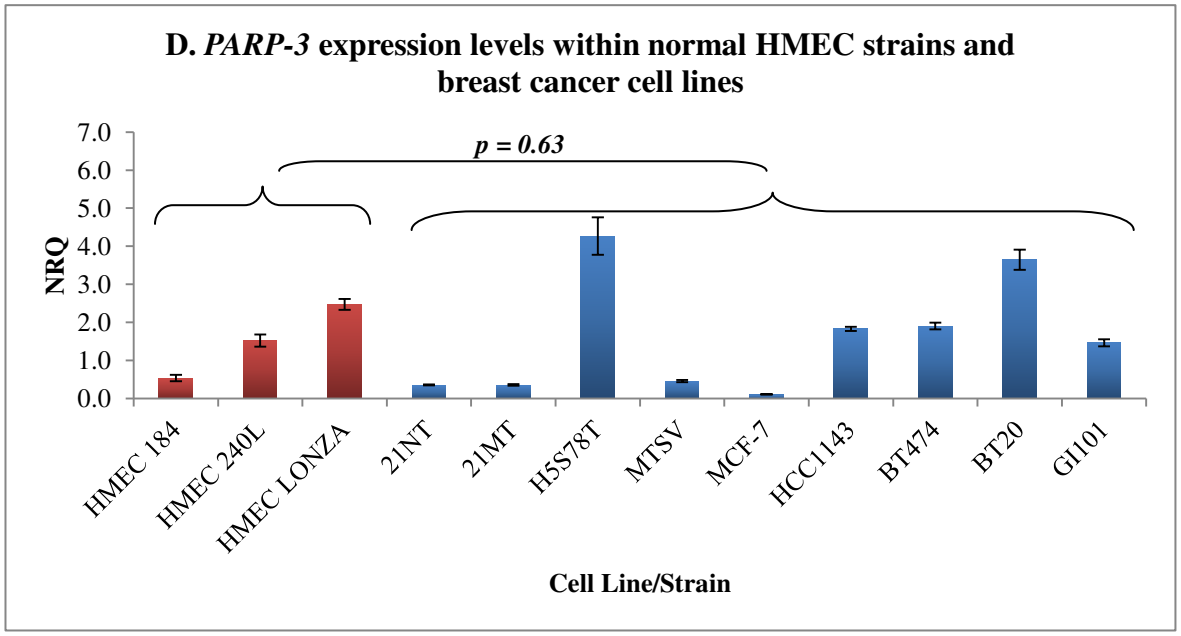


Figure 5.4 - *Normalized relative quantities (NRQ) of candidate genes* ***(A)*** *BAP1,* ***(B)*** *RASSF1,* ***(C)*** *PBRM1 and* ***(D)*** *PARP-3 mRNA levels within a panel of nine breast cancer cell lines (blue) and three normal HMEC strains (red). NRQ values are presented relative to the mean NRQ value of all samples. Error bars represent the standard error of the mean NRQ values from triplicate repeats for each sample. A student's t-test was carried out on log-transformed NRQ values to compare mean candidate gene expression of HMEC strains with breast cancer cell lines.*

Out of all candidate genes examined, only *PBRM1* expression levels were found to be significantly reduced within the majority of breast cancer cell lines relative to normal HMEC strains. Six out of nine (67%) of breast cancer cell lines displayed a 43-90% reduction in

PBRM1 expression levels compared to normal HMEC strains (Figure 5.4). By comparison, only one (11%) breast cancer cell line showed a comparable level of *BAP1* downregulation, while four (44%) showed the same level of reduction in *RASSF1* and *PARP-3* expression relative to normal HMEC strains. These findings provide evidence to show that *PBRM1, RASSF1* and *PARP-3* frequently undergo transcriptional repression within breast cancer cells and may therefore function as important tumour suppressor genes. However, it is important to note that the primers used to detect *RASSF1A* transcripts also detect other protein coding splice variants of the *RASSF1* gene locus including isoforms B, C, D and H (Table 2.3, Chapter 2.3.4). Therefore, the expression levels shown in Figure 5.4B, do not represent the expression levels of the *RASSF1A* transcript isoform alone within cell lines/strains examined.

Interestingly, a sub-set of breast cancer cell lines including 21NT, 21MT and MCF-7 were found to exhibit lower expression levels of all candidate genes relative to other breast cancer cell lines and normal HMECs. The 21NT and 21MT cell lines are derived from the same patient and are known to harbour a *p53* mutation, are *HER2*-positive, have been previously shown to produce tumours when injected into nude mice (Table 4.2, Chapter 4.3.1) and were found have undergone copy number loss of all four candidate genes (Table 5.3). On the other hand, the MCF-7 cell line has been shown to harbour wild-type copies of *p53,* is non-tumorigenic, *HER2*-negative and was not found to have undergone candidate gene copy number loss (Table 5.3). These findings suggest that silencing of tumour suppressor genes within the chromosome 3p21.1-21.3 region occurs within different molecular subtypes of breast cancer and provides further evidence that this region may possess important tumour suppressor genes.

21MT and MCF-7 cell lines are derived from the metastatic lung deposits of two individual breast cancer patients and were found to display a substantial reduction in *PBRM1* and *RASSF1* expression levels relative to HMEC cell strains (Figure 5.4B and C). This could suggest that loss of *PBRM1* and *RASSF1* function may be associated with metastatic spread and more progressive disease. Low levels of *PBRM1* were also observed within primary breast intraductal carcinoma-derived 21NT, HCC1143 and BT20 cell lines relative to normal HMEC strains (Figure 5.4C). Similarly, *RASSF1* expression levels were also found to be markedly reduced within 21NT cells compared with HMEC strains (Figure 5.4B). These results therefore suggest that silencing of *PBRM1* and *RASSF1* expression may occur at an early stage during breast cancer development.

The H5S78T, BT474 and BT20 breast cancer cell lines were consistently found to exhibit higher candidate gene expression levels compared to other breast cancer cell lines and increased levels of *BAP1, RASSF1* and *PARP-3* expression relative to normal HMEC strains (Figure 5.4). A common feature shared by these breast cancer cell lines is the presence of a *p53* gene mutation (Table 4.3, Chapter 4.3.1). As shown in Table 5.3, H5S78T, BT20 and BT474

cell lines have been found to harbour three, two and one allele(s) at candidate gene loci respectively, which suggests that gene copy number loss is not always associated with reduced expression levels.

The H5S78T cell line was found to consistently display higher candidate gene expression levels relative to normal HMEC strains (Figure 5.4). As demonstrated in Table 5.3, this particular cell line was found to have undergone copy number gain at all loci examined, which may provide an explanation as to why the H5S78T cell line exhibited high levels of candidate gene expression. However, the SV40-immortalized MTSV cell line, which was found to harbour three alleles at the majority of loci examined (Table 5.3), exhibited substantially lower levels of *RASSF1, PBRM1* and *PARP-3* expression relative to normal HMEC strains (Figure 5.4B, C and D). These observations also suggest changes in gene copy number are not always associated with concomitant changes in gene expression levels. Other mechanisms of transcriptional silencing may function to inhibit the expression of important tumour suppressor genes within the 3p21.1-p21.3 region in breast cancer cells.

The results presented here demonstrate that the expression levels of several tumour suppressor genes localized to the 3p21.3-21.1 region are de-regulated in breast cancer, which can occur independently of gene copy number alterations. Out of all putative tumour suppressor genes examined, *RASSF1, PBRM1* and *PARP-3* gene expression levels were found to be substantially reduced within 44-67% of breast cancer cell lines employed during this study, which provides additional supporting evidence for the idea that these genes may function as important tumour suppressors in breast cancer cells. A high proportion of breast cancer cell lines were found to display similar to or higher levels of *BAP1* expression relative to normal HMEC strains, which contradicts the hypothesis that this gene may function as a tumour suppressor in breast cancer. However, it is possible that other mechanisms of functional inactivation such as mutation, post-transcriptional or post-translational modification defects, may exist within breast cancer cell lines to abrogate the function of BAP1.

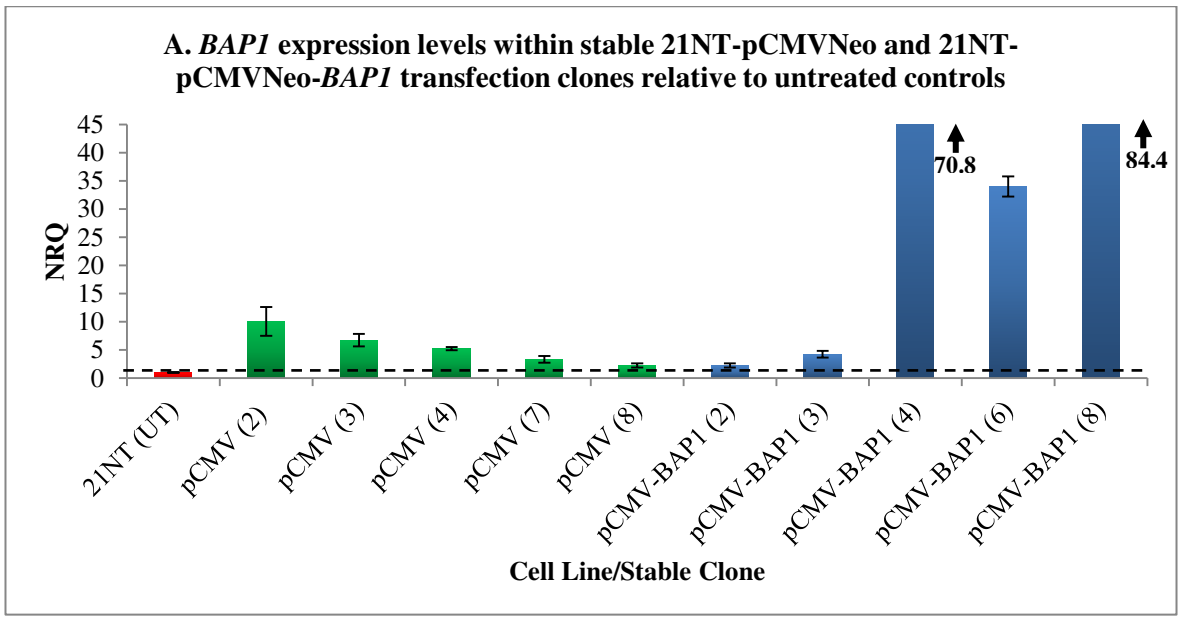
5.3.2 BAP1 AS A CANDIDATE TELOMERASE REPRESSOR AND TUMOUR SUPPRESSOR GENE IN BREAST CANCER CELLS

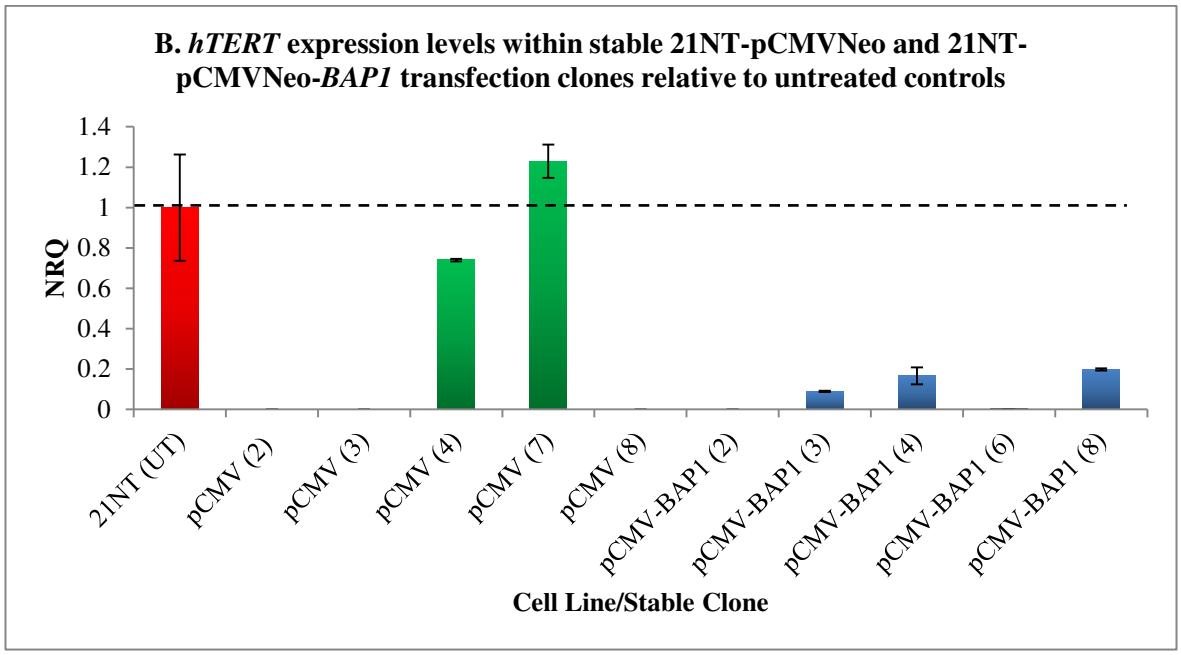
5.3.2.1 The effect of *BAP1* overexpression on *hTERT* expression levels and telomerase activity within the 21NT cell line

Gene expression analysis of *BAP1* within breast cancer cell lines and normal HMEC strains showed that this gene does not frequently undergo transcriptional repression within breast cancer cell lines. Independent studies have shown that reconstitution of wild-type *BAP1* expression suppresses the growth of breast cancer cells *in vitro* and lung cancer cells *in vitro*

and *in vivo*, which provides evidence that *BAP1* may function as a powerful tumour suppressor gene in multiple cancer types (Jensen *et al.,* 1998, Ventii *et al.,* 2008). Therefore, the initial aim of this study was to determine whether *BAP1* could play a functional role as a repressor of *hTERT* gene expression and telomerase activity in breast cancer by forced overexpression of *BAP1* within the 21NT breast cancer cell line. 21NT cells were transfected with either negative control pCMVNeo or target gene pCMVNeo-*BAP1* plasmid vector constructs and cultured in the presence of neomycin for two weeks to select for stable transfection clones. A total of five 21NT-pCMVNeo and five 21NT-pCMVNeo-*BAP1* transfection clones were isolated and propagated as individual cell lines.

As shown in Figure 5.5A, stable 21NT-pCMVNeo-*BAP1* transfection clones were found to exhibit between 2-84 fold higher levels of *BAP1* relative to untreated 21NT cells. These stable clones were also found to display either undetectable or a greater than 80% reduction in *hTERT* expression levels compared to untreated controls (Figure 5.5B). Similarly all stable 21NT-pCMNeo-*BAP1* clones were found to exhibit a greater than 90% reduction in telomerase activity relative to untreated 21NT cells (Figure 5.5C). These findings on their own may appear to suggest that *BAP1* may function as a repressor of *hTERT* and telomerase in 21NT cells. However, curiously, all stable negative control 21NT-pCMVNeo clones were also found to express 2-10 fold higher levels of *BAP1* relative to untreated 21NT cells. Three out of five stable 21NT-pCMVNeo clones were found to display undetectable levels of *hTERT*, while two clones exhibited similar levels of *hTERT* expression to untreated 21NT cells (Figure 5.5B). In addition, a 67-89% reduction in telomerase activity was observed within three stable negative control 21NT-pCMVNeo clones relative to untreated controls (Figure 5.5C). These findings are consistent with those presented in Chapter 4.3.2, where transient and stable expression of the pCMVNeo plasmid vector within 21NT cells was associated with a consistent deregulation in *hTERT* expression and telomerase activity. This was thought to be due to insertional mutagenesis of important *hTERT* regulatory regions. It is therefore unclear whether exogenous expression of *BAP1* is responsible for the observed reduction in *hTERT* expression and telomerase repression within stable 21NT-pCMVNeo-*BAP1* clones. However, stable overexpression of *BAP1* within 21NT cells was found to be associated with a greater and more consistent repression of *hTERT* transcription and telomerase activity compared with stable empty vector controls, which provides evidence to show that *BAP1* may function as a negative regulator of *hTERT* (Figure 5.5).


A. BAP1 expression levels within stable 21NT-pCMVNeo and 21NT-pCMVNeo-BAP1 transfection clones relative to untreated controls
NRQ
45
40
35
30
25
20
15
10
5
0
70.8
84.4
21NT (UT)
pCMV (2)
pCMV (3)
pCMV (4)
pCMV (7)
pCMV (8)
pCMV-BAP1 (2)
pCMV-BAP1 (3)
pCMV-BAP1 (4)
pCMV-BAP1 (6)
pCMV-BAP1 (8)
Cell Line/Stable Clone


B. hTERT expression levels within stable 21NT-pCMVNeo and 21NT-pCMVNeo-BAP1 transfection clones relative to untreated controls
NRQ
1.4
1.2
1
0.8
0.6
0.4
0.2
0
21NT (UT)
pCMV (2)
pCMV (3)
pCMV (4)
pCMV (7)
pCMV (8)
pCMV-BAP1 (2)
pCMV-BAP1 (3)
pCMV-BAP1 (4)
pCMV-BAP1 (6)
pCMV-BAP1 (8)
Cell Line/Stable Clone

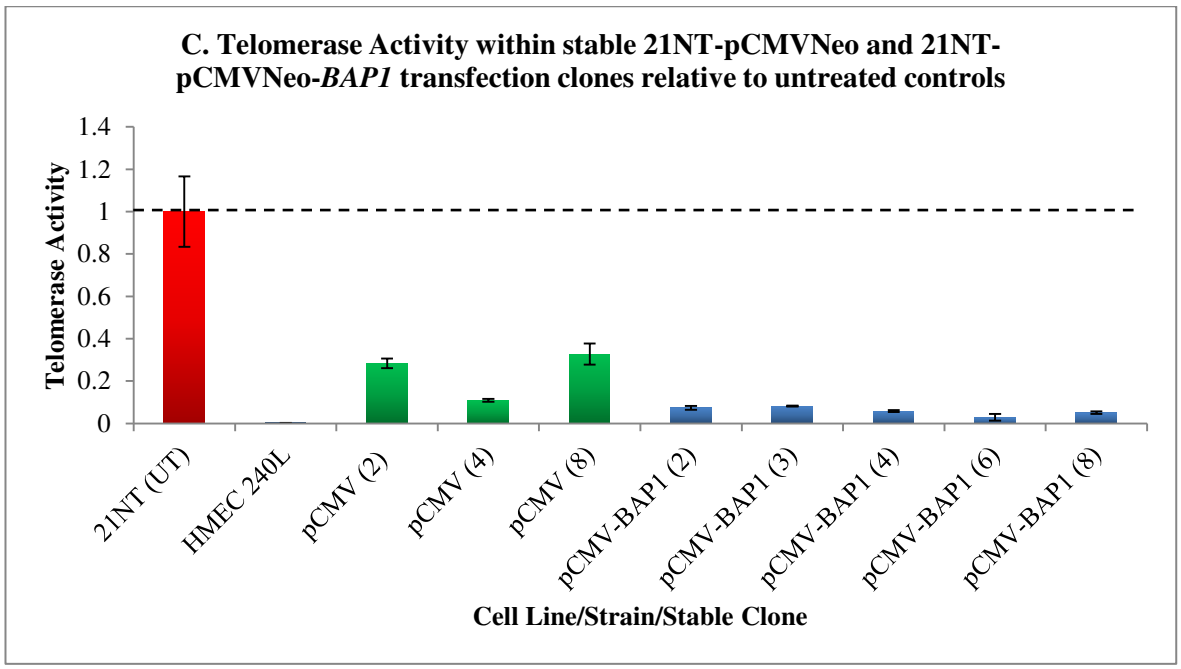
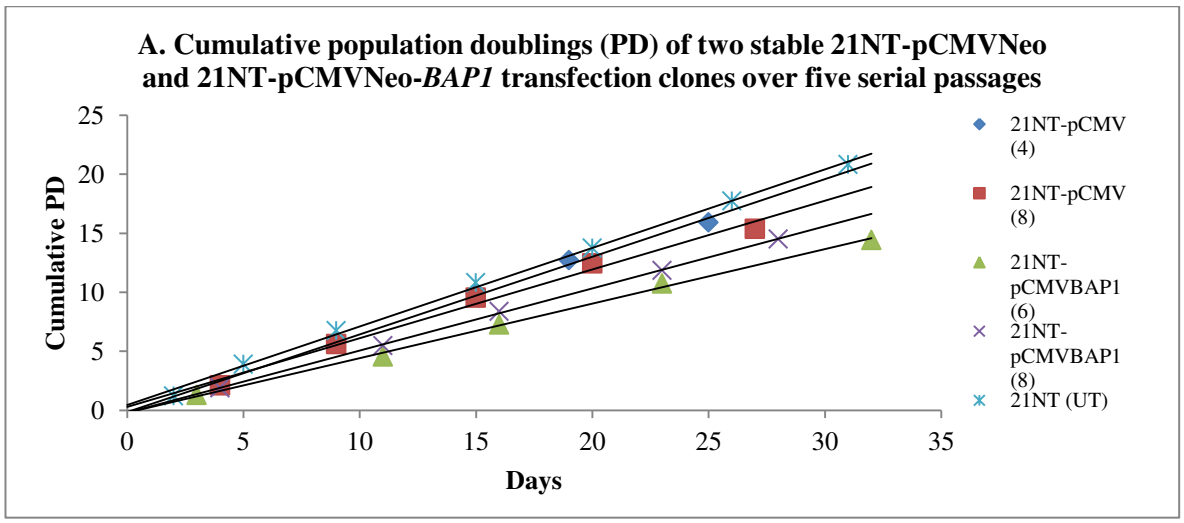
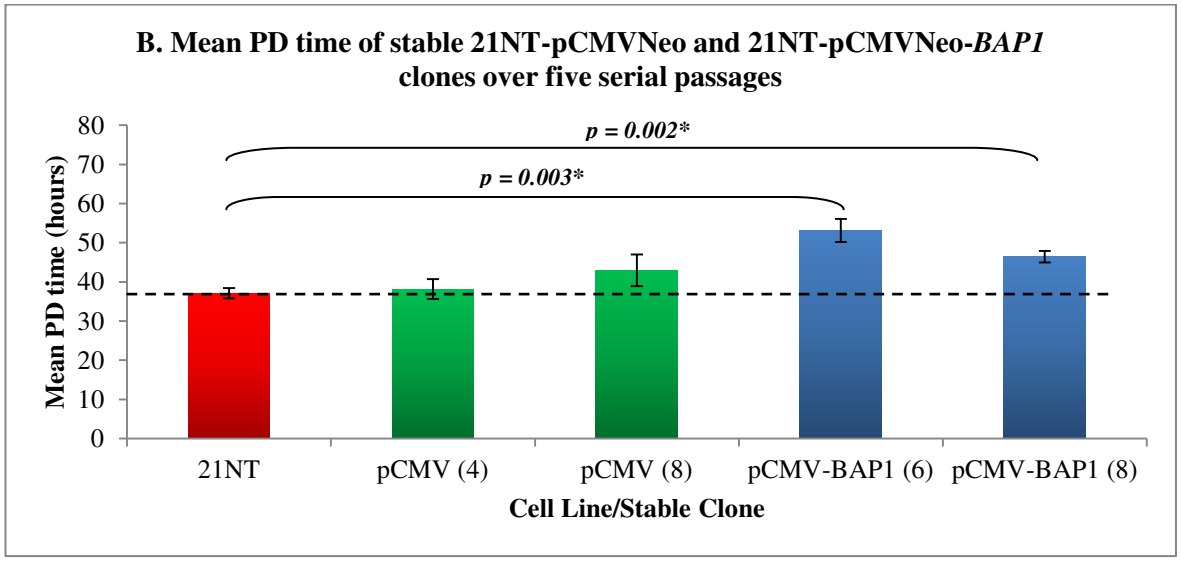


Figure 5.5- *Normalized relative quantities (NRQ) of* ***(A)*** *BAP1 and* ***(B)*** *hTERT mRNA within stable empty vector control 21NT-pCMVNeo (green) and target gene 21NT-pCMVNeo-BAP1(blue) transfection clones relative to the mean ± SD NRQ values of three untreated 21NT cell samples (red). Error bars for stable transfection clones represent the standard error of the mean NRQ values obtained from triplicate repeats from each sample.* ***(C)*** *Telomerase activity within stable 21NT-pCMVNeo and 21NT-pCMVNeo-BAP1 transfection clones relative to untreated 21NT cells. Error bars represent the standard error of the mean telomerase activity of triplicate repeats for each sample. The HMEC 240L cell strain was included to represent a normal human control.*

5.3.2.2 The effect of stable BAP1 overexpression on 21NT cell growth and proliferative capacity

Introduction of a normal copy of human chromosome 3 into the 21NT cell line is associated with a greater than 90% reduction in telomerase activity and delayed permanent growth arrest following 10-12 or 15-18 population doublings (PD) within the majority of hybrid colonies (Cuthbert *et al.,* 1999). The results presented above indicate that exogenous expression of *BAP1* within the 21NT cell line is associated with a substantial and consistent decrease in *hTERT* mRNA levels and a greater than 90% reduction in telomerase activity. In order to examine the effect of *BAP1* overexpression on the growth rate and replicative potential of 21NT cells, the cumulative population doublings (PD) and mean PD time of two stable negative control 21NT-pCMVNeo and two stable 21NT-pCMVNeo-*BAP1* clones were examined over five serial passages.


A. Cumulative population doublings (PD) of two stable 21NT-pCMVNeo and 21NT-pCMVNeo-*BAP1* transfection clones over five serial passages
Cumulative PD
Days
21NT-pCMV (4)
21NT-pCMV (8)
21NT-pCMVBAP1 (6)
21NT-pCMVBAP1 (8)
21NT (UT)


B. Mean PD time of stable 21NT-pCMVNeo and 21NT-pCMVNeo-*BAP1* clones over five serial passages
p = 0.002*
p = 0.003*
Mean PD time (hours)
21NT
pCMV (4)
pCMV (8)
pCMV-BAP1 (6)
pCMV-BAP1 (8)
Cell Line/Stable Clone

C. Cellular morphology of untreated (UT) 21NT cells, stable 21NT-pCMVNeo and 21NT-pCMVNeo-*BAP1* transfection clones

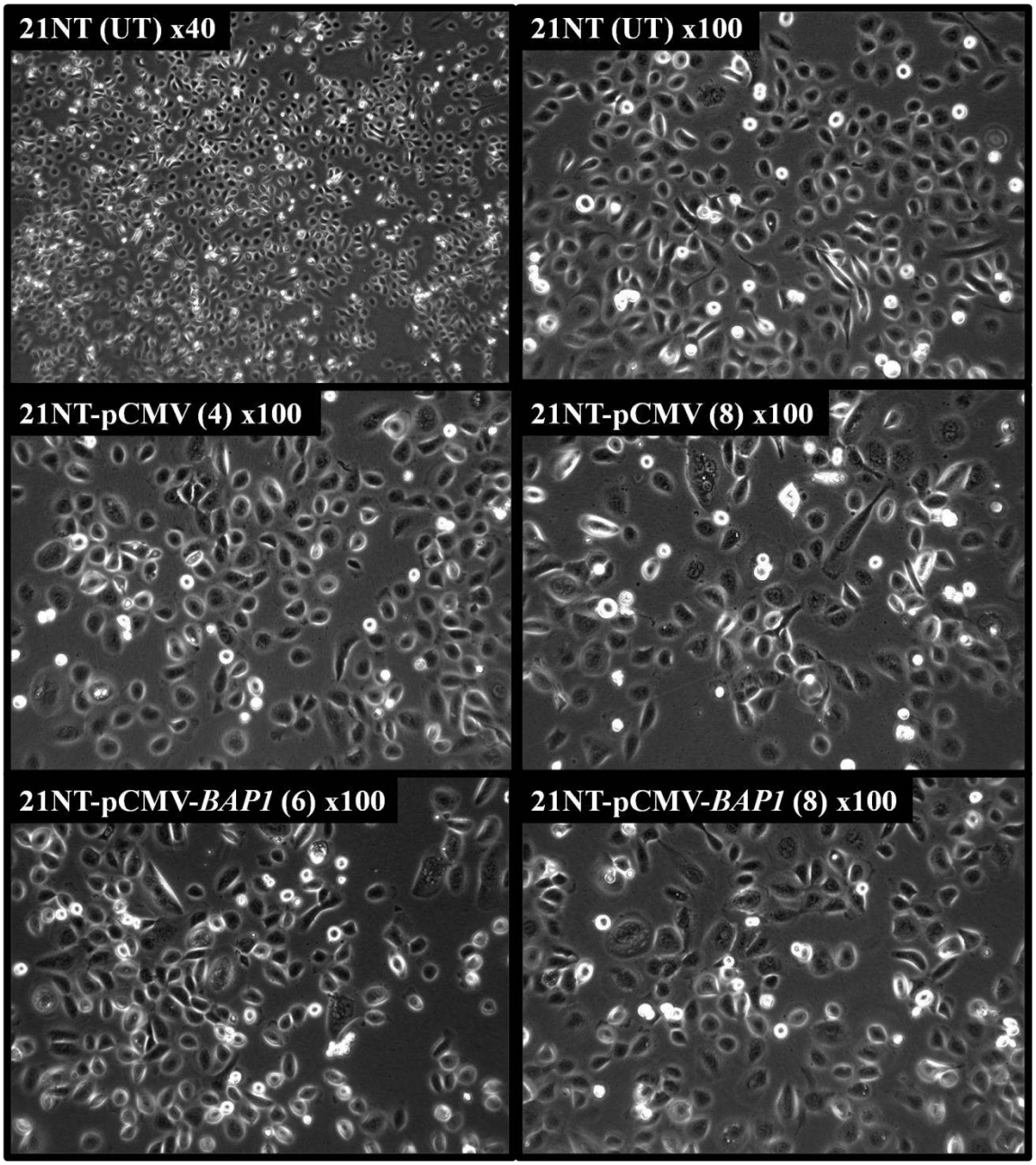


Figure 5.6 - (A) *Cumulative number of population doublings (PD) of untreated 21NT cells, stable 21NT-pCMVNeo transfection clones 4 and 8 and stable 21NT-pCMVNeo-BAP1 transfection clones 6 and 8 over five serial passages.* ***(B)*** *The mean PD time (hours) for all samples across five serial passages. A student's t-test was used to compare the means of sample groups* ***(C)*** *Representative photomicrograph images (x40 or x100 magnification) of untreated 21NT cells, stable empty vector 21NT-pCMVNeo and 21NT-pCMVNeo-BAP1 transfection clones*

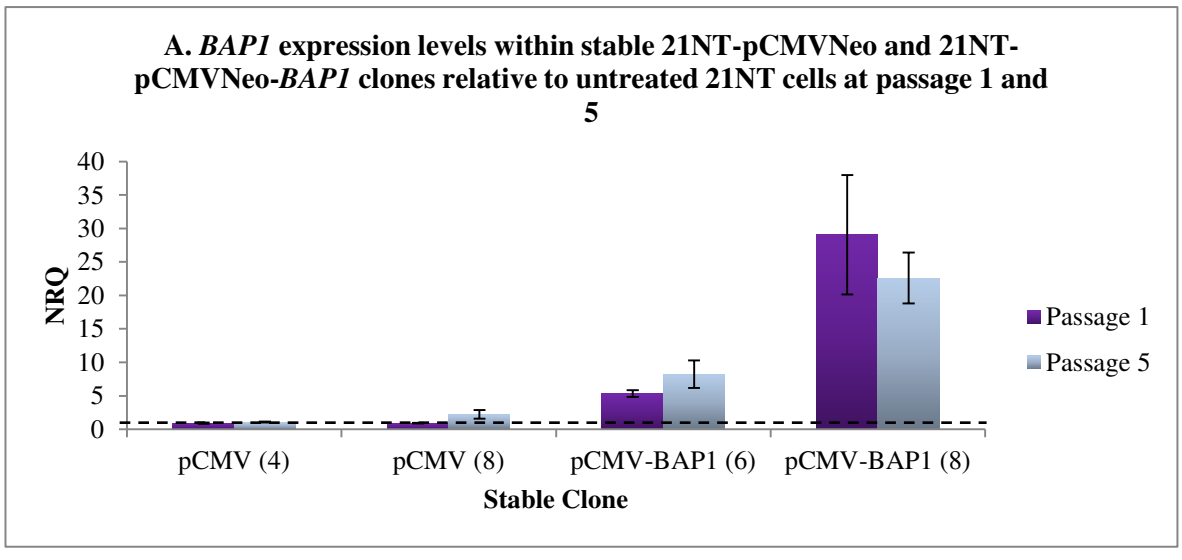
The cell growth rate of all stable transfection clones was examined after the clones were picked and had reached 90% confluence on individual P35 dishes (passage 1). Therefore, colonies had been in culture for 21-25 days prior to cell growth analysis. According to the calculated mean PD time of each individual colony (Figure 5.6B), 21NT-pCMVNeo clones 4 and 8 and 21NT-pCMVNeo-*BAP1* clones 6 and 8 were estimated to have undergone approximately 11-13 PD prior to analysis. As shown in Figure 5.6A and C, stable 21NT-pCMVNeo-*BAP1* clones 6 and 8 were found to proliferate continuously for a further 14 PD with no observable morphological features of senescence or cell growth arrest. This demonstrates that stable overexpression of wild-type *BAP1* was not associated with induction of senescence pathways and permanent cell growth arrest of 21NT cells. However, both 21NT-pCMVNeo-*BAP1* clones 6 and 8 were found to display a moderately (but significantly) longer mean PD time of 53.1 and 46.5 hours respectively compared to untreated 21NT cells, which were found to exhibit a mean PD time of 37.1 hours (Figure 5.6B). As shown in Figure 5.5A, these clones were initially found to display 34 and 84-fold higher levels of *BAP1* expression relative to untreated 21NT cells. Therefore, exogenous *BAP1* expression within 21NT cells is associated with a significant reduction in cell growth rate. Stable control 21NT-pCMVNeo clones 4 and 8 were also found to proliferate continuously for a further 15-16 PD and display a similar mean PD time as untreated 21NT cells (Figure 5.6A and B). These clones were initially found to exhibit 2-5-fold higher levels of *BAP1* and a substantial reduction in *hTERT* expression levels and telomerase activity relative to untreated 21NT cells (Figure 5.5). Therefore, induction of endogenous *BAP1* expression within 21NT cells was not associated with cell growth arrest or a reduction in growth rate. These results could also suggest that a greater than 5-fold increase in *BAP1* expression levels may be required to suppress 21NT breast cancer cell growth.

The findings presented here so far show that 21NT-pCMVNeo-*BAP1* transfection clones 6 and 8 were initially found to exhibit a greater than 90% reduction in telomerase activity but were also found to proliferate continuously for approximately 25-27 PD. Cuthbert *et al.,* (1999) demonstrated that 21NT-chromosome 3 hybrids exhibiting a greater than 90% reduction in telomerase activity entered delayed permanent growth arrest, which was thought to be due to progressive telomere shortening and induction of cellular replicative senescence pathways. Therefore, in order to determine whether the observed levels of telomerase repression initially exhibited by 21NT-pCMVNeo-*BAP1* clones 6 and 8 were maintained over the course of five serial passages, *BAP1* expression levels and telomerase activity was examined at passages 1 and 5. As shown in Figure 5.7A, stable 21NT-pCMVNeo-*BAP1* transfection clones 6 and 8 were found to display substantially higher levels of *BAP1* expression relative to untreated 21NT cells and negative control 21NT-pCMVNeo clones at passages 1 and 5, indicating that higher levels of *BAP1* expression were maintained over five serial passages. However, *BAP1* expression levels within 21NT-pCMVNeo-*BAP1* clones 6 and 8 were found to be substantially lower in

absolute terms than initially observed (Figure 5.5A), which may reflect the inter-clonal variability of *BAP1* expression exhibited by wild type 21NT cells.

As demonstrated within Figure 5.7B, both 21NT-pCMVNeo-*BAP1* clones were found to exhibit approximately 85% lower levels of telomerase activity relative to untreated 21NT cells at passage 1, which is similar to initial findings presented in Figure 5.5C. Interestingly, both stable 21NT-pCMVNeo-*BAP1* clones were found to display approximately three-fold higher levels of telomerase activity at passage 5 compared to passage 1, which indicates that telomerase repression was not maintained over the course of five serial passages. Later-passage stable 21NT-pCMVNeo-*BAP1* clones were found to exhibit similar levels of telomerase activity to stable negative control 21NT-pCMVNeo clones (Figure 5.7B).

These findings demonstrate that forced overexpression of *BAP1* within 21NT cells harboring a single copy of *BAP1,* is associated with transient telomerase repression within 21NT cells. The observed increase in telomerase activity within later passage 21NT-pCMVNeo-*BAP1* clones may have prevented progressive telomere shortening and induction of cell growth arrest. Early-passage 21NT-pCMVNeo-*BAP1* stable clones were found to display substantially lower levels of *hTERT* expression and telomerase activity compared to untreated 21NT cells and stable negative control 21NT-pCMVNeo clones (Figure 5.5B and C and Figure 5.7B), which suggests that *BAP1* may function as a negative regulator of *hTERT*. It is possible that, in addition to *BAP1,* other co-factors or transcription factors may be required to maintain stable *hTERT* repression. Ultimately, these findings provide evidence to show that *BAP1* is unlikely to be responsible for the strong telomerase repression and cell growth arrest observed within the majority of 21NT-chromosome 3 hybrids generated in the Cuthbert *et al.,* (1999) study. However, these results suggest that *BAP1* may function as an important component of a telomerase regulatory pathway.



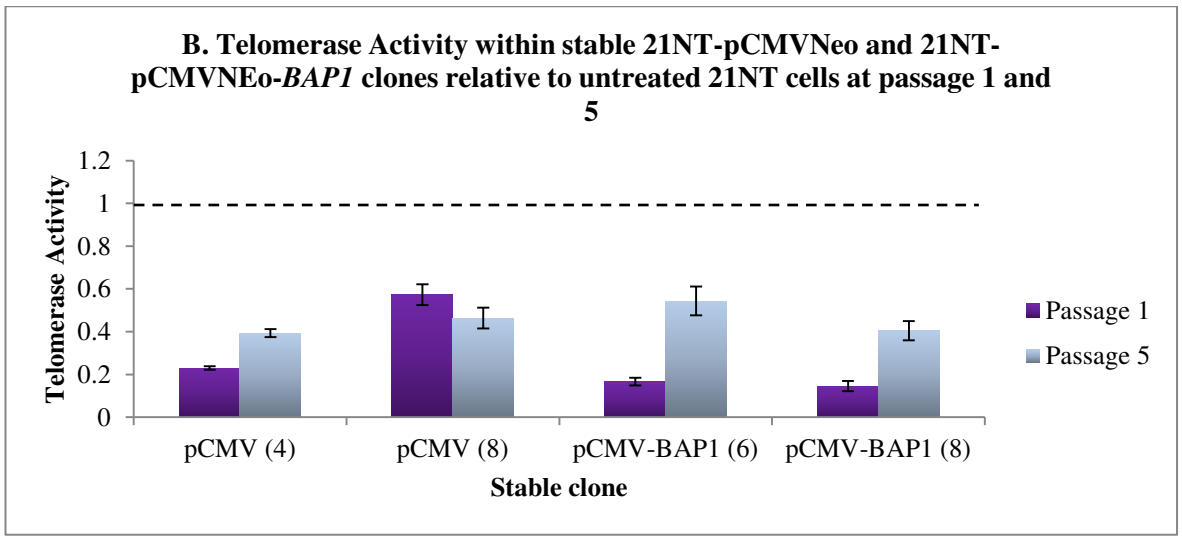
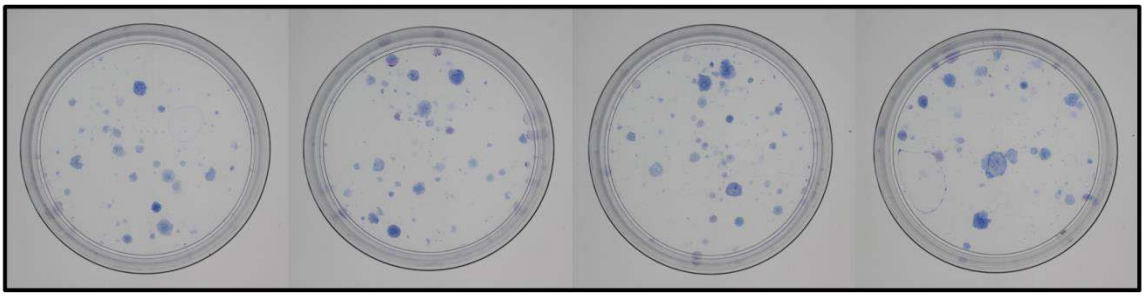


Figure 5.7 - (A) *Normalized relative quantities (NRQ) of BAP1 mRNA within two stable 21NT-pCMVNeo and two 21NT-pCMVNeo-BAP1 transfection clones relative to three individual untreated 21NT cell samples (indicated by a dotted line) at passage 1 and following five serial passages. Error bars represent the standard error of the mean for triplicate repeats of each sample.* ***(B)*** *Telomerase activity within the same samples relative to untreated 21NT cells (indicated by a dotted line). Error bars represent the standard deviation of the mean for triplicate repeats of each sample.*

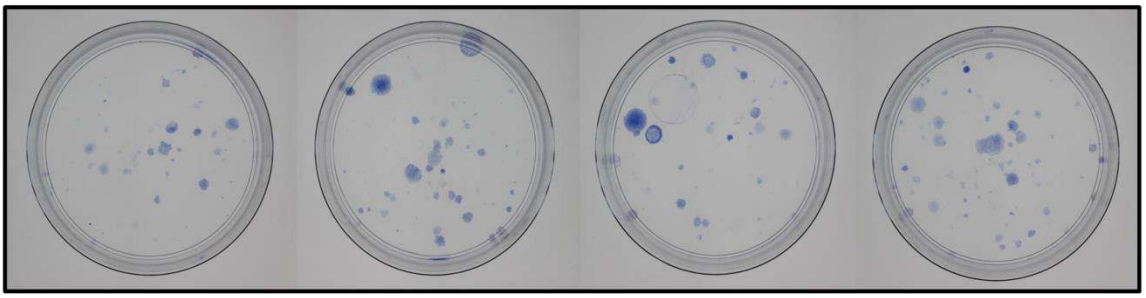
Studies have shown that forced wild-type *BAP1* overexpression within MCF-7 and HCC1937 breast carcinoma cell lines is associated with cell growth suppression (Jensen *et al.,* 1998, Ventii *et al.,* 2008). As shown in Figure 5.6, overexpression of *BAP1* within 21NT cells was associated with a significant reduction in cell growth rate compared with untreated controls. Therefore, in order to examine the effect of wild type *BAP1* overexpression on the survival and proliferation of 21NT breast cancer cells, the number and size of stable negative control 21NT-pCMVNeo and 21NT-pCMVNeo-*BAP1* cell clones that emerged following two weeks of neomycin selection (post-transfection) was examined. As shown in Figure 5.8A and B, no significant difference was observed between the number of stable negative control 21NT-pCMVNeo and 21NT-pCMVNeo-*BAP1* cell clones that emerged, indicating that exogenous expression of *BAP1* is not associated with changes in the clonogenic survival of 21NT cells. In contrast to results presented in Figure 5.6, both 21NT-pCMVNeo and 21NT-pCMVNeo-*BAP1* stable clones were found to display a similar average clone diameter, indicating that forced overexpression of B*AP1* within the 21NT cell line is not associated with a significant change in colony cell growth (Figure 5.8C and D).

A. Neomycin-resistant cell clones that emerged 2 weeks following transfection of 21NT cells with pCMVNeo or pCMVNeo-*BAP1* plasmid vectors

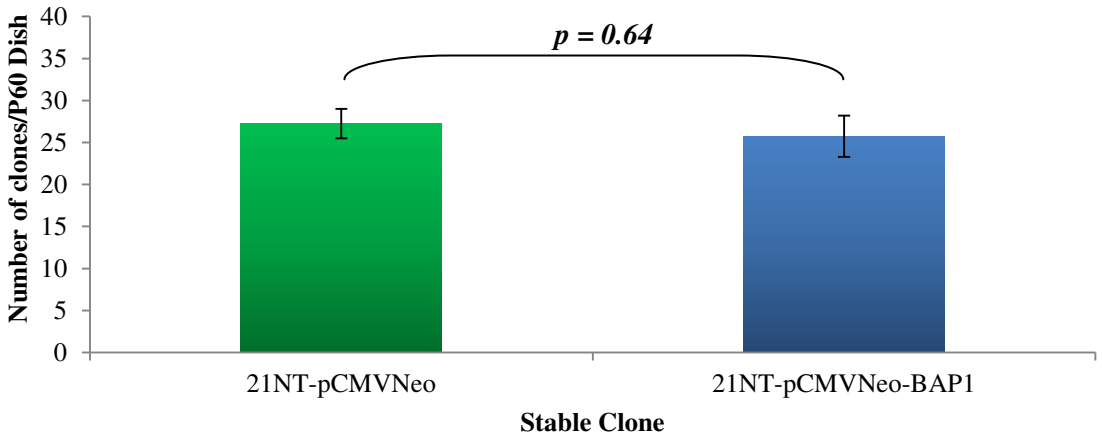
(i) 21NT+pCMVNeo



(ii) 21NT+pCMVNeo-*BAP1*



B. Average number of stable 21NT-pCMVNeo and 21NT-pCMVNeo-*BAP1* clones that emerged following two weeks of neomycin selection



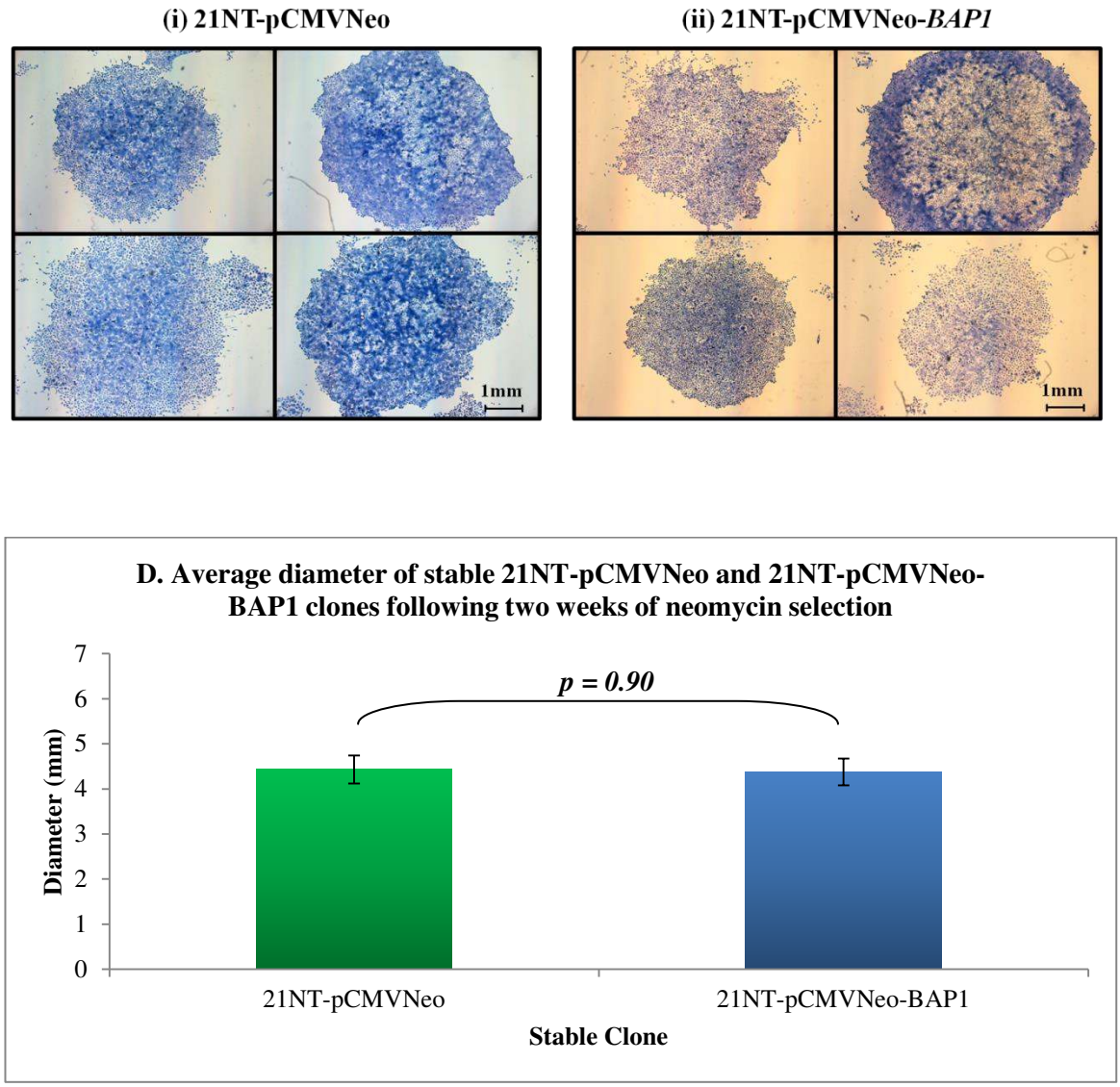


Figure 5.8 - ***(A)*** *Photographs of P60 dishes containing fixed and stained stable* ***(i)*** *negative control 21NT-pCMVNeo and* ***(ii)*** *target gene 21NT-pCMVNeo-BAP1 colonies that emerged (post-transfection) following two weeks of neomycin selection.* ***(B)*** *The mean number of negative control (green) and BAP1 target gene (blue) stable colonies present across four individual P60 dishes for each plasmid construct.* ***(C)*** *Representative photomicrographs (2.5x magnification) of fixed and stained stable* ***(i)*** *21NT-pCMVNeo and* ***(ii)*** *21NT-pCMVNeo-BAP1 clones that emerged following two weeks of neomycin selection.* ***(D)*** *The average diameter (mm) of 11-13 individual 21NT-pCMVNeo (green) and 21NT-pCMVNeo-BAP1 (blue) clones. Error bars represent the standard error of the mean number of clones/clone diameter for each group. A student's t-test was used to compare the mean of both groups.*

5.3.3 *RASSF1A* AS A CANDIDATE TELOMERASE REPRESSOR AND TUMOUR SUPPRESSOR GENE IN BREAST CANCER CELLS

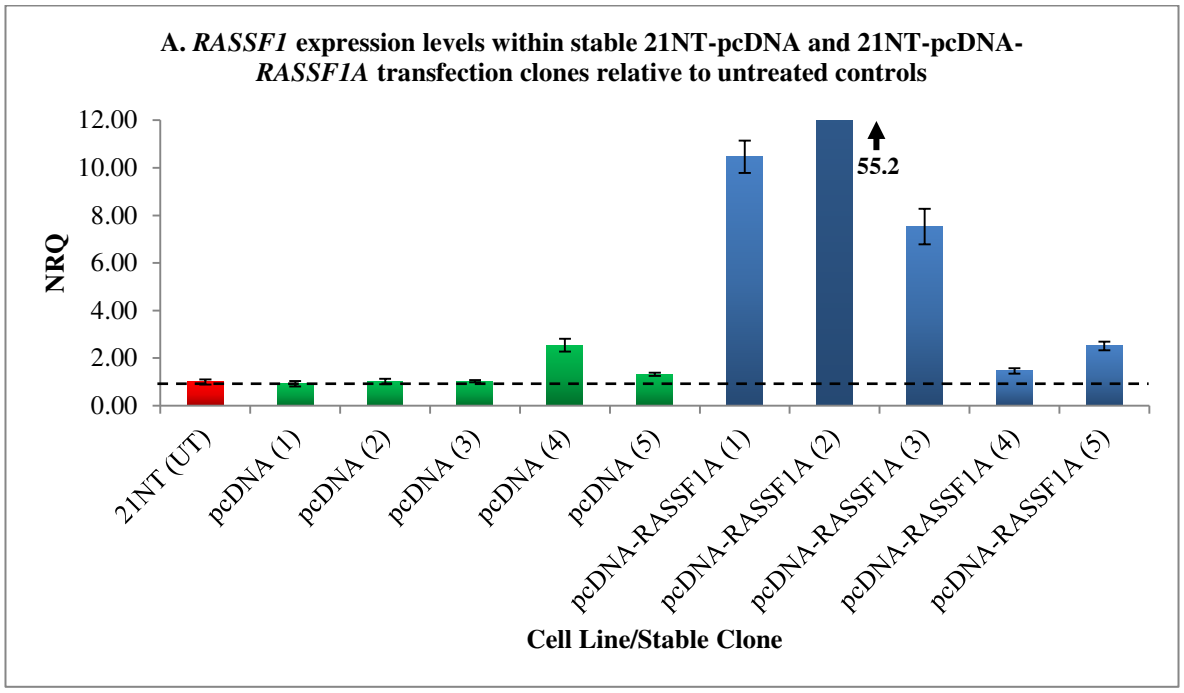
5.3.3.1 The effect of *RASSF1A* overexpression on *hTERT* expression levels and telomerase activity within 21NT cells

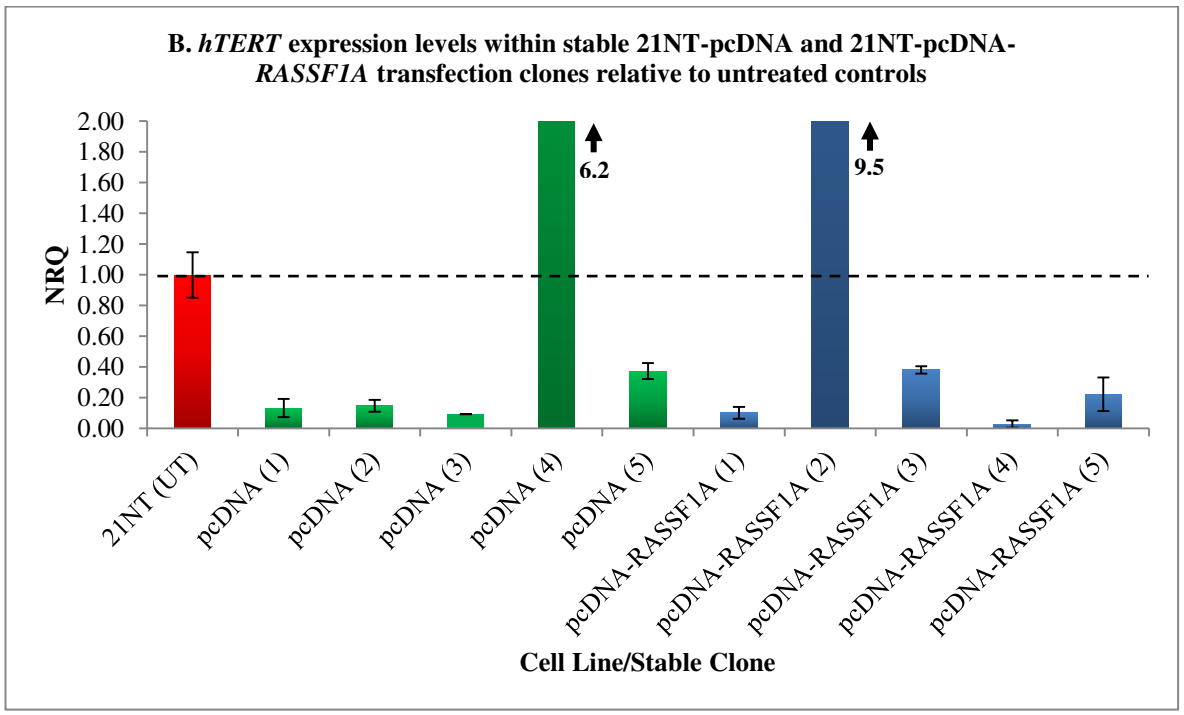
The findings presented above provide some evidence to show that *BAP1* may function as a transcriptional repressor of *hTERT*. However, it is unlikely that *BAP1* is responsible for the observed telomerase repression and cell growth arrest within 21NT-chromosome 3 hybrids generated in the Cuthbert *et al.,* (1999) study. Therefore, the functional role of *RASSF1A* as a candidate repressor of *hTERT* expression and telomerase activity within the 21NT breast cancer cell line was investigated. To do this, individual dishes of 21NT cells were transfected with the pcDNA3.1/V5-His-TOPO/*LacZ* (pcDNA) plasmid vector or a pcDNA3.1(+)-*RASSF1A* plasmid construct containing the human *RASSF1A* cDNA (pcDNA-*RASSF1A*, kindly provided by Professor Reinhard Damman, Justus-Liebig University). 48 hours following transfection, cells were cultured in the presence of neomycin for two weeks to select for stable clones. A total of five negative control 21NT-pcDNA and five target gene 21NT-pcDNA-*RASSF1A* clones were isolated and propagated as individual cell lines.

As shown in Figure 5.9A, three out of five stable 21NT-pcDNA-*RASSF1A* clones (1, 2 and 3) were found to exhibit 8-55 fold higher levels of *RASSF1* expression relative to untreated 21NT cells. The remaining two 21NT-pcDNA-*RASSF1A* stable clones (4 and 5) were found to display similar levels of *RASSF1A* expression to negative control 21NT-pcDNA and untreated 21NT cells. As shown in Figure 5.9B, four out of five 21NT-pcDNA-*RASSF1A* clones were found to exhibit a 62-97% reduction in *hTERT* expression levels relative to untreated controls; however only half of these clones were found to display increased levels of relative *RASSF1A* expression. 21NT-pcDNA-*RASSF1A* clone 2, which showed the highest level of *RASSF1A* expression relative to untreated controls, was found to exhibit a 9.5-fold increase in *hTERT* expression levels (Figure 5.9B). These findings demonstrate that forced overexpression of *RASSF1A* within 21NT cells is not always associated with a reduction in *hTERT* expression. Moreover, four out of five negative control stable 21NT-pcDNA clones were also found to exhibit a 63-87% reduction in *hTERT* expression levels relative to untreated controls. Therefore, the increase in *RASSF1A* expression was unlikely to have been responsible for the observed transcriptional repression of *hTERT* within 21NT-pcDNA-*RASSF1A* clones 1 and 3 (Figure 5.9A and B).

Two out of five stable 21NT-pcDNA-*RASSF1A* clones (2 and 4) were found to exhibit a 43-47% reduction in telomerase activity relative to untreated 21NT cells, while the remaining three clones displayed a similar level of telomerase activity to untreated 21NT cells and negative control 21NT-pcDNA stable clones (Figure 5.9C). 21NT-pcDNA-*RASSF1A* clone 2,

but not clone 4, was found to express substantially higher levels of *RASSF1* expression (55-fold higher) relative to untreated controls and negative control 21NT-pcDNA stable clones (Figure 5.9A). 21NT-pcDNA-*RASSF1A* clones 1 and 3, which were found to express over 8-fold higher levels of *RASSF1* (Figure 5.9A) were found to exhibit no significant change in telomerase activity relative to untreated controls (Figure 5.9C), Therefore, consistent with the effect on *hTERT* transcription, forced overexpression of *RASSF1* within 21NT cells is not always associated with a reduction in telomerase activity levels. These findings therefore provide some evidence to show that *RASSF1A* is unlikely to be responsible for the observed telomerase repression exhibited by 21NT-chromosome 3 hybrids within the Cuthbert *et al.,* (1999) study.





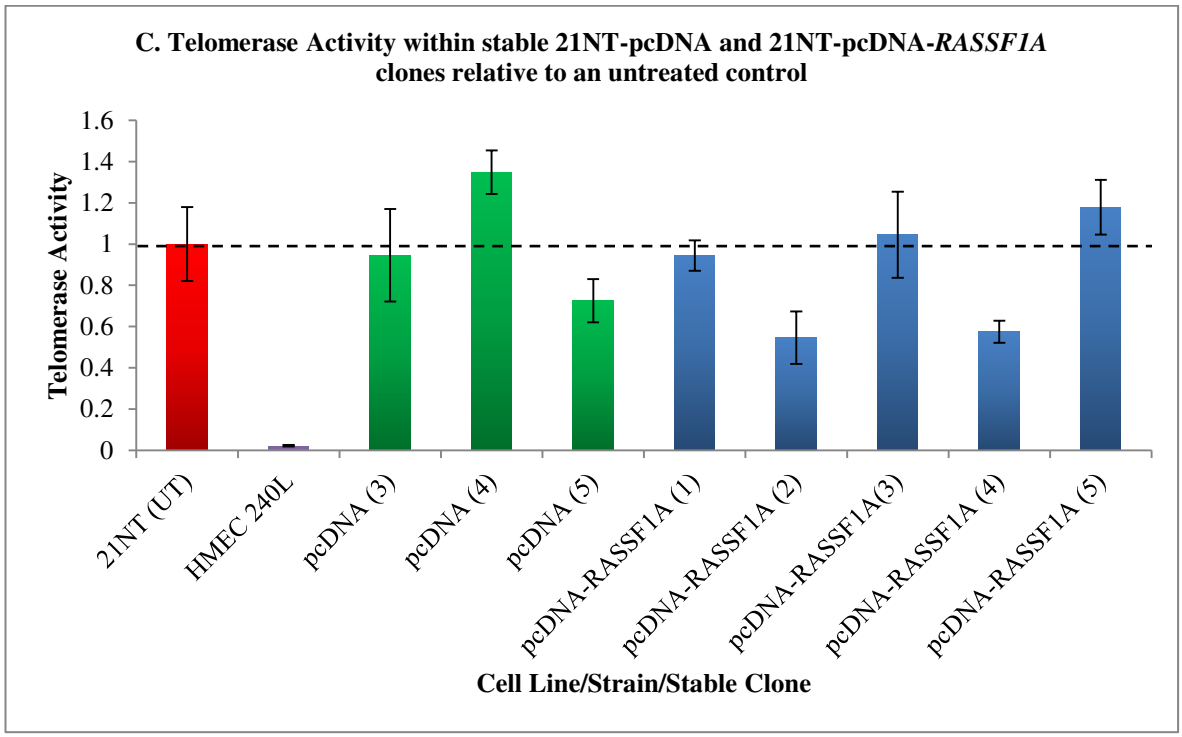


Figure 5.9- *Normalized relative quantities (NRQ) of* ***(A)*** *RASSF1 and* ***(B)*** *hTERT mRNA within stable empty vector control 21NT-pcDNA (green) and target gene 21NT-pcDNA-RASSF1A (blue) transfection clones relative to the mean ± SD NRQ values of three untreated 21NT cell samples (red). Error bars for stable transfection clones represent the standard error of the mean NRQ values obtained from triplicate repeats from each sample.* ***(C)*** *Telomerase activity within stable 21NT-pcDNA and 21NT-pcDNA-RASSF1A transfection clones relative to untreated 21NT cells. Error bars represent the standard error of the mean telomerase activity of triplicate repeats for each sample. The HMEC 240L cell strain was included to represent a normal human control.*

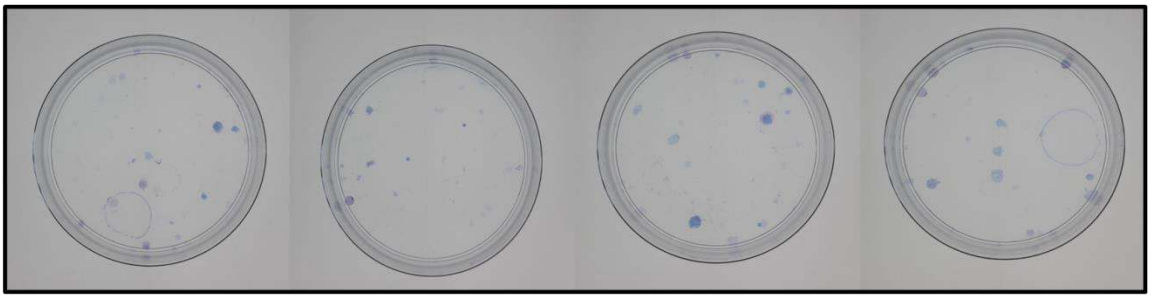
5.3.3.2 The effect of *RASSF1A* overexpression on the clonogenic survival and growth rate of 21NT cells

Around 62% of primary breast carcinomas have been found to undergo transcriptional repression of *RASSF1A* through CpG island promoter hypermethylation (Damman *et al.,* 2001). Transcriptional repression of *RASSF1A* is thought to occur at an early point during breast cancer development (Strunnikova *et al.,* 2005, Dumont *et al.,* 2009). Independent studies have shown that forced overexpression of *RASSF1A* within multiple tumour cell lines including NSCLC, prostate, lung, nasopharyngeal and breast and is associated with a significant reduction in cell colony formation, proliferation and tumorigenicity of cancer cells both *in vitro* and *in vivo* (Damman *et al.,* 2000, Burbee *et al.,* 2001, Chow *et al.,* 2004, Dreijerink *et al.,* 2001, Kuzmin *et al.,* 2002, Thaler *et al.,* 2012).

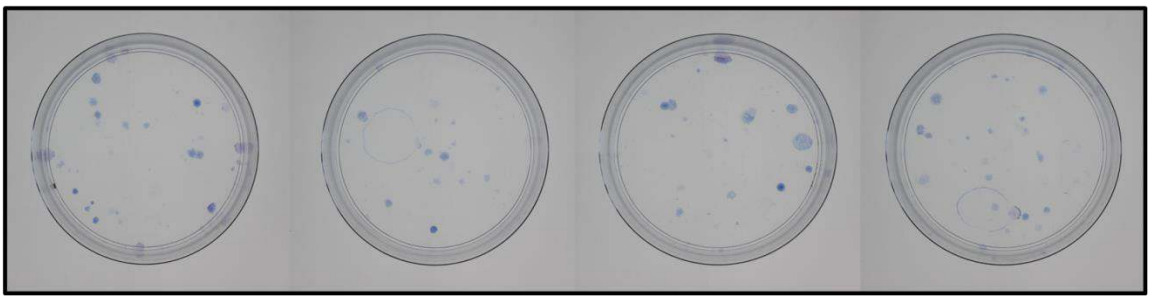
The 21NT primary breast cancer cell line was found to exhibit a 72% reduction in *RASSF1* expression relative to normal HMEC strains and is also likely to have undergone copy number loss of a minimal 5.5Mb region within 3p21.1-21.3, which encompasses the *RASSF1A* genomic locus (Figure 5.4B and Table 5.3). Therefore, in order to examine the tumour suppressive properties of *RASSF1A* within the 21NT breast cancer cell line, the effect of forced wild-type *RASSF1A* overexpression on the clonogenic survival and proliferation of 21NT cells was investigated. To do this, 21NT cells were transfected with negative control pcDNA plasmid vectors or target gene pcDNA-*RASSF1A* constructs and the number and size of stable cell clones that emerged following two weeks of neomycin selection were compared.

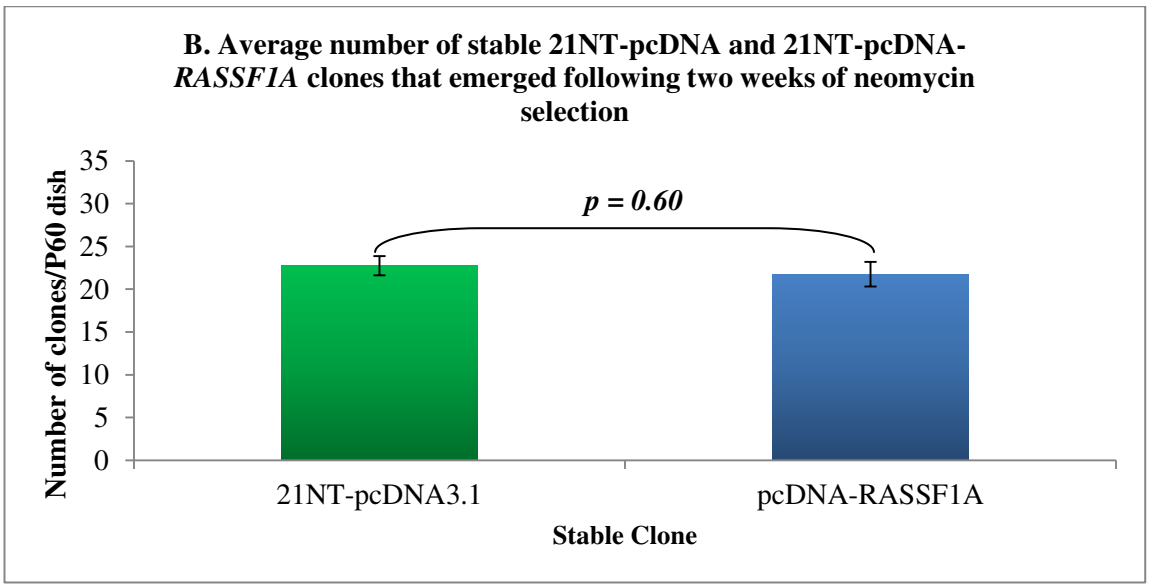
A. Neomycin-resistant clones that emerged 2 weeks following transfection of 21NT cells with pcDNA or pcDNA-*RASSF1A* plasmid vectors

(i) 21NT+pcDNA



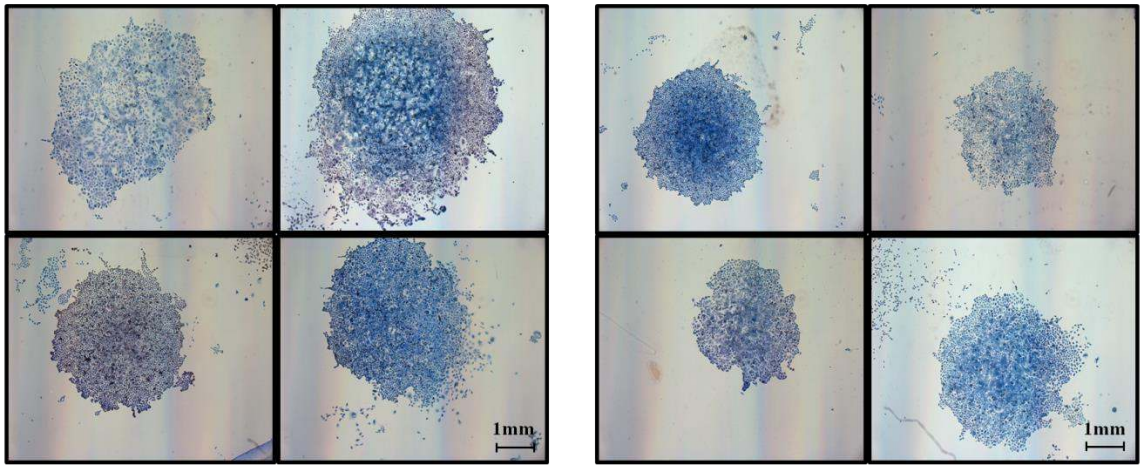
(ii) 21NT+pcDNA-*RASSF1A*





C. Examination of Average Stable 21NT-pcDNA and 21NT-pcDNA-*RASSF1A* Clone Diameters

(i) 21NT-pcDNA **(ii) 21NT-pcDNA –*RASSF1A***



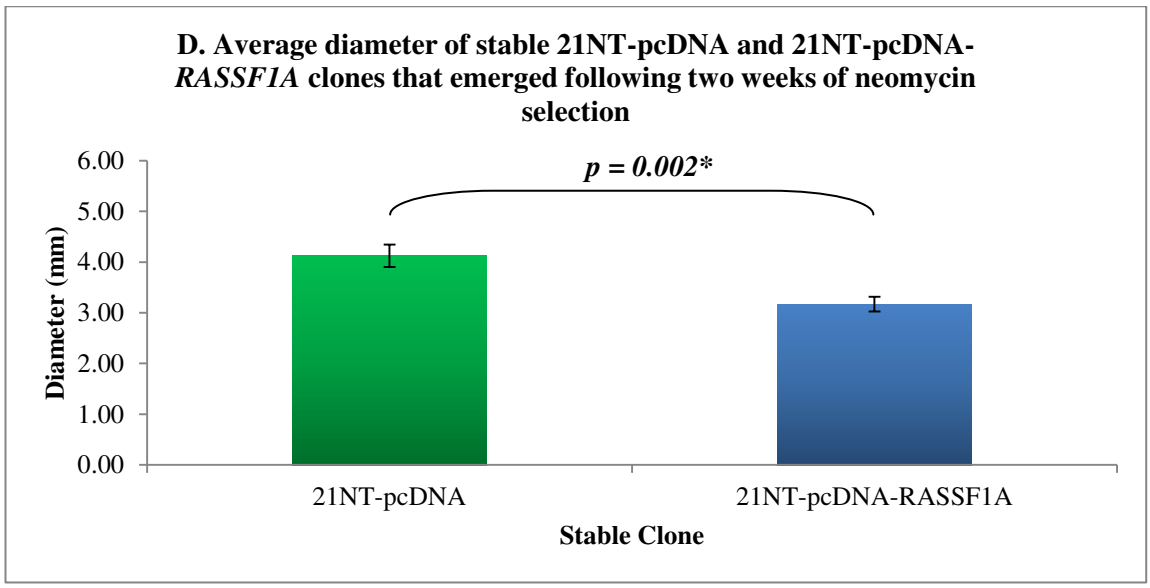


Figure 5.10- ***(A)*** *Photographs of P60 dishes containing fixed and stained stable* ***(i)*** *negative control 21NT-pcDNA and* ***(ii)*** *target gene 21NT-pcDNA-RASSF1A colonies that emerged (post-transfection) following two weeks of neomycin selection.* ***(B)*** *The mean number of negative control (green) and RASSF1A target gene (blue) stable colonies present across four individual P60 dishes for each plasmid construct.* ***(C)*** *Representative photomicrographs (2.5x magnification) of fixed and stained stable* ***(i)*** *21NT-pcDNA and* ***(ii)*** *21NT-pcDNA-RASSF1A clones that emerged (post-transfection) following two weeks of neomycin selection.* ***(D)*** *The average diameter (mm) of 11-17 individual 21NT-pcDNA (green) and 21NT-pcDNA-RASSF1A (blue) clones. Error bars represent the standard error of the mean number of cell clones/clone diameter for each group. A student's t-test was used to compare the mean of both groups.*

As shown in Figure 5.10A and B, no significant difference in the average number of stable negative control 21NT-pcDNA and target gene 21NT-pcDNA-*RASSF1A* clones was observed, which indicates that forced overexpression of *RASSF1A* is not associated with a significant decrease in the clonogenic survival of 21NT breast cancer cells. However, stable 21NT-pcDNA-*RASSF1A* clones were found to exhibit a significant reduction in average clone diameter compared with negative control stable 21NT-pcDNA clones, which indicates that *RASSF1A* overexpression within 21NT cells is associated with a significant decrease in cell proliferation.

5.3.4 PBRM1 AS A CANDIDATE TELOMERASE REPRESSOR AND TUMOUR SUPPRESSOR GENE IN BREAST CANCER CELLS

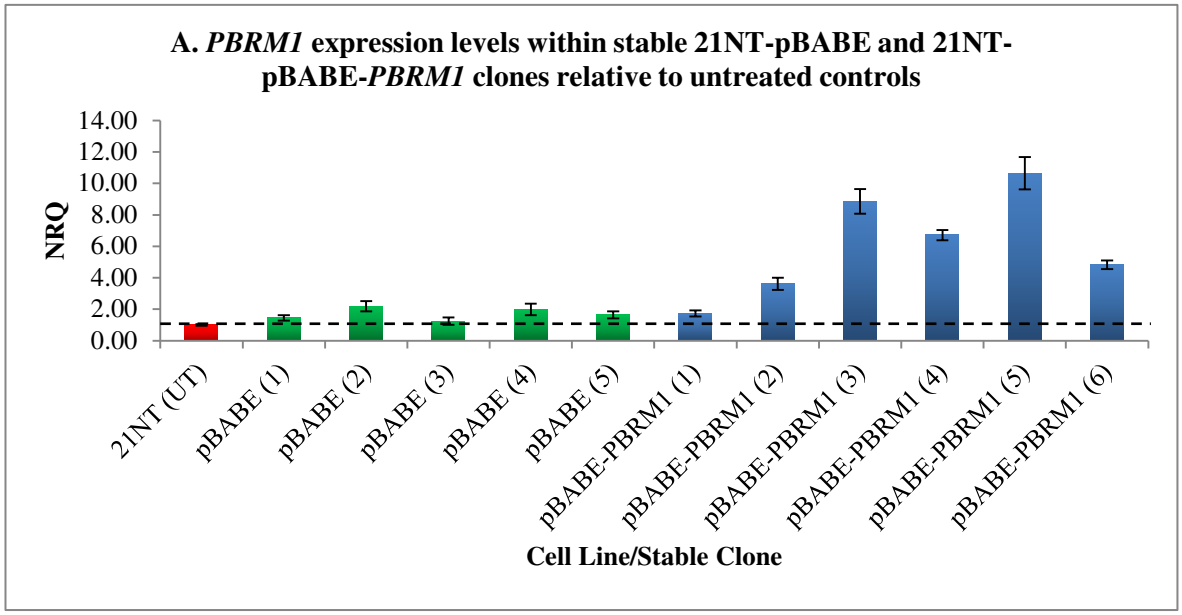
5.3.4.1 The effect of *PBRM1* overexpression on *hTERT* expression levels and Telomerase Activity within 21NT cells

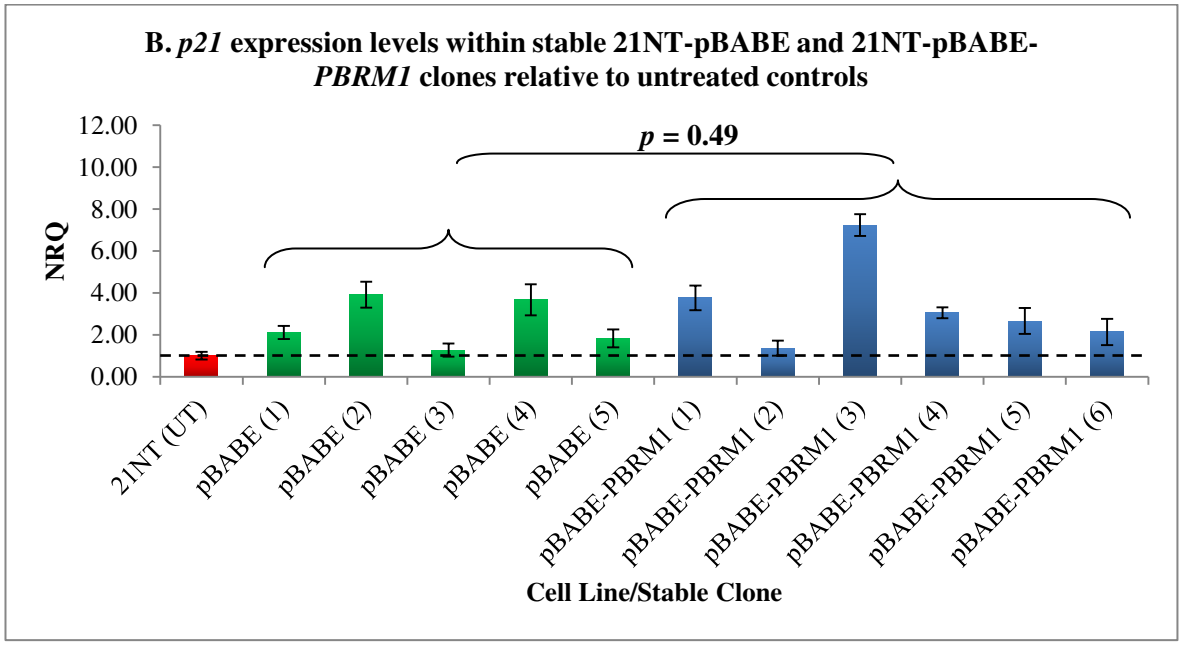
PBRM1 has been found to play an important functional role in the transcriptional regulation of basal and induced *p21* expression within normal breast epithelial and cancer cells (Xia *et al.,* 2008). Forced overexpression of *PBRM1* within two *PBRM1*-mutant breast cancer cell lines has been associated with an increase in p21 protein levels (Xia *et al.,* 2008). In addition, lack of *PBRM1*-mediated transcriptional activation of *p21* was thought to have been responsible for the delayed induction of replicative senescence pathways observed following knockdown of *PBRM1* within normal human fibroblasts (Burrows *et al.,* 2010). *p21* has been found to play an important functional role in the transcriptional repression of *hTERT* in breast cancer cells (Shats *et al.,* 2004). Therefore, in order to determine whether an increase in *PBRM1* expression levels is associated with repression of *hTERT* expression and telomerase activity through induction of *p21* expression, the effect of forced wild-type *PBRM1* overexpression within the 21NT cell line was examined. To do this, 21NT cells were transfected with the puromycin-selectable, negative control pBABE plasmid vector or a pBABE-*PBRM1* vector construct containing the 4961bp *PBRM1* wild-type cDNA. Two days following transfection of plasmid vectors, cells were cultured in the presence of puromycin to select for stable clones. Following two weeks of puromycin selection, five stable empty vector 21NT-pBABE clones and six target gene 21NT-pBABE-*PBRM1* clones were isolated and propagated as individual cell lines.

As shown in Figure 5.11A, stable 21NT-pBABE-*PBRM1*clones were found to exhibit 2-11 fold higher levels of *PBRM1* expression relative to untreated controls. By comparison, two out of five (40%) stable empty vector control 21NT-pBABE clones were found to display around 2-fold higher levels of endogenous *PBRM1* expression. Therefore, five out of six (83%) 21NT-pBABE-*PBRM1* clones were found to display consistently higher *PBRM1* expression levels than stable empty vector 21NT-pBABE clones. As shown in Figure 5.11B, all stable empty vector control and *PBRM1*-transfected 21NT clones were found to exhibit increased levels of *p21* expression relative to untreated controls. However, on average, stable 21NT-pBABE-*PBRM1* clones were not found to exhibit significantly higher levels of *p21* expression compared to stable empty vector 21NT-pBABE controls (p = 0.49, Figure 5.11B). As demonstrated in Figure 5.11A, the majority of stable 21NT-pBABE-*PBRM1* clones were found to express substantially higher levels of *PBRM1* expression relative to untreated 21NT cells and stable empty vector controls. Therefore, forced overexpression of wild-type *PBRM1* within 21NT cells is not associated with a significant increase in *p21* expression levels compared to

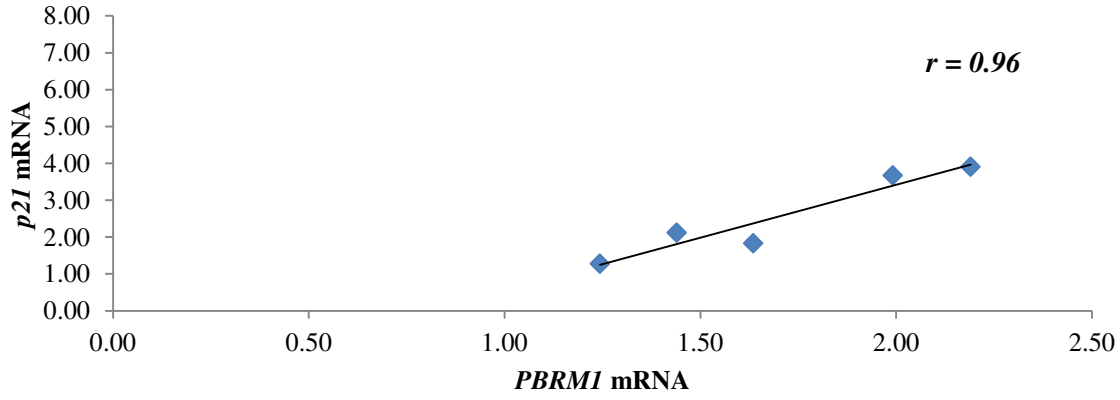
empty vector controls. Despite these observations, a positive correlation between *PBRM1* and *p21* expression levels was observed within both stable empty vector 21NT-pBABE and 21NT-pBABE-*PBRM1* clones (r = 0.96 and r = 0.35 respectively, Figure 5.11C and D). Consistent with Xia., *et al.,* (2008), these findings provide evidence to show that there may be a functional link between *PBRM1* and *p21* expression in breast cancer cells. However, these findings also indicate that forced overexpression of *PBRM1* within 21NT cells is not associated with a significant induction of *p21* expression compared to empty vector controls. This does not exclude the possibility that a greater than 11-fold increase in *PBRM1* may be required to induce high levels of *p21* expression.

Four out of six (67%) stable 21NT-pBABE-*PBRM1* clones were found to exhibit a greater than 50% reduction in *hTERT* expression levels relative to untreated 21NT cells (Figure 5.11E). Out of these, two (33%) displayed a greater than 90% reduction in relative *hTERT* expression levels. The remaining two (33%) 21NT-pBABE-*PBRM1* clones displayed 3-4 fold higher levels of *hTERT* relative to untreated controls (Figure 5.11E). Interestingly, stable 21NT-pBABE-*PBRM1* clones that exhibited the highest levels of *PBRM1* expression were also found to display higher levels of *hTERT* expression relative to untreated 21NT cells. 21NT-pBABE-*PBRM1* clones that exhibited a greater than 50% reduction in *hTERT* were found to display a 2-7 fold increase in *PBRM1* expression levels relative to untreated controls. These findings demonstrate that an increase in *PBRM1* expression is not always associated with repression of *hTERT* transcription. Interestingly, the results presented in Figure 5.11F show that all stable 21NT-pBABE-*PBRM1* clones exhibited a 59-89% reduction in telomerase activity relative to untreated controls. However, stable empty vector 21NT-pBABE clones were also found to display a substantial reduction in telomerase activity. Therefore, it is unclear whether stable forced overexpression of *PBRM1* was responsible for the reduction in telomerase activity observed within 21NT-pBABE-*PBRM1* clones.

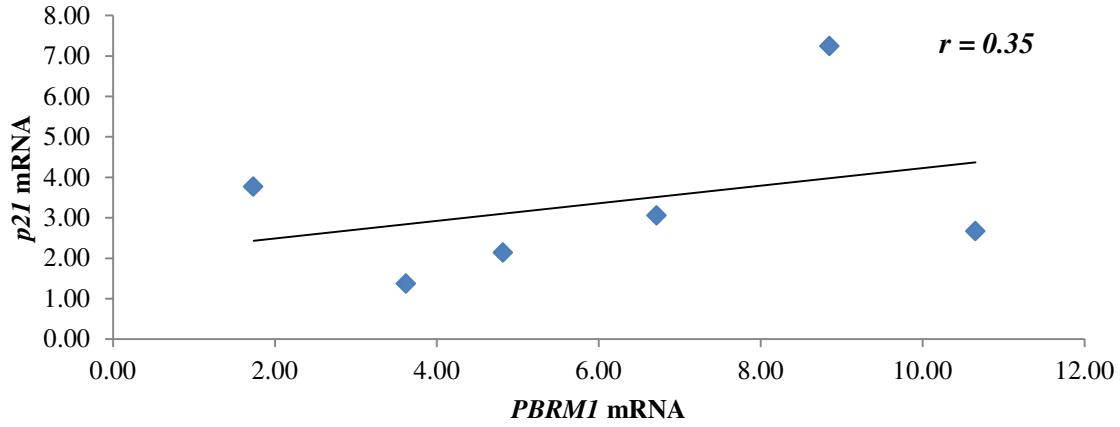

A. PBRM1 expression levels within stable 21NT-pBABE and 21NT-pBABE-PBRM1 clones relative to untreated controls
NRQ
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
21NT (UT)
pBABE (1)
pBABE (2)
pBABE (3)
pBABE (4)
pBABE (5)
pBABE-PBRM1 (1)
pBABE-PBRM1 (2)
pBABE-PBRM1 (3)
pBABE-PBRM1 (4)
pBABE-PBRM1 (5)
pBABE-PBRM1 (6)
Cell Line/Stable Clone


B. p21 expression levels within stable 21NT-pBABE and 21NT-pBABE-PBRM1 clones relative to untreated controls
p = 0.49
NRQ
12.00
10.00
8.00
6.00
4.00
2.00
0.00
21NT (UT)
pBABE (1)
pBABE (2)
pBABE (3)
pBABE (4)
pBABE (5)
pBABE-PBRM1 (1)
pBABE-PBRM1 (2)
pBABE-PBRM1 (3)
pBABE-PBRM1 (4)
pBABE-PBRM1 (5)
pBABE-PBRM1 (6)
Cell Line/Stable Clone

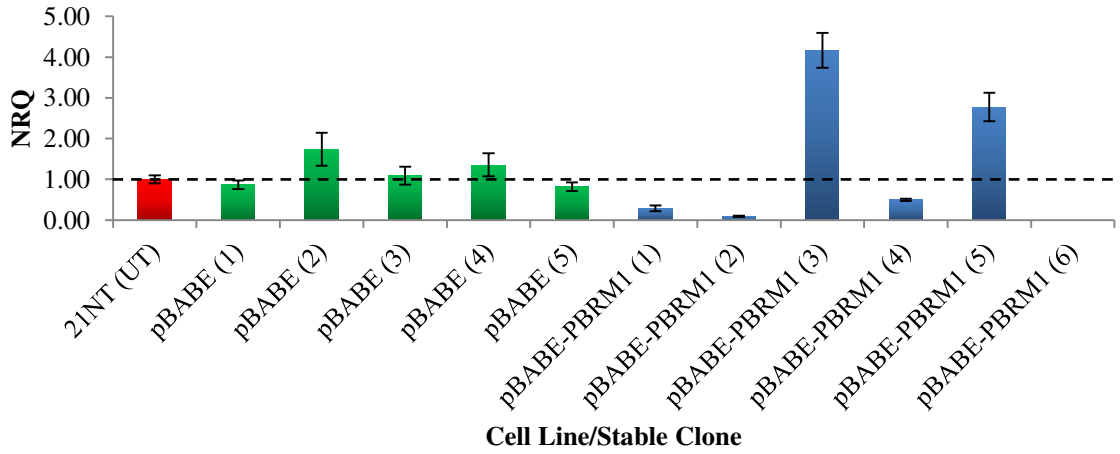
C. Relationship between relative *PBRM1* and *p21* expression levels within stable empty vector control 21NT-pBABE clones



D. Relationship between relative *PBRM1* and *p21* expression levels within stable 21NT-pBABE-*PBRM1* clones



E. *hTERT* expression levels within stable 21NT-pBABE and 21NT-pBABE-*PBRM1* clones relative to untreated controls



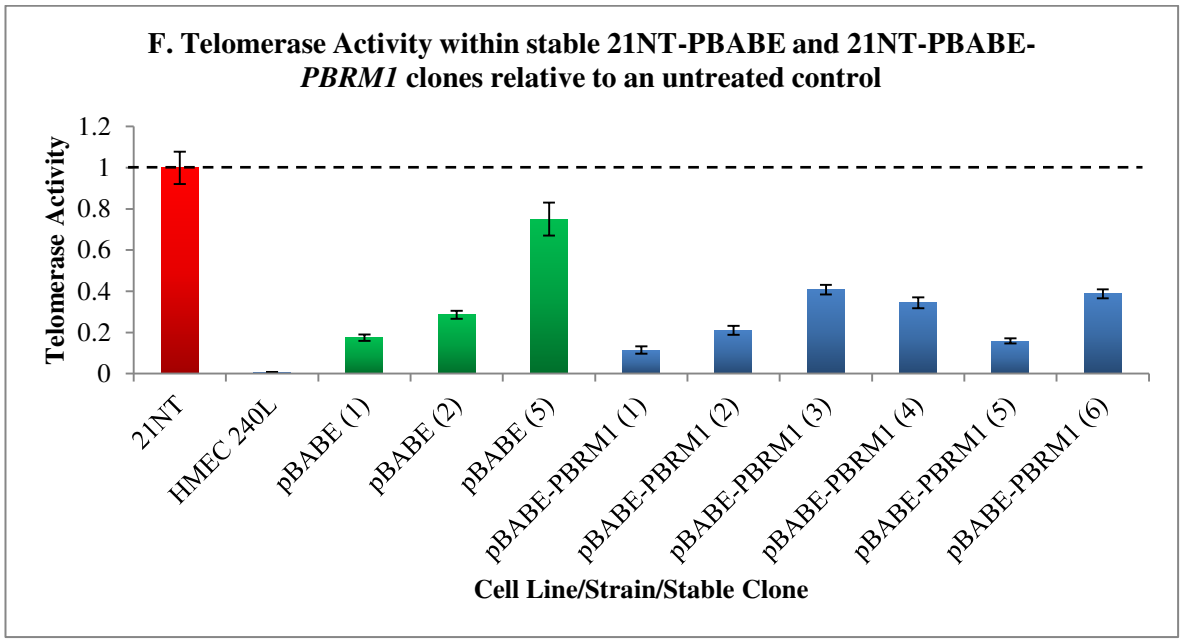
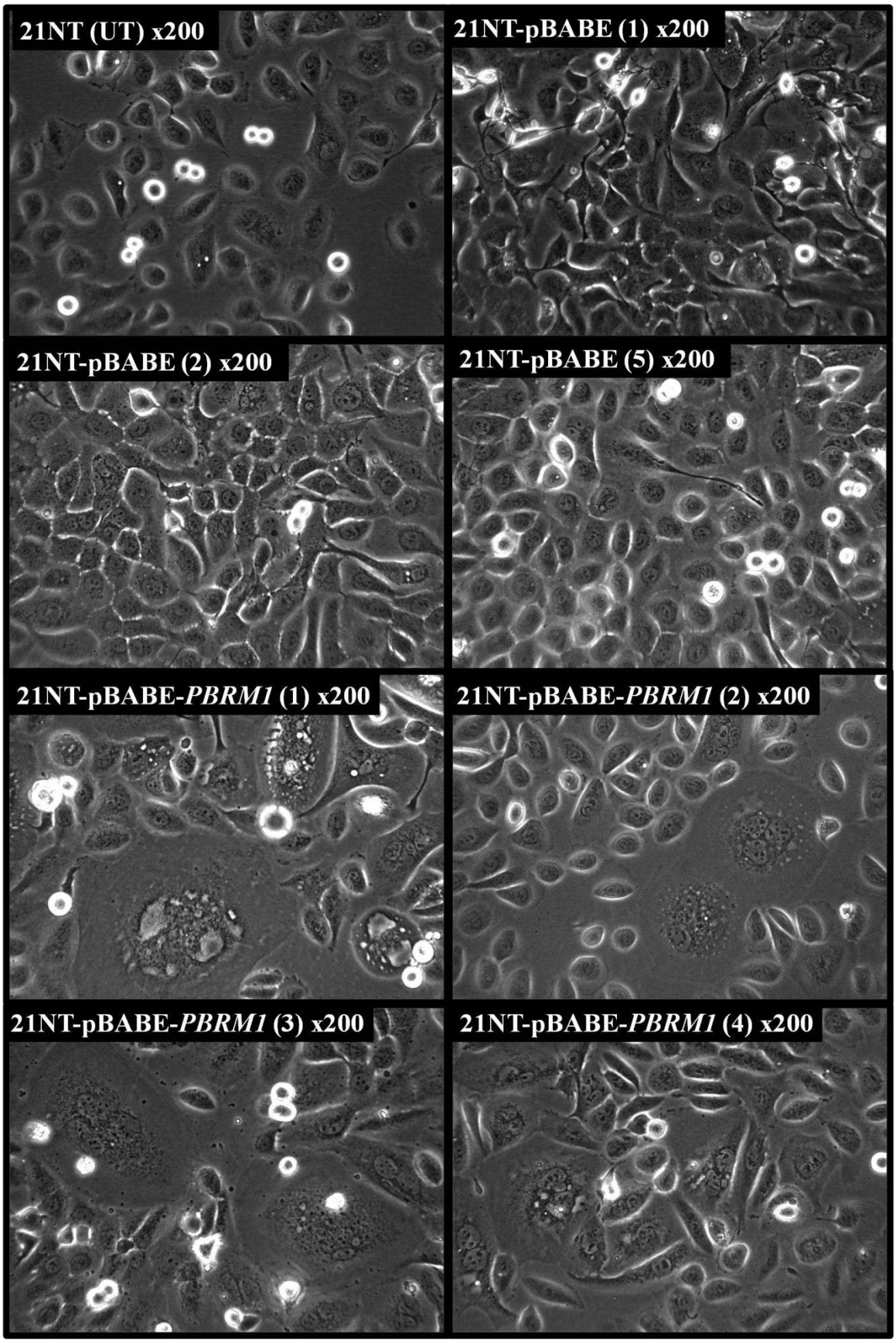


Figure 5.11 - *Normalized relative quantities (NRQ) of* ***(A)*** *PBRM1 and* ***(B)*** *p21 mRNA within stable empty vector control 21NT-pBABE (green) and target gene 21NT-pBABE-PBRM1 (blue) transfection clones relative to the mean ± SD NRQ values of three untreated 21NT cell samples (red). The relationship between PBRM1 and p21 expression levels within* ***(C)*** *21NT-pBABE and* ***(D)*** *21NT-pBABE-PBRM1 clones, relative to untreated 21NT cells. The Pearson Correlation Coefficient (r) was used to quantify the strength of the relationship between both variables.* ***(E)*** *NRQ of hTERT mRNA within the same samples relative to mean ± SD NRQ values of three untreated 21NT cell samples.* ***(F)*** *Telomerase activity within stable 21NT-pBABE and 21NT-pBABE-PBRM1 transfection clones relative to untreated 21NT cells. The HMEC 240L cell strain was included to represent a normal human control. Error bars represent the standard error of the mean NRQ value or telomerase activity of triplicate repeats for each sample.*

Cellular morphology of untreated (UT) 21NT cells, stable 21NT-pBABE and 21NT-pBABE-*PBRM1* transfection clones



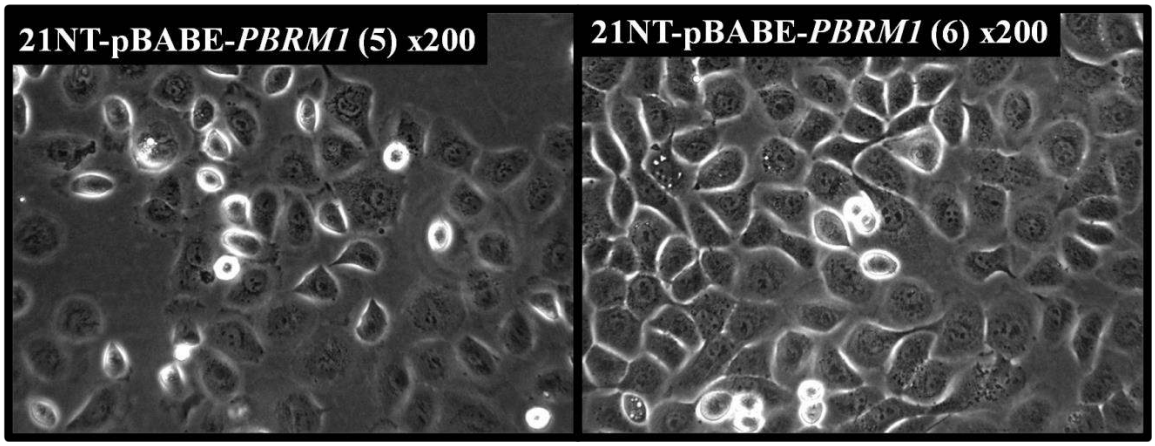


Figure 5.12- *Representative photomicrographs (magnification x200) of untreated (UT) 21NT cells and a subset of stable control 21NT-pBABE and target gene 21NT-pBABE-PBRM1 clones.*

Stable empty vector control 21NT-pBABE and target gene 21NT-pBABE-*PBRM1* clones were propagated in culture for around three weeks before they were cryopreserved. During this time none of the stable *PBRM1*-transfected 21NT clones were found to enter complete growth arrest. However, four out of six (67%) 21NT-pBABE-*PBRM1* clones were found to contain cells exhibiting characteristic features of senescence including cytoplasmic enlargement, vacuolation and multinucleation (Figure 5.12). By comparison, stable empty vector 21NT-pBABE clone cells were found to exhibit a similar morphology to untreated 21NT cells. Interestingly, 21NT-pBABE clones 1 and 2, which were found to exhibit similarly low levels of telomerase activity relative to untreated cells as 21NT-pBABE-*PBRM1* clones, were not found to contain cells that displayed features of senescence. This suggests that, independent of telomerase status, forced expression of wild-type *PBRM1* within 21NT cells is associated with the appearance of cells exhibiting senescent features.

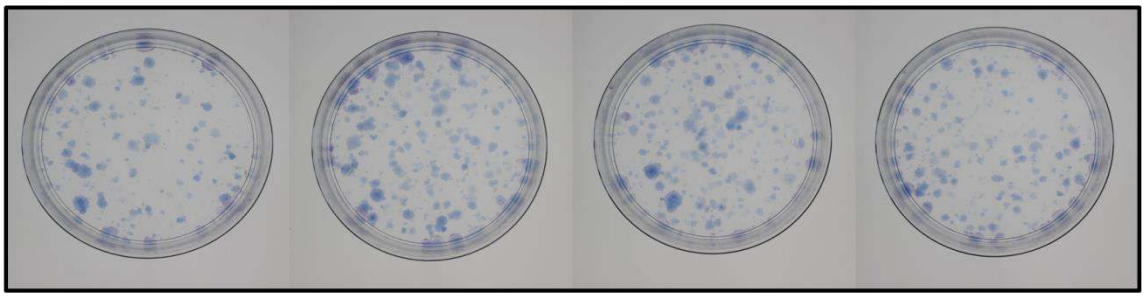
5.3.4.2 The effect of *PBRM1* overexpression on the clonogenic survival and growth rate of 21NT cells

Overexpression of wild-type *PBRM1* within the *PBRM1*-deficient HCC1143 breast cancer cell line has been associated with a significant reduction in the number and size of stable *PBRM1*-transfected cell colonies compared to negative controls (Xia *et al.,* 2008). Therefore, the effect of exogenous wild-type *PBRM1* expression on the clonogenic survival and proliferation of 21NT cells was examined within this study. To do this, 21NT cells were transfected with empty vector pBABE plasmids or pBABE-*PBRM1* constructs and cultured in the presence of puromycin for two weeks to select for stable clones. The number and size of puromycin-resistant stable 21NT-pBABE and 21NT-pBABE-*PBRM1* cell clones that emerged was then examined.

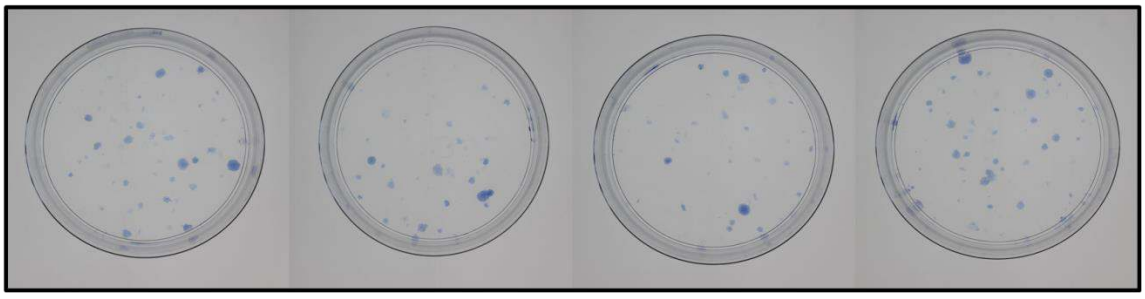
As shown in Figure 5.13A and B, the average number of stable 21NT-pBABE-*PBRM1* clones that emerged was significantly lower than the average number of 21NT-pBABE empty vector controls ($p = 1.5x10^{-5}$*), which demonstrates that forced expression of wild-type *PBRM1* is associated with a significant decrease in the clonogenic survival and growth of 21NT breast cancer cells. However, surviving stable 21NT-pBABE-*PBRM1* clones were found to display a similar average clone diameter to stable empty vector 21NT-pBABE clones (Figure 5.13C and D), which could suggest that a critical threshold of *PBRM1* expression required for cell growth inhibition was not reached or the pBABE-*PBRM1* plasmid construct did not remain intact upon stable integration within a proportion of 21NT-pBABE-*PBRM1* clones. As outlined in Chapter 4.2.3.1, genetic recombination events that occur during stable integration of plasmid DNA into the host genome, may involve important sites required for target gene expression within mammalian cells.

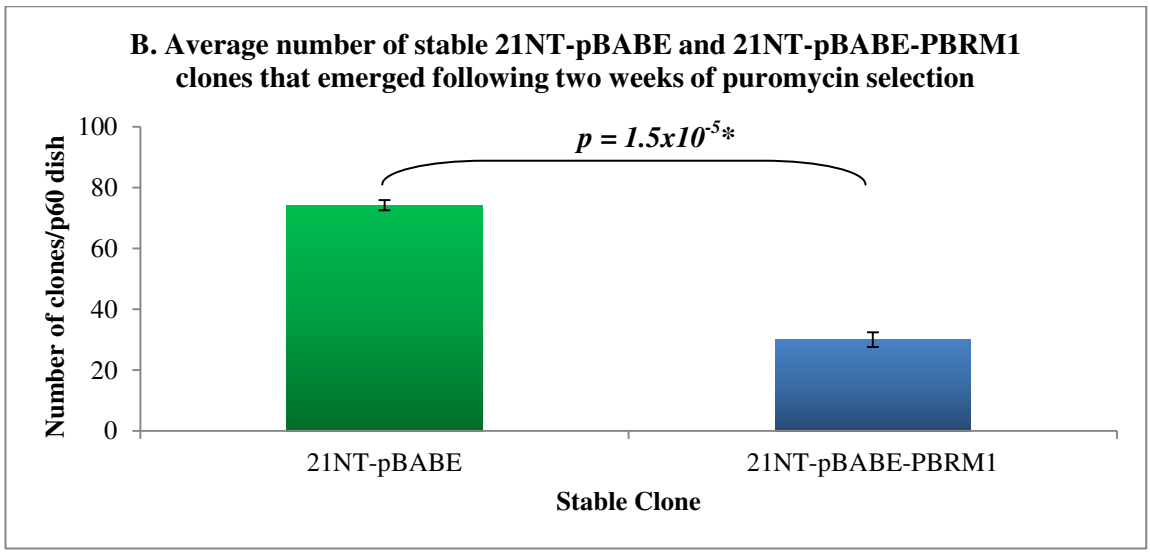
A. Puromycin-resistant clones that emerged 2 weeks following transfection of 21NT cells with pBABE or pBABE-*PBRM1* plasmid vectors

(i) 21NT+pBABE



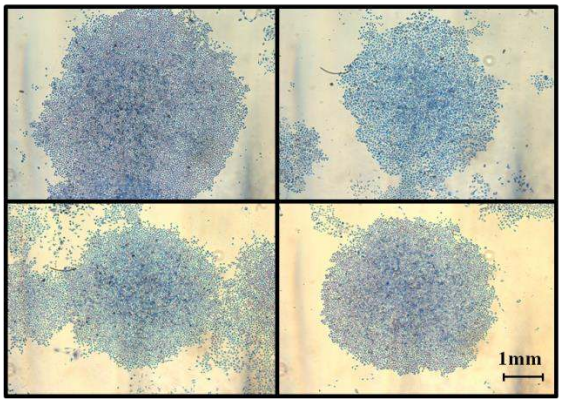
(ii) 21NT+pBABE-*PBRM1*



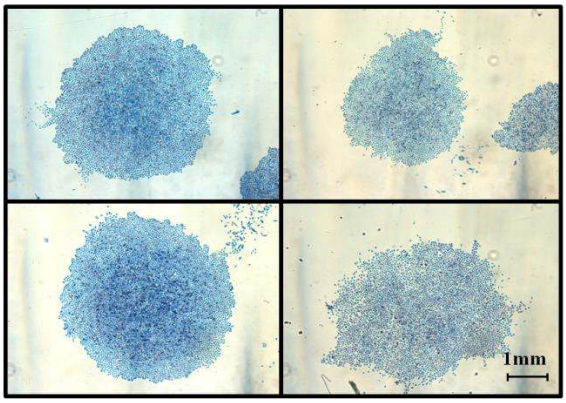


C. Examination of Average Stable 21NT-pBABE and 21NT-pBABE-*PBRM1* Clone Diameters

(i) 21NT-pBABE



(i) 21NT-pBABE-*PBRM1*



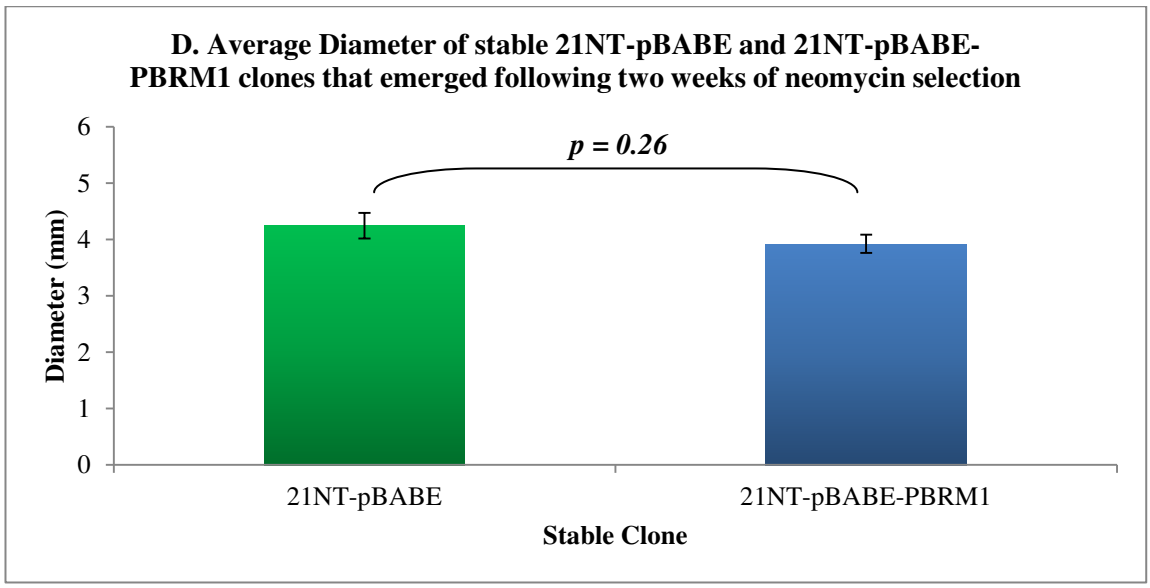


Figure 5.13- ***(A)*** *Photographs of P60 dishes containing fixed and stained stable* ***(i)*** *negative control 21NT-pBABE and* ***(ii)*** *target gene 21NT-pBABE-PBRM1 colonies that emerged (post-transfection) following two weeks of puromycin selection.* ***(B)*** *The mean number of negative control (green) and PBRM1 target gene (blue) stable colonies present across four individual P60 dishes for each plasmid construct.* ***(C)*** *Representative photomicrographs (2.5x magnification) of fixed and stained stable* ***(i)*** *21NT-pBABE and* ***(ii)*** *21NT-pBABE-PBRM1 clones that emerged (post-transfection) following two weeks of puromycin selection.* ***(D)*** *The average diameter (mm) of 13 individual 21NT-pBABE (green) and 21NT-pBABE-PBRM1 (blue) clones. Error bars represent the standard error of the mean number of cell clones/clone diameters for each group. A student's t-test was used to compare the mean of both groups.*

5.4 SUMMARY

5.4.1 BAP1

Independent studies have shown that the *BAP1* de-ubiquitylase enzyme plays a tumour suppressor role in multiple cancer types including non-small cell lung cancer (NSCLC) and breast cancer (Jensen *et al.,* 1998, Ventii *et al.,* 2008). *BAP1* mutations have been identified within these cancer types and are associated with poor cancer-specific survival in ccRCC patients (Hakimi *et al.,* 2013). Germline *BAP1* mutation carriers have an increased risk of developing mesothelioma, uveal melanoma and other malignancies including lung, breast and renal cancer, which demonstrates that this gene may play an important role in preventing tumorigenesis. *BAP1* resides within a common region of loss of heterozygosity (LOH) in multiple cancer types including renal, lung and breast cancers (reviewed by Angeloni *et al.,* 2007). In support of this, the results presented within my study demonstrated that over half (56%) of breast cancer cell lines examined had undergone copy number loss involving the *BAP1*

genomic locus, while the remaining cell lines (44%) were found to harbour either two (22%) or three (22%) alleles. In contrast, over half (56%) of breast cancer cell lines were found to exhibit either similar or higher levels of *BAP1* expression compared with normal HMEC strains. This demonstrates that changes in *BAP1* gene copy number are not always associated with concomitant changes in gene expression levels. Only the MCF-7 breast cancer cell line was found to exhibit a substantial (60%) reduction in *BAP1* expression levels. Interestingly, Jensen *et al.,* (1998) found that the MCF-7 cell line does not harbour mutations in the open reading frame (ORF) of *BAP1*. However, forced overexpression of *BAP1* within the MCF-7 cell line was associated with significant cell growth suppression, which indicates that high levels of *BAP1* expression is associated with a powerful tumour suppressor effect even in the presence of endogenous wild-type *BAP1* (Jensen *et al.,* 1998). Northern blot analysis of *BAP1* transcripts within 31 small cell lung cancer (SCLC) and 27 NSCLC cell lines showed that only two cell lines were found to display undetectable levels of *BAP1* expression; one of which was due to the presence of a frameshift mutation within the *BAP1* ORF leading to a truncated protein product. Similarly, during a screen of *BAP1* mutations within breast, prostate, colorectal and gastric cancers, only one colon carcinoma-derived tumour sample was found to harbour a *BAP1* mutation, indicating that somatic *BAP1* mutations are a rare occurrence in several cancer types including breast cancer (Je *et al.,* 2012). The results presented within my study demonstrating that breast cancer cell lines exhibit similar or higher *BAP1* expression levels relative to normal HMECs, suggest that transcriptional repression of *BAP1* is uncommon in breast cancer cells. Interestingly, undetectable levels of BAP1 protein have been observed within RCC tumour samples harboring both mutant and wild-type copies of *BAP1* (Pena-Llopis *et al.,* 2012). Together, these findings suggest that loss of BAP1 function in cancer cells may involve post-transcriptional processing defects or perhaps lack of transcriptional activators of *BAP1*.

BAP1 is a nuclear-localized chromatin-bound protein that forms multi-protein complexes with chromatin-modifying proteins and transcription factors (Machida *et al.,* 2009, Yu *et al.,* 2010). A common binding partner of BAP1 is Host Cell Factor-1 (HCF-1), which is known to regulate the expression levels of target genes involved in cell cycle control by recruiting chromatin modifying complexes to promoter regions. BAP1 has been found to interact with and augment the cell growth suppressive effect of BRCA1; a previously identified *hTERT* repressor protein in breast cancer (Jensen *et al.,* 1998, Xiong *et al.,* 2003). *BAP1* is located close to the 3p21.3-p22 region that is thought to harbour a powerful telomerase repressor sequence in breast cancer cells (Cuthbert *et al.,* 1999). Therefore, a principle aim of my study was to investigate the putative role of *BAP1* in *hTERT* regulation. I found that forced overexpression of *BAP1* within the 21NT breast cancer cell line was associated with a greater than 80% reduction in *hTERT* expression levels and 85-90% reduction in telomerase activity relative to untreated controls. Stable *BAP1*-overexpressing clones were also found to exhibit a

greater reduction in *hTERT* expression and telomerase activity than empty vector controls, which demonstrates that *BAP1* may play a functional role in regulating telomerase activity in breast cancer cells. Despite these observations, stable 21NT-pCMVNeo-*BAP1* transfection clones were found to proliferate continuously for approximately 25-27 PD, without entering growth arrest or exhibiting morphological features of senescence. However, 21NT-pCMVNeo-*BAP1* clones were found to exhibit a significant reduction in cell growth rate compared with untreated 21NT cells, indicating that forced *BAP1* overexpression is associated with 21NT cell growth suppression. Cuthbert *et al.,* (1999) demonstrated that 21NT cells posses short mean telomere restriction fragment (TRF) lengths of 3 kilobases (kb) and introduction of a normal copy of chromosome 3 into the 21NT cell line was associated with a greater than 90% reduction in telomerase activity and cell growth arrest within 10-12 or 15-18 PD. By comparison, telomerase repression within RCC23-chromosome 3 hybrids was associated with cell growth arrest within 31-41 PD with a progressive reduction in mean TRF lengths from 6kb to 2.5kb (Horikawa *et al.,* 1998). Therefore, following abolishment of telomerase activity, the number of PDs that occur prior to cell growth arrest appears to be dependent on mean telomere lengths within the parental cell line. Taking this into account, the telomerase-repressed stable 21NT-pCMVNeo-*BAP1* clones obtained within my study had therefore exceeded the number of PD that had occurred between telomerase repression and growth arrest of 21NT-chromosome 3 hybrids within the Cuthbert *et al.,* (1999) study. A comparison of early (14PD) and late passage (25-27 PD) stable 21NT-pCMVNeo-*BAP1* clones revealed that telomerase activity had increased over progressive passages, to a similar level displayed by empty vector control stable clones. This is likely to have prevented progressive telomere shortening and induction of cell growth arrest pathways. *BAP1* expression levels were substantially higher relative to untreated 21NT cells within both early and late passage 21NT-pCMVNeo-*BAP1* stable clones, therefore the observed increase in telomerase activity was unlikely to be due to changes in *BAP1* expression.

In a similar fashion to forced overexpression of *BAP1* within 21NT breast cancer cells, in published work by others, stable overexpression of the *BAP1* binding partner *BRCA1,* within DU-145 prostate cancer and T47D breast cancer cells was associated with a significant reduction in *hTERT* expression levels and telomerase activity (Xiong *et al.,* 2003). Stable *BRCA1*-DU-145 and *BRCA1*-T47D transfection clones were also found to exhibit a reduction in cell growth rate and progressive telomere shortening from 3-3.3kb to 1.9kb and 2.3-4.0kb to 2.0kb respectively. Interestingly, despite observing a significant reduction in telomere length, stable *BRCA1*-DU-145 and *BRCA1*-T47D clones were found to proliferate continuously without induction of cell growth arrest. Telomere length analysis of *BRCA1*-expressing DU145/*BRCA1*-Tet-off cells, which express wild-type *BRCA1* in the absence of tetracycline (TCN), showed that longer telomere lengths ranging from 2.3-6kb were lost within 3 days and maintained at 2.3kb

for around 31 days. A further reduction in mean TRF lengths to 1.8-2kb was observed at around 60 days, with no signs of senescence or apoptosis. Therefore, expression of wild-type *BRCA1* is associated with an initial period of rapid telomere erosion, followed by continued gradual telomere shortening without induction of cell growth arrest pathways. This was thought to either be due to the presence of residual levels of telomerase activity, or the presence of *pRb* and/or *p53* mutations within parental cell lines (Xiong *et al.,* 2003). BRCA1 was found to repress *hTERT* transcription through inhibition of c-Myc-mediated transcriptional activation of *hTERT* and also independently of c-Myc (Xiong *et al.,* 2003). Similarly, Li *et al.,* (2002) found that Nmi recruits BRCA1 to c-Myc and prevents c-Myc-mediated transcriptional activation of *hTERT* within breast cancer cells. Transfection of *BRCA1* harboring mutations associated with hereditary breast cancer, failed to inhibit c-Myc induced *hTERT* transcriptional activation within the T47D breast cancer cell line. These findings indicate that BRCA1 may play an important tumour suppressor role in early in breast cancer development by preventing c-Myc induced activation of *hTERT* expression and telomerase activity.

Mean TRF lengths of stable 21NT-pCMVNeo-*BAP1* transfection clones generated in my study were not ascertained. However, forced overexpression of *BAP1* the 21NT breast cancer cell line was associated with a similar effect on *hTERT* expression levels, telomerase activity, cell growth and proliferative capacity as that resulting from stable overexpression of *BRCA1* within prostate and breast cancer cell lines (Xiong *et al.,* 2003). BAP1 is known to interact with the RING finger domain of BRCA1, which was found to be essential for *BRCA1*-mediated *hTERT*-repression by Xiong *et al.,* (2003). Therefore, it is possible that the repression of *hTERT* expression and telomerase activity observed within stable 21NT-pCMVNeo-*BAP1* clones in my study may be linked to BRCA1. This is supported by the finding that *BAP1* has been found to enhance the cell growth suppressive effect of *BRCA1* in breast cancer (Jensen *et al.,* 1998) and *BAP1* mutations have been found to occur early in breast tumorigenesis (Newman *et al.,* 2013, Stephens *et al.,* 2012). Needless to say, further investigation is necessary to establish a direct functional link between *BAP1* and *BRCA1*-mediated *hTERT* transcriptional repression.

In conclusion, forced overexpression of *BAP1* within the 21NT breast cancer cell line was not associated with any significant change in clonogenic survival, but was associated with an initial but unsustained repression of *hTERT* transcription and telomerase activity and continuous cellular proliferation at a significantly reduced growth rate. Due to the observed telomerase repression and induction of replicative senescence pathways within 21NT-chromosome 3 hybrids generated by Cuthbert *et al.,* (1999), the findings presented within my study suggest that *BAP1* is unlikely to be responsible for mediating this effect. However, due to the observed similarities between these findings and those presented by Xiong *et al.,* (2003), it

is possible that *BAP1* may be linked to BRCA1-mediated *hTERT* transcriptional repression, which presents another possible tumour suppressive role of *BAP1* in cancer. These findings also suggest that there may be multiple genes, including *BAP1,* located on the short arm of chromosome 3 that are functionally responsible for maintaining stable telomerase repression within normal human somatic cells.

5.4.2 RASSF1A

The *RASSF1* isoform A (*RASSF1A)* gene is one of the most frequently inactivated genes in human cancer (Agathanggelou *et al.,* 2005, Donninger *et al.,* 2007). *RASSF1A* mutations have been identified within nasopharyngeal carcinomas (Pan *et al.,* 2005) but the most common mechanism of inactivation identified within over 30 different cancer types is CpG island promoter hypermethylation, which has been shown to correlate with loss of *RASSF1A* expression (Agathanggelou *et al.,* 2005, Donninger *et al.,* 2007, Damman *et al.,* 2000, Burbee *et al.,* 2001). The *RASSF1* genomic locus is located within an overlapping homozygous 120kb region of deletion on chromosome 3p21.3 in three SCLC cell lines and one breast cancer cell line (Sekido *et al.,* 1998). Independent studies have reported that 30%-69% of breast tumour samples and breast cancer cell lines exhibit allele loss within 3p21.3, which suggests that important tumour suppressor genes may be present within this region (Maitra *et al.,* 2001, Chen *et al.,* 1994, De Oliveira *et al.,* 2012). Consistent with these findings, the results presented within my study show that 56% of breast cancer cell lines examined were found to have undergone consistent copy number loss of five genes spanning a 5.5Mb region within 3p21.1-p21.3 (47.1Mb-52.6Mb from the telomere), which encompasses the *RASSF1* locus. Allele loss within this region was found to have occurred within 21NT and 21MT breast cancer cells lines, which are derived from the primary breast tumour and metastatic deposit of the same patient respectively. This provides additional evidence to show that the 3p21.1-p21.3 region may have been lost at an early point during breast tumorigenesis and may harbour important tumour suppressor genes. According to the Database of Genomic Variants (DGV, MacDonald *et al.,* 2013), allele loss at the *RASSF1* locus has been observed within normal healthy individuals. Within my study, three normal HMEC strains derived from reduction mammoplasty tissue samples of individual healthy female patients were used to calibrate gene copy number within breast cancer cell lines in the absence of matched normal tissue samples. Therefore, copy number alterations at the *RASSF1* locus could not be determined. However, *RASSF1* is located 50.3Mb from the telomere, which is in the center of the putative 5.5Mb region of allele loss observed within over half of breast cancer cell lines examined. Therefore, it is likely that the *RASSF1* genomic locus was lost along with neighbouring gene loci within each sample analyzed.

Normal breast epithelial cells have been found to express both *RASSF1A* and *RASSF1C* transcript isoforms, which are under the control of independent promoters within the *RASSF1* locus (Damman *et al.,* 2000, Damman *et al.,* 2001, Burbee *et al.,* 2001). *RASSF1A* and *RASSF1C* encode 340 and 270 amino acid proteins respectively (Damman *et al.,* 2000). The last four exons are common to both *RASSF1A* and *RASSF1C* and encode the RAS-association (RA) domain. Unlike *RASSF1C, RASSF1A* encodes an N-terminal putative SH3 domain and a diacylglycerol/phorbol ester binding domain (DAG). Around 60% of breast cancer cell lines have been found to undergo *RASSF1A* CpG island promoter hypermethylation, which is strongly associated with complete loss of *RASSF1A* expression (Damman *et al.,* 2001, Burbee *et al.,* 2001). *RASSF1A* promoter methylation has been observed within pre-malignant breast lesions and immortal non-tumorigenic breast cancer cell lines, which indicates that loss of *RASSF1A* may drive breast tumorigenesis (Dumont *et al.,* 2009). Unlike *RASSF1A*, *RASSF1C* transcripts have been detected within breast and lung cancer cell lines and has not been found to undergo CpG island promoter hypermethylation in NSCLC and SCLC cell lines (Damman *et al.,* 2000, Damman *et al.,* 2001, Burbee *et al.,* 2001). It is therefore thought that the *RASSF1A* isoform, but not *RASSF1C,* functions as a critical tumour suppressor gene in breast cancer. However, Vos *et al.,* (2000) found that 67% of transformed ovarian cell lines exhibited undetectable levels of *RASSF1C* expression. In addition, forced overexpression of *RASSF1C* was found to suppress the growth of transformed T-293 human embryonic kidney cells, which was thought to be due to induction of pro-apoptotic signalling pathways. Similarly, Li *et al.,* (2004) found that *RASSF1C* suppressed the growth of KRC/Y renal cell carcinoma and LNCaP prostate cancer cell lines. Therefore, the *RASSF1C* isoform may harbour tissue-specific tumour suppressor functions.

The primer sequences used to examine the expression levels of *RASSF1* within breast cancer cell lines and normal HMEC strains in my study, were designed to detect all known protein coding transcript isoforms *A, B, C, D* and *H.* Exon 1 is common to *RASSF1* transcripts *A, D* and *H,* which suggests that these isoforms are likely to be under the control of the same promoter that has been found to be frequently hypermethylated in breast cancer cell lines (NCBI, Damman *et al.,* 2000, Damman *et al.,* 2001, Burbee *et al.,* 2001). The *RASSF1B* isoform appears to be expressed only within normal haematopoietic cells (Damman *et al.,* 2000). My results demonstrate that 44% of breast cancer cell lines examined exhibit a 52-72% reduction in *RASSF1* expression levels relative to normal HMEC strains. Damman *et al.,* (2001) and Burbee *et al.,* (2001) did not observe any detectable levels of *RASSF1A* expression within breast cancer cell lines. In contrast, all breast cancer cell lines examined within my study showed detectable levels of *RASSF1* expression. However, this is likely to be due to the presence of *RASSF1C* transcripts, which are detectable within breast cancer cell lines regardless of *RASSF1A* expression (Burbee *et al.,* 2001). Therefore, it is possible that the observed reduction in *RASSF1*

expression levels within breast cancer cell lines in my study is associated with loss of *RASSF1A* expression. In support of this, Damman *et al.,* (2000) and Damman *et al.,* (2001) detected both *RASSF1A* and *RASSF1C* transcripts within the H5S78T breast carcinoma cell line, whereas the MCF-7 cell line was found to display *RASSF1C* but undetectable levels of *RASSF1A* expression. Consistent with these findings, the results presented within my study showed that H5S78T and MCF-7 breast cancer cell lines exhibited 257% and 40% *RASSF1* expression levels relative to normal HMEC strains respectively. Therefore, it is likely that the 21NT, 21MT and MTSV cell lines, which exhibited a greater than 50% reduction in *RASSF1* expression relative to normal HMEC strains, may have also undergone transcriptional silencing of *RASSF1A*. These findings therefore provide additional evidence that deregulation of *RASSF1* is a frequent event in breast cancer cells. Independent studies have shown that *RASSF1A* promoter hypermethylation is associated with poor disease-free survival and more progressive disease (Muller *et al.,* 2003, Martins *et al.,* 2011, Hagrass *et al.,* 2013, Xu *et al.,* 2012). In support of these observations, I found that breast cancer cell lines derived from the metastatic deposits of two breast cancer patients (21MT and MCF-7), were among those that exhibited the lowest levels of *RASSF1* expression relative to normal HMECs.

RASSF1 is located within the 3p21.3 region, which is thought to harbour a telomerase repressor sequence that may be lost in breast cancer cells (Cuthbert *et al.,* 1999). In support of the hypothesis that *RASSF1A* may function as a putative telomerase repressor gene, variant HMEC (vHMEC) cells transformed with the Ha-Ras-V12 oncogene were found to exhibit increased levels of *RASSF1A* promoter methylation and telomerase activity (Dumont *et al.,* 2009). In addition, forced overexpression of *RASSF1A* within the A549 lung cancer cell line has been associated with cell cycle arrest and senescence, which was found to be mediated by increased *p21* expression and activation of pRb (Thaler *et al.,* 2009). *p53*-mediated transcriptional repression of *hTERT* in breast cancer cells has been found to be mediated by *p21* and recruitment of the histone-modifying E2F-pRb-HDAC complex to the *hTERT* promoter (Shats *et al.,* 2004). Szutorisz *et al.,* (2003) demonstrated that the chromosome 3-encoded *hTERT* repressor sequence mediates transcriptional repression of *hTERT* through altering the chromatin state around intron 2. Within my study, forced overexpression of *RASSF1A* within the 21NT breast cancer cell line was not associated with consistent transcriptional repression of *hTERT* or a reduction in telomerase activity compared to untreated or empty vector controls. These findings are consistent with those presented by Tanaka *et al.,* (2005), demonstrating that transfection of wild-type *RASSF1A* within the RCC23 renal cell carcinoma cell line was not associated with any observable change in *hTERT* promoter activity. Similarly to 21NT breast cancer cells, introduction of a normal copy of human chromosome 3 into RCC23 cells by MMCT was associated with *hTERT* transcriptional repression, a significant reduction in telomerase activity and cell growth arrest (Ohmura *et al.,* 1998). Together, these findings

provide evidence to show that *RASSF1A* is unlikely to function as a transcriptional regulator of *hTERT* in breast or renal cancer cells.

Reconstitution of *RASSF1A* expression within *RASSF1A*-deficient lung, nasopharyngeal, kidney, prostate and breast cancer cell lines has been associated with significant cell growth inhibition and decreased tumorigenicity *in vitro* and *in vivo* (Damman *et al.,* 2000, Burbee *et al.,* 2001, Chow *et al.,* 2004, Dreijerink *et al.,* 2001, Kuzmin *et al.,* 2002, Thaler *et al.,* 2012). In my study, transfection of wild-type *RASSF1A* within the 21NT breast cancer cell line was not associated with any observable change in colony formation when compared to empty vector controls. However, a significant reduction in average colony size of stable 21NT-pcDNA-*RASSF1A* clones relative to empty vector controls was observed, which suggests that *RASSF1A* may suppress the proliferation but not the clonogenic survival of 21NT cells. These results contrast findings presented by similar studies demonstrating that *RASSF1A* overexpression within cancer cell lines is associated with a significant reduction in cell survival. Stable overexpression of *RASSF1A* within H1299 NSCLC cells was associated with 40-60% decrease in anchorage-dependent growth, a 90% reduction in anchorage-independent colony formation and a significant reduction in tumour formation in nude mice, compared with empty vector controls (Burbee *et al.,* 2001). Interestingly, several stable *RASSF1A* overexpressing H1299 clones were found to proliferate well *in vitro*, but were found to exhibit a significant reduction in anchorage-independent growth, which demonstrates that *RASSF1A* may have multiple independent functions that suppress cancer cell growth and malignant potential. Therefore, based on the observed lack of cell growth inhibition within stable 21NT-pcDNA-*RASSF1A* clones within my study, it would be interesting to determine whether overexpression of *RASSF1A* is associated with any changes in the tumorigenicity of 21NT cells.

5.4.3 PBRM1

The polybromo-1 (PBRM1) protein member of the SWI/SNF complex B (PBAF) chromatin remodelling complex has been found to play important functional roles in regulating gene expression, cell cycle control, replicative senescence and more recently, genome stability in human cells (Xia *et al.,* 2008, Burrows *et al.,* 2010, Brownlee *et al.,* 2014, Karkarougkas *et al.,* 2014). *PBRM1* mutations have been identified within a variety of cancer types including breast, stomach, urinary tract and is the second most commonly mutated gene in renal cell carcinoma (www.cancer.sanger.ac.uk, Forbes *et al.,* 2014, Varela *et al.,* 2011). Loss of *PBRM1* in RCC is also associated with advanced tumour stage and poor disease prognosis (Pawlowski *et al.,* 2012). These findings demonstrate that *PBRM1* may function as an important tumour suppressor gene in multiple tissue types.

According to the COSMIC database, only 0.95% of 1156 breast carcinoma samples have been found to harbour *PBRM1* mutations, the majority of which are missense mutations located within PBRM1 bromodomains BD3, BD4, BD5, BD6, the protein-protein interaction bromoadjacent homology domain 1 (BAH1) and the DNA-binding high mobility group (HMG) domain (Forbes *et al.,* 2014). Out of 43 breast cancer cell lines, Xia *et al.,* (2008) identified four truncating mutations within the bromodomains of PBRM1 of four samples (Xia *et al.,* 2008). The presence of *PBRM1* mutations within these cell lines was also associated with loss of *PBRM1* expression. These findings demonstrate that *PBRM1* mutations are uncommon in breast cancer and can occur within all three major functional domains. However, Xia *et al.,* (2008) demonstrated that almost half (48%) of 52 paired breast tumour samples showed loss of heterozygosity at two sites flanking the *PBRM1* genomic locus. Similarly, the results presented within my study showed that 56% of breast cancer cell lines examined were found to have undergone allele loss within the *PBRM1* locus. In addition, *PBRM1* copy number variation (CNV) analysis of the HCC1143 breast cancer cell line confirmed the results presented by Xia *et al.,* (2008) demonstrating the presence of a homozygous deletion within *PBRM1*. Interestingly, two breast cancer cell lines (22%) were found to possess a different number of alleles at the *PBRM1* genomic locus when compared with neighbouring gene loci situated upstream of *PBRM1* (*BAP1, PARP-3, FLJ* and *SETD2).* The SV40-immortalized MTSV breast cell line was found to have gained a single allele at all loci examined apart from *PBRM1*, which was found to be present at two copies. Similarly, the HCC1143 breast cancer cell line showed loss of a single allele at all loci examined apart from *PBRM1,* which was not present within HCC1143 genomic DNA. This demonstrates that allele loss within the *PBRM1* locus is a frequent event in breast cancer and provides additional evidence to show that *PBRM1* may function as an important tumour suppressor gene.

The high frequency of allele loss at the *PBRM1* locus in breast cancer cells suggest that gene copy number alterations may influence *PBRM1* function. In support of this, *PBRM1* expression levels within breast cancer cell lines that had undergone allele loss at the *PBRM1* locus were, on average, significantly lower than normal human mammary epithelial cell (HMEC) strains. However, both MCF-7 and BT20 breast cancer cell lines, which harbour two *PBRM1* alleles, were also found to exhibit a greater than 50% reduction in *PBRM1* expression levels relative to normal HMEC strains. These findings suggest that both gene copy number loss and transcriptional repression may contribute to deregulation of *PBRM1* in breast cancer cells. In contrast to the findings presented within my study, DeCristofaro *et al.,* (2001) showed that 21NT, BT474, BT20, H5S78T and MCF-7 breast cancer cell lines display similar protein levels of SWI/SNF complex A and B members, including PBRM1 to the human Jurkat leukemic T-cell line. Only 2 out of 21 (9.5%) breast cancer cell lines, were found to exhibit significantly lower levels of PBRM1 protein compared to Jurkat T-cells, which suggests that the majority of

breast cancer cell lines may possess normal levels of PBRM1 protein. However, mammalian SWI/SNF complexes have been found to exhibit cell-type specific differences in protein sub-unit composition and expression levels, which is thought to be due to tissue-specific SWI/SNF chromatin remodelling functions (Wang *et al.,* 1996). Therefore, taking into account the frequency of *PBRM1* allele loss and the observed transcriptional deregulation of *PBRM1* within breast cancer cell lines, it is possible that PBRM1 protein levels may also be significantly reduced when compared with normal HMEC strains.

Depletion of *PBRM1* within normal human fibroblasts has been associated with delayed induction of replicative senescence pathways and a reduction in the expression levels of multiple *p53* target genes, including *p21* (Burrows *et al.,* 2010). Transient overexpression of *PBRM1* within *PBRM1*-deficient HCC1143 breast cancer cell line has also been associated with induced *p21* expression and cell cycle arrest in G1 phase. *PBRM1* has been found to interact directly with the *p21* promoter and mediate transcriptional activation in response to DNA damage-induced signalling pathways (Xia *et al.,* 2008). *p53* has been found to mediate transcriptional repression of *hTERT* within breast cancer cells through activation of *p21* and recruitment of E2F-pRB pocket protein-HDAC chromatin remodelling complexes (Shats *et al.,* 2004). Interestingly, the chromsome-3 encoded telomerase repressor gene within telomerase-repressed 21NT-chromosome 3 hybrid colonies has been found to mediate transcriptional repression of *hTERT,* in part, through chromatin remodelling at intron 2 of the *hTERT* promoter (Szutorisz *et al.,* 2003). Taking these findings into account, the putative role of *PBRM1* in regulating *hTERT* expression and telomerase activity through induction of *p21* was investigated within my study. Consistent with Xia *et al.,* (2008) a positive correlation between *PBRM1* and *p21* expression levels was observed within stable 21NT-*PBRM1* transfection clones and empty vector controls. However, a 2-11 fold increase in stable *PBRM1* expression within 21NT cells, was not associated with a significant increase in *p21* expression levels compared to empty vector controls. Xia *et al.,* (2008) demonstrated a substantial up-regulation in p21 protein 16-22 hours following transient transfection of HCC1143 and SUM1315 breast cancer cell lines with wild-type *PBRM1*. It is possible that transient overexpression of *PBRM1*-plasmid constructs produced a greater than 11-fold increase in *PBRM1* transcript levels within breast cancer cells in the Xia *et al.,* (2008) study. Therefore, high levels of *PBRM1* expression may be required to induce *p21* transcription within breast cancer cells.

In my study, stable overexpression of *PBRM1* was not associated with a consistent reduction in *hTERT* expression levels within 21NT cells. Despite observing a substantial decline in telomerase activity within stable 21NT-pBABE-*PBRM1* transfection clones, empty vector controls were also found to display a similar decrease in telomerase activity levels relative to untreated 21NT cells. Therefore, it remains unclear whether *PBRM1* functions as a regulator of *hTERT* transcription or telomerase activity in breast cancer cells. Four out of six (67%) stable

PBRM1-transfected 21NT clone populations contained cells that exhibited morphological features of senescence. However, none of the stable clone populations were found to enter complete growth arrest within around five weeks of continuous culture. Cuthbert *et al.,* (1999) found that the majority of telomerase-repressed 21NT-chromosome 3 hybrids entered growth arrest around 3-5 weeks following MMCT-mediated transfer of a normal copy of chromosome 3 into 21NT cells. Therefore, it is unlikely that *PBRM1* is responsible for the observed telomerase repression and cell growth arrest of 21NT-chromosome 3 hybrids within the Cuthbert *et al.,* (1999) study.

Forced overexpression of wild-type *PBRM1* within the HCC1143 breast cancer cell line was associated with a significant reduction in the number and size of stable colonies that emerged following two weeks of antibiotic selection, compared to empty vector controls (Xia *et al.,* 2008). This was found to be due, in part, to induction of *p21* expression and cell cycle arrest in G1 phase. Using the same plasmid vector constructs as Xia *et al.,* (2008), I found that forced overexpression of *PBRM1* within the 21NT breast cancer cell line was also associated with a significant reduction in stable cell colony formation relative to empty vector controls. Further investigation would be necessary to establish whether *PBRM1*-associated 21NT cell growth inhibition was also due cell cycle arrest in G1 phase. These findings provide additional evidence in support of the idea that that *PBRM1* functions as an important tumour suppressor gene in breast cancer cells.

5.4.4 PARP-3

The human poly(ADP-ribose) polymerase enzyme PARP-3 has been implicated in multiple signalling pathways including DNA repair, cell cycle progression, telomere stability and has recently been shown to play a role in telomerase regulation (Boehler *et al.,* 2010, Rulten *et al.,* 2010, Beck *et al.,* 2014, Augustin *et al.,* 2003, Fernandez-Marcelo *et al.,* 2014). Around 10% of primary invasive breast tumour samples have been found to exhibit significantly lower levels of *PARP-3* mRNA relative to normal breast tissue samples (Bieche *et al.,* 2011). Underexpression of *PARP-3* was also more frequently associated with a high histopathological tumour grade and the presence of oestrogen and progesterone receptors. Overall, the majority of invasive breast tumour specimens were found to display similar expression levels of *PARP-3* to normal breast tissue, with only a small proportion (1.4%) exhibiting significant overexpression (Bieche *et al.,* 2011). These findings suggest that a deregulation of *PARP-3* expression occurs within a sub-set of invasive breast cancer cases and may be associated with more progressive disease. Within my study, 44% of breast cancer cell lines were found to exhibit a greater than 70% reduction in *PARP-3* mRNA levels relative to normal HMEC strains. These included the 21NT and 21MT cell lines derived from the primary tumour and metastatic deposit of a patient

diagnosed with breast IDC, the MCF-7 metastatic breast adenocarcinoma cell line and the SV40-immortalized breast luminal epithelial cell line MTSV. Both metastatic deposit-derived 21MT and MCF-7 cell lines were found to exhibit the lowest levels of *PARP-3* expression, which is consistent with the findings presented by Bieche *et al.,* (2011) demonstrating an association between *PARP-3* underexpression and more progressive disease. The observed downregulation in *PARP-3* expression within cell lines derived from primary breast tumours and metastatic deposits suggests that loss of *PARP-3* expression may promote breast tumour development and progression.

Within my study, normal HMEC strains were found to exhibit a wide range of *PARP-3* expression levels. Johnsson *et al.,* (1999) demonstrated that *PARP-3* transcripts can be detected within multiple normal human tissues, however certain tissue types including the placenta and thymus exhibit particularly low levels of *PARP-3* mRNA. Similarly, immunohistochemical analysis of Cynomolgous Monkey tissues demonstrated that PARP-3 exhibits a tissue and cell-type specific expression pattern (Rouleau *et al.,* 2009). High levels of PARP-3 expression were observed primarily within the ductal epithelial cells of the prostate, pancreas and salivary glands and moderately high levels within mammary secretory and ductal epithelial cells. In addition, high levels of PARP-3 protein were observed within well-differentiated cells compared with immature cells such as oocytes or spermatogenic cells. Due to the observed interaction between *PARP-3* and members of the PcG complex (Rouleau *et al.,* 2007), it was proposed that high levels of *PARP-3* may be required to repress the transcription of genes involved in early development (Rouleau *et al.,* 2009). PARP-3 protein was undetectable within human (HeLa and A549) and monkey cell lines, which suggests that actively proliferating cells generally exhibit low levels of PARP-3 expression both *in vitro* and *in vivo.* Normal early-passage pre-stasis HMEC strains consist of a mixture of breast luminal, myoepithelial and progenitor epithelial cells (Garbe *et al.,* 2012). With increasing passages, the proportion of multipotent progenitor and luminal epithelial cells within the population have been found to increase. Therefore, the variation in *PARP-3* expression levels observed within normal HMEC strains within my study may be due to differences in the passage number and composition of immature progenitor and differentiated epithelial cell types that existed within the population at the time of analysis.

Within my study, *PARP-3* gene copy number variation analysis of nine breast cancer cell lines revealed that 56% had undergone allele loss within this locus. The majority of these breast cancer cell lines were derived from primary breast IDC tumours known to harbour *p53* mutations. The remaining four breast cancer cell lines were found to harbour two alleles (22%) or three alleles (22%) and were derived from primary breast IDC, metastatic breast adenocarcinoma, primary breast carcinosarcoma and an SV40-immortalized luminal epithelial cell line. These findings demonstrate that allele loss within the *PARP-3* genomic locus may be a frequent event in breast cancer. Interestingly, I found that *PARP-3* gene copy number was loss

was not always associated with a reduction in mRNA levels. BT474, HCC1143 and GI101 breast cancer cell lines harbouring only one *PARP-3* allele were found to exhibit similar levels of *PARP-3* expression to normal HMEC strains. Moreover, both MCF-7 and MTSV cell lines, which harbour two and three *PARP-3* alleles respectively, were among those breast cancer cell lines exhibiting the lowest levels of *PARP-3* expression. Therefore, *PARP-3* copy number alterations in breast cancer are not always associated with changes in *PARP-3* expression and other mechanisms of transcriptional repression may also be responsible for the observed deregulation in *PARP-3* expression levels. However, these results demonstrate that *PARP-3* copy number loss is a frequent event in breast cancer, which is consistent with the notion that this gene may function as a tumour suppressor.

The presence of high levels of telomerase activity within NSCLC tumour samples has been associated with low levels of *PARP-3* expression (Frias *et al.,* 2007). Within my study, all breast cancer cell lines examined were found to possess detectable levels of telomerase activity (Chapter 4.3.1.3). Interestingly, apart from BT20, breast cancer cell lines exhibiting higher levels of telomerase activity than the telomerized PC3-hTERT cell line were found to have either lost one allele at the *PARP-3* locus, or were found to exhibit low *PARP-3* mRNA levels relative to normal HMEC strains. In addition, the H5S78T breast carcinosarcoma cell line, which was found to exhibit the highest levels of *PARP-3* expression, also exhibited low levels of telomerase activity levels when compared to other breast cancer cell lines examined. However, in contrast to Frias *et al.,* (2007), not all breast cancer cell lines exhibiting the highest levels of telomerase activity were found to display a considerable decrease in *PARP-3* expression levels relative to normal HMECs. The protein levels or functional status of PARP-3 within breast cancer cell lines was not examined within this study, therefore there is insufficient evidence to show that high levels of telomerase activity are associated with loss of PARP-3 function in breast cancer. However, within the majority of breast cancer cell lines examined, deregulation of *PARP-3* due to gene copy number loss or reduced expression levels was found to be associated with higher levels of telomerase activity.

Transient overexpression of *PARP-3* within A549 lung adenocarcinoma cells has been associated with a significant decrease in telomerase activity relative to empty vector controls (Fernandez-Marcelo *et al.,* 2014). In addition, shRNA-mediated depletion of *PARP-3* within Saos-2 osteosarcoma cells, was associated with a significant increase in telomerase activity compared with non-targeting controls. Consistent with these findings, recent preliminary experiments carried out by my colleague Dr. Terry Roberts at Brunel University, have demonstrated that stable overexpression of *PARP-3* within the 21NT breast cancer cell line is associated with a reduction in *hTERT* expression levels when compared with empty vector controls (Dr. Terry Roberts, unpublished data). *PARP-3* is located within the 3p21.2 region, which is close to the 3p21.3-p22 region that is thought to contain a telomerase repressor

sequence in breast cancer (Cuthbert *et al.,* 1999). The results presented within my study show that the majority of breast cancer cell lines exhibiting higher levels of telomerase activity have either undergone allele loss within *PARP-3* locus, or display a substantial reduction in *PARP-3* expression levels. In addition, Boehler *et al.,* (2010) demonstrated that loss of PARP-3 within normal human lung fibroblasts was associated with spontaneous sister telomere fusions and loss, indicating that PARP-3 may play a functional role in maintaining telomere stability (Boehler *et al.,* 2010). These observations, together with the findings presented by Fernandez-Marcelo *et al.,* (2014) and Dr. Roberts provide evidence to show that *PARP-3* may function as a negative regulator of *hTERT* transcription in breast and lung cancer cells. Future experiments will aim to determine whether stable *PARP-3* overexpression within the 21NT cell line is associated with telomerase repression and induction of cellular senescence pathways.

CHAPTER 6

6 INVESTIGATING CHROMOSOME 3P STRUCTURE DURING THE MULTI-STEP PROCESS OF NORMAL HUMAN MAMMARY EPITHELIAL CELL (HMEC) IMMORTALIZATION

6.1 INTRODUCTION

The transformation of normal human somatic cells into fully malignant tumour cells is thought to be a multi-step process involving the acquisition of multiple genetic alterations that allow bypass of intrinsic barriers to uncontrolled and unlimited cell growth (reviewed by Newbold., 2002, Stampfer and Yaswen., 2002). Studies examining the immortalization of normal finite lifespan, human epithelial cells *in vitro* have identified mechanisms by which these cells are able to bypass intrinsic barriers to proliferation (Stampfer and Yaswen., 2002). Normal HMECs, derived from reduction mammoplasty tissues of healthy individuals, normally proliferate for around 15-45 population doublings (PD) before entering growth arrest termed stasis (Figure 6.1, Brenner *et al.,* 1998, Romanov *et al.,* 2001). At this point, cells display expression of senescence-associated β-galactosidase (SA-β-gal), are karyotypically normal and exhibit significantly increased expression levels of the cyclin-dependent kinase inhibitor (CKDI) $p16^{INK4A}$ (Brenner *et al.,* 1998). Romanov *et al.,* (2001) demonstrated that pre-stasis HMECs do not express detectable levels of telomerase activity and telomere attrition prior to growth arrest is approximately 30bp/PD. Once the cells had reached stasis, mean telomere restriction fragment (TRF) lengths were around 6kbp, which would indicate that stasis is a telomere-length independent barrier. Furthermore, exogenous *hTERT* expression within pre-stasis HMEC cells was not associated with stasis bypass or cellular immortalization in the majority of cases (Stampfer *et al.,* 2000). Only one immortal *hTERT*-transduced pre-stasis HMEC clone emerged, which was found to have lost *p16* expression. It is thought that stasis occurs in response to stressful stimuli, which leads to induction of *p16* expression, inhibition of pRb phosphorylation and cell cycle arrest in G1 (Brenner *et al.,* 1998, Stampfer and Yaswen., 2002). In support of this, HMECs cultured in serum-free media proliferate continuously for only 2-3 passages before displaying increased levels of *p16* expression and entering growth arrest (Hammond *et al.,* 1984).

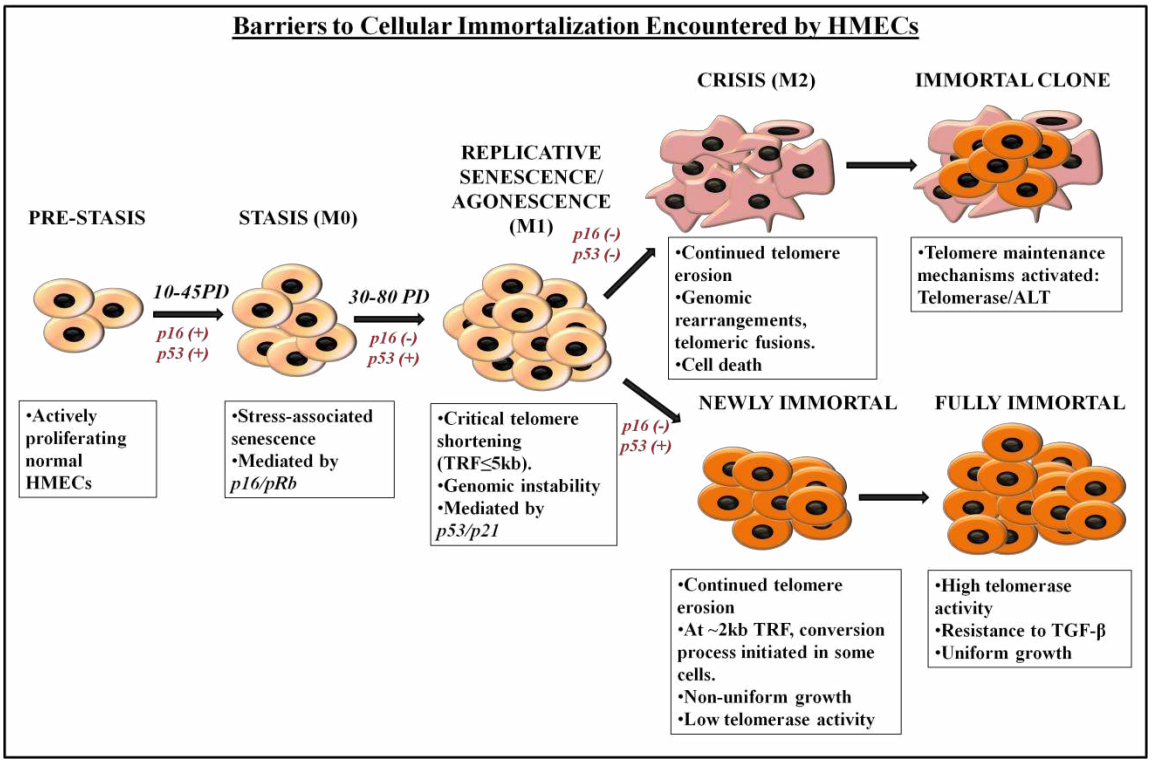


Figure 6.1 - *Model of the barriers to indefinite proliferation encountered by normal human mammary epithelial cells (HMEC) and how these are bypassed/overcome (adapted from Stampfer and Yaswen., 2003). Normal primary HMECs undergo 10-45 population doublings (PD) before entering the p16/pRb-mediated stress-associated senescence barrier known as stasis (M0). Loss of p16 function through mutation or promoter hypermethylation allows stasis bypass. Post-stasis HMECs continue to divide for 30-80PD until mean telomere lengths reach ≤5kb. Cells then enter replicative senescence/agonescence (M1), where cells exhibit abnormal changes in chromosome structure. In the absence of functional p53, HMECs undergo further telomere shortening and enter crisis (M2), which is characterised by extensive genomic instability and cell death. Following a rare mutational/epigenetic event during crisis, telomere maintenence mechanisms are activated and immortal clones emerge. In the presence of functional p53, post-stasis HMECs can become immortalized through a process termed conversion, which is characterized by the gradual acquisition of telomerase activity and resistance to TGF-β-induced growth arrest. Abbreviations: TRF (Telomere Restriction Fragment), ALT (Alternative Lengthening of Telomeres), TGF-β (Transforming Growth Factor-Beta).*

Small subpopulations of proliferating cells have been found to emerge sporadically from senescent cultures of HMECs maintained in both serum-containing and serum-free media (Brenner *et al.,* 1998). Unlike normal human mammary fibroblasts, spontaneous emergence from stasis occurs more frequently and is associated with loss of *p16* expression (Huschtscha *et al.,* 1998, Brenner *et al.,* 1998, Romanov *et al.,* 2001). A common mechanism of *p16* silencing observed within post-stasis HMECs is CpG island promoter methylation. Treatment of these

cells with the demethylating agent 5-aza-2-deoxycytidine was found to be associated with re-expression of *p16* and induction of senescence pathways (Brenner *et al.,* 1998). Loss of *p16* is a frequent event in breast cancer, with around 50% of breast tumours exhibiting undetectable levels of p16 expression (Geradts and Wilson., 1996). In addition, variant HMECs (vHMECs), which possess hypermethylated and silenced *p16* expression, have been observed within histologically normal mammary tissue sections derived from healthy women, indicating that post-stasis mammary epithelial cells are already present *in vivo* (Holst *et al.,* 2003). These findings demonstrate that *p16* is a key mediator of stress-associated stasis and that loss of *p16* expression and bypass of the stasis barrier may be an early event in breast carcinogenesis.

Post-stasis, *p16*-deficient HMECs also known as post-selection or extended life HMECs, have been found to proliferate for up to 80 PD before entering a second growth plateau known as replicative senescence or agonescence (Figure 6.1, Romanov *et al.,* 2001, Stampfer and Yaswen., 2002). Despite the presence of cells positive for SA-β-gal, HMECs at this growth plateau exhibit significantly different characteristics from cells at stasis, including a heterogeneous morphology, higher proliferative index and increased cell death (Romanov *et al.,* 2001). During the second exponential growth phase, HMECs undergo progressive telomere shortening and exhibit mean TRF lengths of around 3.5kbp upon reaching the replicative senescence barrier. This is accompanied by increasing levels of genomic instability including chromosomal deletions, translocations, rearrangements, telomeric fusions and γ-H2AX foci (Romanov *et al.,* 2001, Garbe *et al.,* 2009). The major mediator of this proliferative barrier is *p53,* which activates DNA damage response pathways and cell growth arrest in response to critical telomere shortening (Stampfer and Yaswen., 2002). Garbe *et al.,* (2007) found that post-stasis HMECs transduced with the dominant-negative *p53* suppressor element GSE22 entered crisis, characterized by massive cell death and mean TRF lengths of around 3.1kbp. As expected, untreated *p53*-postive controls were found to enter replicative senescence. Ectopic expression of *hTERT* within post-stasis HMECs is sufficient to bypass replicative senescence pathways and lead to immortal transformation (Stampfer *et al.,* 2000). These findings demonstrate that both replicative senescence and crisis are important telomere length-dependent barriers to proliferation and the presence of *p53* within post-stasis HMECs is associated with induction of viable cell growth arrest.

Spontaneous emergence of immortal cells from replicative senescence has never been found to occur *in vitro* (Stampfer and Yaswen., 2002). Studies have shown that abrogation of *p53* function through introduction of specific dominant-negative p53 mutants or transduction with the SV40 Large T-Antigen is associated with the rare emergence of immortal clones from crisis (Gao *et al.,* 1996, Gollahan and Shay., 1996, Van Der Haegen and Shay., 1993). Spontaneous immortalization from crisis has also been observed within HMECs derived from a patient with Li Fraumeni syndrome, which harbour germline mutations in *p53* (Shay *et al.,*

1994). However, stable transduction of post-stasis HMECs with the *p53* inhibitor GSE22 was associated with weak or no telomerase activity both prior to and as cells entered crisis, which demonstrates that loss of *p53* function alone is not sufficient for de-repression of endogenous telomerase activity and cellular immortalization (Garbe *et al.,* 2007). It is thought that a rare mutational event occurs during crisis that is responsible for telomerase reactivation (Shay., 1993).

Interestingly, Stampfer *et al.,* (1997) demonstrated that loss of *p53* is not a necessary event for the immortal transformation of HMECs. Following benzo(a)pyrene (BaP)-treatment of primary HMEC cultures, two immortal cell lines 184A1 and 184B5 emerged (Stampfer and Bartley., 1985). They were found to harbour functional *p53* but possess numerous chromosomal aberrations including deletions and translocations and were found to be non-tumorigenic. Early-passage 184A1 and 184B5 cultures maintained continuous, non-uniform growth, progressive telomere shortening and no detectable telomerase activity for around 10-30PD. Once mean TRF lengths had reached around 2kbp, some surviving cells were found to express low levels of telomerase activity and displayed a limited ability to maintain growth in the presence of TGF-β (Stampfer *et al.,* 1997). TGF-β induces growth arrest within normal human epithelial cells, whereas resistance to TGF-β is a common feature of malignant carcinoma cells and is associated with invasive properties (Fynan and Reiss., 1993). Over subsequent passages, both telomerase activity levels and resistance to TGF-β were found to increase, until mass cultures exhibited uniform growth patterns and were fully immortalized. It is thought that critical telomere shortening within early passage, conditionally immortal HMECs 184A1 and 184B5, triggered a 'conversion' process whereby telomerase was re-activated gradually through an epigenetic mechanism. These findings contrast previous observations that telomerase re-activation occurs as a rapid event due to a rare mutation during crisis (Shay *et al.,* 1993). Therefore, normal human epithelial cells may possess several mechanisms of telomerase-repression that prevent immortal transformation. Interestingly, transduction of 184A1 cells with the *p53*-inhibitor GSE22, was associated with a more rapid increase in *hTERT* expression, telomerase activity and TGF-β resistance compared with untreated controls (Stampfer *et al.,* 2003). Therefore, loss of *p53* function is insufficient to induce telomerase reactivation, but instead may accelerate the acquisition of rare mutational events required for telomerase de-regulation and immortal transformation. In support of this, early-passage immortal *p53*-deficient HMECs have been found to exhibit higher levels of genomic instability with further genomic aberrations observed over increasing passages, than conditionally immortal HMECs harbouring functional *p53* (Stampfer *et al.,* 2003).

Transduction of *hTERT* within post-stasis HMECs is sufficient for bypass of replicative senescence barriers and complete immortal transformation (Stampfer *et al.,* 2000). In addition, telomerase-positive cancer cell lines have been found to express *hTERT* transcripts, whereas

telomerase-negative normal human primary lung fibroblasts do not (Ducrest *et al.,* 2001). Therefore, deregulation of *hTERT* transcription may be the primary mechanism by which telomerase is reactivated as a rare event during crisis. As discussed in earlier Chapters, introduction of a normal copy of human chromosome 3 within the 21NT breast cancer cell line was associated with a 30-fold reduction in endogenous *hTERT* expression and strong telomerase repression (Cuthbert *et al.,* 1999, Ducrest *et al.,* 2001). Deletion mapping of telomerase-positive segregant 21NT-chromosome 3 hybrids identified two regions on the short arm of chromosome 3 (3p21.3-p22 and 3p12-21.1) that may harbour putative *hTERT* repressor sequences (Cuthbert *et al.,* 1999). Furthermore, LOH within chromosome 3p has been found to occur within 52-87% of breast tumour samples and 45% of pre-neoplastic breast lesions, which demonstrates that critical tumour suppressor genes may be located within this region that are lost as a frequent and early event during breast carcinogenesis (Matsumoto *et al.,* 1997, Martinez *et al.,* 2001, Maitra *et al.,* 2001, Chen *et al.,* 1994, De Oliveira *et al.,* 2012). Taking these observations into account, the primary aim of this study was to investigate whether genomic deletions within chromosome 3p coincide with de-repression of *hTERT* and telomerase re-activation during the immortal transformation of normal HMECs.

6.2 MATERIALS AND METHODS

6.2.1 CELL LINES/STRAINS

This study was carried out in collaboration with my colleague at Brunel University Dr. Hemad Yasaei, who was responsible for cultivating all HMEC cell strains, their derivatives and for generating immortal clones. The normal HMEC 184D strain (Stampfer and Bartley., 1984), derived from reduction mammoplasty tissue of a 21-year old female Caucasian patient was employed for the purposes of this study (Table 2.1, Chapter 2.1.2.1). To generate immortal clones, pre-stasis HMEC 184D cells were treated with nickel chloride ($NiCl_2$) to silence *p16* expression and bypass the stasis barrier (Figure 6.2 - 184D-Ni). Nickel chloride is known to mediate its carcinogenic effects through DNA methylation and heterochromatization of genes (Lee *et al.,* 1995). Finite lifespan, post-stasis 184D-Ni cells were then transfected with short-hairpin RNA (shRNA) that targets *p53* (a kind gift from Professor E. K. Parkinson). Following selection of stable transfection clones, cells were found to enter crisis. After 6-12 weeks, spontaneous immortal clones were found to emerge, which were isolated and propagated as individual cell lines (Figure 6.2 - 184D-Ni*p53*sh clones 2.1, 2.3, 3.3, 6.1, 6.2 and 6.3). Non-targeting control 184D-Ni cells entered replicative senescence and no immortal clones were found to emerge spontaneously. In addition, immortal 184D-*p16*sh and 184D-hT1 cell line derivatives were also employed for the purposes of this study (Figure 6.2). The 184D-hT1 cell line was derived through electroporation of pre-stasis HMEC 184D cell cultures with *hTERT*

cDNA plasmid vectors (Dr. Yasaei, unpublished data). Transfected cells were found to undergo non-clonal bypass of both stasis and replicative senescence barriers (Figure 6.2). The 184D-*p16*sh cell line was derived from transduction of pre-stasis 184D cells, maintained in low serum and low stress medium, with shRNA targeting *p16* (Garbe *et al.,* 2014). Following stasis bypass, immortal 184D-*p16*sh clones were reported to have emerged from crisis (Figure 6.2). As a normal telomerase-negative control, the finite lifespan, pre-stasis HMEC 184D strain was used (Stampfer and Bartley., 1984). The 21NT cell line, which is derived from a primary intraductal carcinoma of the breast, was included within this study to represent a telomerase-positive malignant breast tumour cell line (Band *et al.,* 1990). This cell line was cultivated by me using the methods and culture conditions described previously (Table 2.2, Chapter 2.1.2).

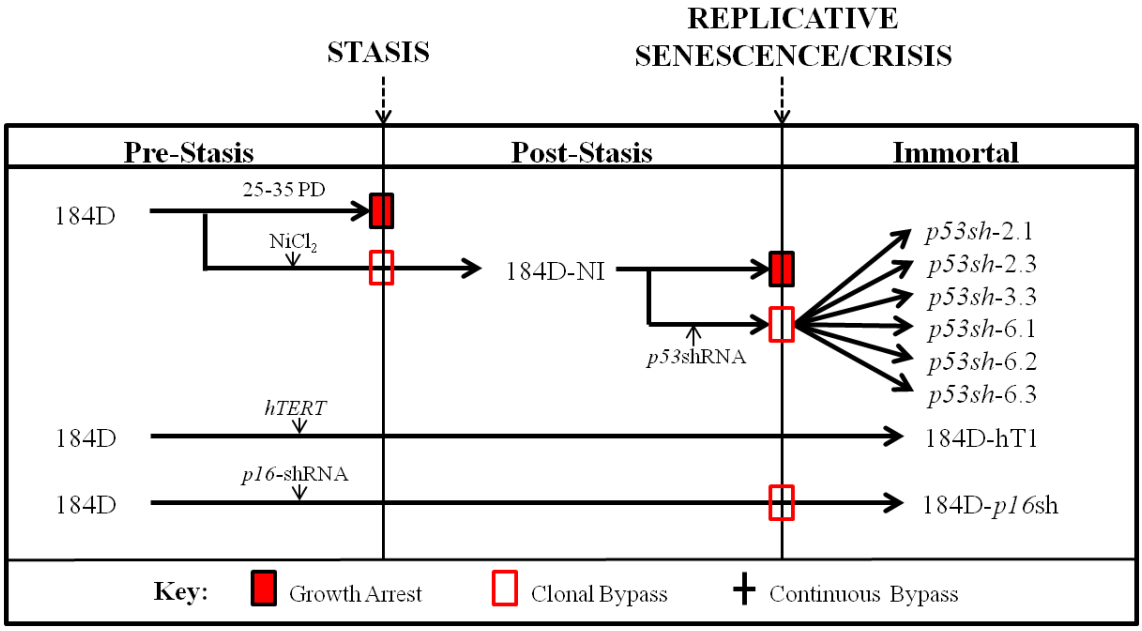


Figure 6.2 - *A model showing the derivation of HMEC 184D cell strains/lines used during this study. HMEC 184D (Stampfer and Bartley 1984), 184D-p16sh (Garbe et al., 2014) 184D-Ni, immortal p53sh clones and the immortalized 184D-hT1 cell line were cultivated by Dr. Yasaei at Brunel University. The HMEC 184D cell strain and immortal 184D-p16sh cell line was obtained directly from the Human Mammary Epithelial Cell (HMEC) Bank, Lawrence Berkeley Laboratory, University of California (http://hmec.lbl.gov/mindex.html).*

All HMEC 184D strains and their derivatives were provided to me upon request as cell pellets obtained from mass cultures growing in log-phase. These were immediately stored at -80ºC until required. 21NT cells growing in log-phase were cultured under normal growth conditions (Chapter 2.1.2) in P100 dishes until sub-confluent. Cells were then harvested using the methods described in Chapter 2.1.3 and pelleted by centrifugation at 15,000xg for 5 minutes before storing at -80ºC until required.

6.2.2 GENE EXPRESSION ANALYSIS

The expression levels of *p16, p53* and *hTERT* were examined within finite lifespan pre-stasis HMEC 184D, post-stasis 184D-Ni, immortal HMEC 184D derivatives (184D-*p16*sh, 184D-hT1 and 184D-Ni*p53*sh clones 2.1, 2.3, 3.3, 6.1, 6.2, 6.3) and the 21NT cell line. Sample cell pellets were removed from -80ºC and thawed on wet ice. All cell lines/strains underwent RNA extraction, DNAseI-treatment and cDNA synthesis using the methods described in Chapter 2.3. Using the appropriate primer sequences and thermal cycling parameters described in Table 2.3 and Table 2.4 (Chapter 2.3.4), the gene expression levels of *p16, p53* and *hTERT* were examined as described in Chapter 2.3.4. Target gene expression levels were normalized against *TOP1* and *YWHAZ* endogenous control genes. The qbase^{PLUS} (Biogazelle) data analysis software program was used to calculate normalized relative quantities (NRQ) of target gene mRNA for all samples as described in Chapter 2.3.5.

6.2.3 COPY NUMBER VARIATION (CNV) ANALYSIS

Physical mapping of human chromosome 3p structure within finite lifespan HMEC 184D strains, immortal derivatives and the 21NT breast cancer cell line was determined by CNV analysis of five annotated genes and one region (termed *FHIT*-prox henceforth) spanning the length of chromosome 3p including, *SUMF1*, *RAR-β*, *SETD2*, *PBRM1*, *FHIT*-prox and *CADM2* (Table 6.1). *FHIT*-prox is located at 3p14.2, which is approximately 1.4kbp downstream from *FHIT*.

Table 6.1 - Details of target genes/region selected for physical chromosome 3p mapping by CNV analysis

Gene	Full Name	NCBI Location: Distance from Telomere (Mb)
SUMF1	Sulfatase modifying factor 1	3p26.1c: 4.4
RAR-β	Retinoic acid receptor-β	3p24.2a: 25.5
SETD2	SET domain containing 2	3p21.31f: 47.1
PBRM1	Polybromo-1	3p21.1e: 52.7
***FHIT*-prox**	Proximal to the fragile histidine triad	3p14.2b: 61.3
CADM2	Cell adhesion molecule 2	3p12.1a: 85.9

Abbreviations: NCBI (National Centre for Biotechnology Information, http://www.ncbi.nlm.nih.gov/)

Sample cell pellets were first removed from -80ºC and thawed on wet ice. Genomic DNA (gDNA) was extracted from each sample as described in Chapter 2.2 and target gene CNV

analysis was carried out using pre-designed Taqman® Copy Number Assay primers developed by ABI (Applied Biosystems, US) according to manufacturer's guidelines (Chapter 2.5). Details of gene primer sets used to determine target gene copy number are described in Table 2.6 (Chapter 2.5). Normal pre-stasis HMEC 184D cell gDNA extracts was used to calibrate gene copy number within all samples. The Copy Caller™ Software data analysis program was used to calculate target gene copy number within all samples (Chapter 2.5).

6.3 RESULTS

6.3.1 CHARACTERIZATION OF HMEC 184D AND IMMORTAL DERIVATIVES

In order to determine whether changes in chromosome 3p structure occur during the immortal transformation of normal HMECs, the primary HMEC 184D cell strain together with post-stasis and immortal derivatives (cultivated and developed by my colleague Dr. Hemad Yasaei, Brunel University), were investigated (Table 6.2). These included pre-stasis and post-stasis finite lifespan HMEC 184D cell strains, six immortal 184D-Ni*p53*shRNA clones that had emerged from crisis following stable knockdown of *p53*, an immortal 184D cell line derived from transfection of pre-stasis 184D cells with *hTERT* and an immortal *p16*-shRNA treated 184D cell line derivative (Figure 6.2 and Table 6.2). To generate immortal 184D-Ni*p53*sh clones, pre-stasis HMEC 184D primary cultures had been treated with nickel chloride to induce epigenetic silencing of *p16* and bypass the stasis barrier (Dr. Yasaei, unpublished data). Following treatment of finite lifespan 184-Ni cells with shRNA targeting *p53,* cells entered crisis and immortal clones were found to emerge at a frequency of around $2x10^{-7}$ (184D-Ni*p53*sh clones: 2.1, 2.3, 3.3, 6.1, 6.2, 6.3). According to recent data, these clones are telomerase positive and have been found to proliferate continuously for over 110 PD (Dr. Yasaei, unpublished data). It is thought that telomerase re-activation occurs due to a rare mutational event during cell crisis (Shay *et al.,* 1993). However the findings presented by Stampfer *et al.,* (1997) demonstrate that loss of *p53* is not required for telomerase re-activation to occur and that *p53*-independent mechanisms of telomerase repression may exist within normal HMECs. Therefore, 184D-hT1 (Dr. Yasaei, unpublished data) and 184-*p16*sh (Garbe *et al.,* 2014) cell line derivatives, which did not acquire an immortal phenotype through enforced abrogation of *p53* function, were also employed for the purposes of this study. The 184D-*p16*sh cell line was found to emerge spontaneously from replicative senescence, whereas 184D-hT1 was found to exhibit non-clonal bypass of both stasis and replicative senescence barriers (Figure 6.2). The 21NT cell line, derived from a malignant primary intraductal carcinoma of the breast, was also included within this study. This particular cell line has been found to express *hTERT* transcripts (Ducrest *et al.,* 2001, Szutorisz *et al.,* 2003, Chapter 4.3.2) is telomerase positive (Cuthbert *et al.,* 1999, Chapter 3.3.2) and was previously shown to have undergone allele loss of

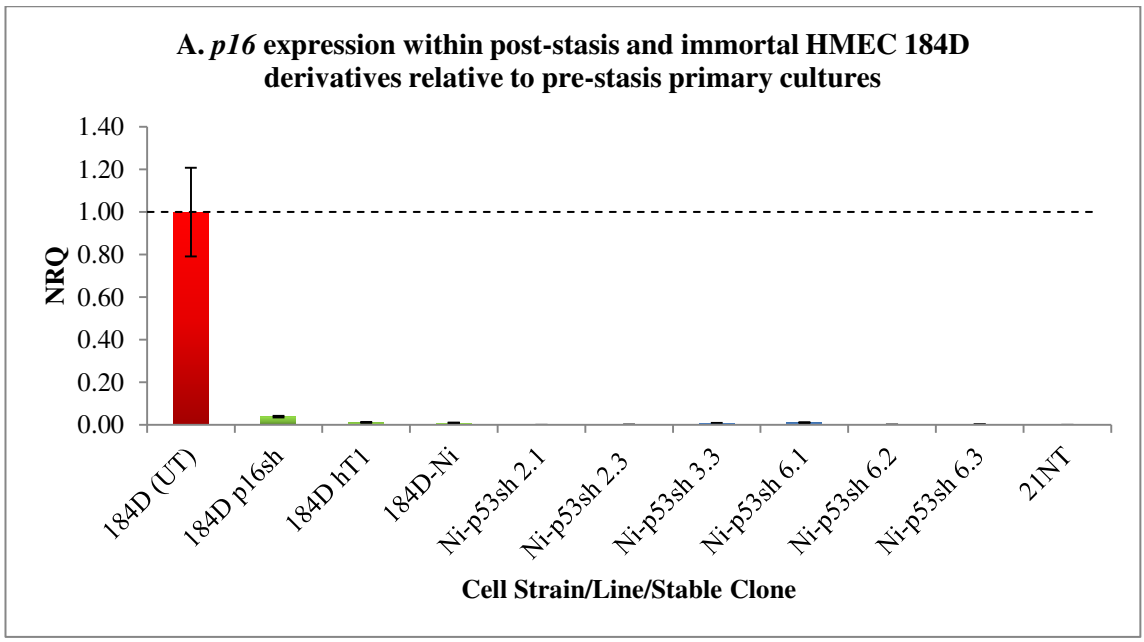
a 5.5Mb region within 3p21.1-p21.3 (Chapter 5.3.1). Importantly, the 21NT cell line is thought to have lost a critical telomerase repressor gene on chromosome 3p, as introduction of a normal chromosome 3 copy by MMCT is associated with strong telomerase repression and induction of cell growth arrest (Cuthbert *et al.,* 1999).

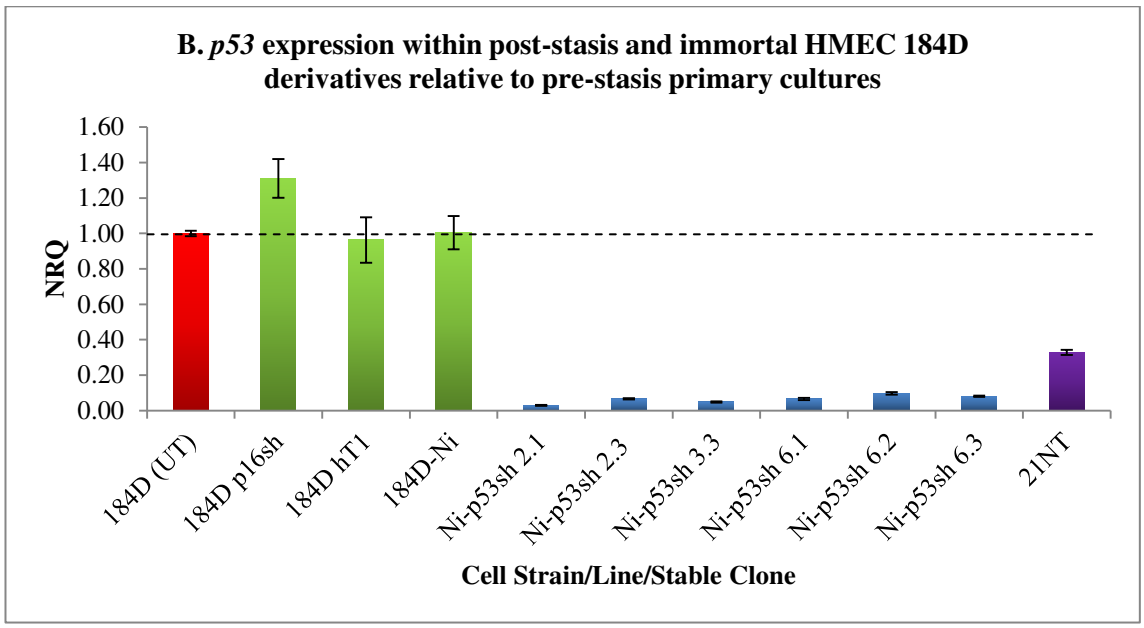
Table 6.2 - A summary of HMEC cell strains, immortal derivatives and breast cancer cell lines employed for the purposes of this study

Finite Lifespan		**Indefinite Lifespan**	
Pre-Stasis HMEC	**Post-Stasis HMEC**	**Immortal HMECs**	**Malignant Breast Cancer Cell Line**
184D (P5)	184D-Ni (P22)	184D-Ni*p53*sh clones: 2.1 (P34), 2.3 (P34), 3.3 (P34), 6.1 (P34), 6.2 (P32), 6.3 (P34)	21NT (P45)
		184D-hT1 (P32)	
		184D-*p16*sh (P32)	

Abbreviations: P (passage number)

To verify the stage of immortal transformation of each cell strain/line, the expression levels of *p16, p53* and *hTERT* were examined. As shown in Figure 6.3A, the nickel-treated 184D-Ni strain and all immortal 184D variants were found to exhibit either low or undetectable levels of *p16* expression relative to pre-stasis 184D cells, which confirms that these cell strains/lines had bypassed the stasis barrier. Loss of *p16* expression was also observed within the 21NT breast cancer cell line. All HMEC 184D-Ni*p53*sh clones were found to exhibit a greater than 90% reduction in *p53* expression relative to pre-stasis 184D and post-stasis 184D-Ni cell strains (Figure 6.3B). Both 184D-*p16*sh and 184D-hT1 immortal cell lines were found to display similar to or higher levels of *p53* relative to normal pre-stasis 184D cells, which suggests that *p53* may be functional within these immortal 184D derivatives. The 21NT cell line, which is known to harbour a frameshift mutation within the coding region of *p53* (Liu *et al.,* 1994), was found to display a 67% reduction in *p53* expression levels relative to the normal pre-stasis 184D cell strain (Figure 6.3B).


A. p16 expression within post-stasis and immortal HMEC 184D derivatives relative to pre-stasis primary cultures
NRQ
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
184D (UT)
184D p16sh
184D hT1
184D-Ni
Ni-p53sh 2.1
Ni-p53sh 2.3
Ni-p53sh 3.3
Ni-p53sh 6.1
Ni-p53sh 6.2
Ni-p53sh 6.3
21NT
Cell Strain/Line/Stable Clone


B. p53 expression within post-stasis and immortal HMEC 184D derivatives relative to pre-stasis primary cultures
NRQ
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
184D (UT)
184D p16sh
184D hT1
184D-Ni
Ni-p53sh 2.1
Ni-p53sh 2.3
Ni-p53sh 3.3
Ni-p53sh 6.1
Ni-p53sh 6.2
Ni-p53sh 6.3
21NT
Cell Strain/Line/Stable Clone

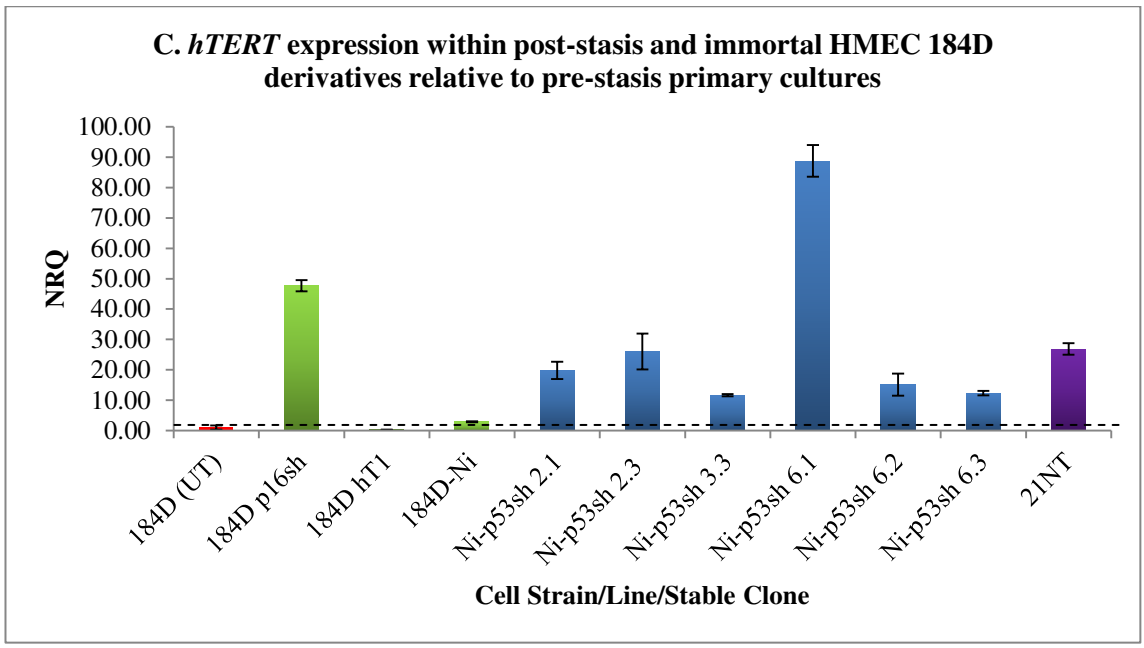


Figure 6.3 - *Normalized relative quantities (NRQ) of* ***(A)*** *p16,* ***(B)*** *p53* and ***(C)*** *hTERT mRNA within nickel chloride-treated post-stasis 184D-Ni cells, immortalized 184D-hT1, spontaneously immortalized 184-p16sh and 184D-p53sh clones and the 21NT breast cancer cell line relative to the pre-stasis primary 184D cell strain. NRQ values represent the mean ± standard error (SE) of triplicate repeats for each sample.*

As shown in Figure 6.3C, all 184D-Ni*p53*sh clones that emerged from crisis were found to exhibit substantially higher levels of *hTERT* expression relative to the normal pre-stasis 184D cell strain and the post-stasis 184D-Ni strain. This indicates that de-repression of endogenous *hTERT* expression had occurred following stable knockdown of *p53* and emergence from crisis, and not following nickel chloride treatment of primary 184D cells and clonal bypass of stasis. Around 50-fold higher levels of *hTERT* expression were observed within immortal 184D-*p16*sh cells, which confirms that stable knockdown of *p16* and clonal bypass of replicative senescence was associated with *hTERT* de-repression and immortal transformation of this cell line. As shown in Figure 6.3C, immortalized 184D-hT1 cells were found to display undetectable levels of *hTERT* expression. The primer sequences used to determine *hTERT* expression levels within this study were previously designed by Ducrest *et al.,* (2001) to detect only pre-spliced immature *hTERT* transcript molecules by amplifying a region within the exon 2-intron 2 boundary and therefore do not detect exogenous *hTERT* cDNA. The immortal 184D-hT1 cell line was derived from transduction of pre-stasis HMEC 184D cells with *hTERT* cDNA (Garbe *et al.,* 2014). This accounts for the lack of pre-spliced *hTERT* mRNA expression within 184D-hT1 cells and confirms that re-activation of endogenous *hTERT* did not occur during immortal transformation of this cell line.

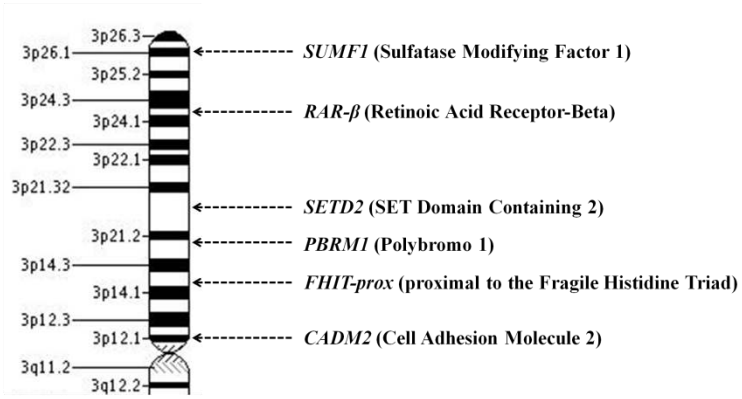
6.3.2 *Physical Mapping of Chromosome 3p Structure at Different Stages of HMEC Immortal Transformation*

To investigate changes in chromosome 3p structure during the immortal transformation of normal HMEC cells, copy number variation analysis (CNV) of five genes and one region proximal to the fragile histidine triad (*FHIT*) gene (*FHIT*-prox), which together span chromosome 3p12.1-26.1, were examined within the primary HMEC 184D strain, nickel chloride-treated post-stasis 184D-Ni cells, immortal 184D-Ni*p53*sh clones, immortal 184D-hT1 and 184D*p16*sh derivatives and the primary breast carcinoma cell line 21NT.

As shown in Figure 6.4, both primary pre-stasis HMEC 184D and nickel chloride-treated post-stasis 184D-Ni strains were found to harbour two alleles at all genomic loci examined, which suggests that gross structural changes in chromosome 3p did not occur following nickel chloride treatment of primary 184D cells and clonal bypass of stasis. Interestingly, four out of six (67%) immortal 184D-Ni*p53*sh clones were found to have undergone allele loss at one or more genomic loci examined. This demonstrates that genomic deletions within chromosome 3p are a common event following functional inactivation of *p53,* clonal emergence from crisis, *hTERT* transcriptional derepression and acquisition of an immortal phenotype. However, two immortal 184D-Ni*p53*sh clones (6.2 and 6.3) and the immortal 184D-*p16*sh and 184D-hT1 cell line derivatives were found to exhibit no change in allele number at all loci examined, compared to primary pre-stasis HMEC184D cells (Figure 6.4). This demonstrates that immortal transformation through clonal bypass of crisis or replicative senescence barriers is not always associated with gross structural losses of chromosome 3p. However, these findings do not exclude the possibility that smaller structural alterations may have occurred in regions between each of the genomic loci examined in this study. Allele loss at all genomic loci was observed within malignant 21NT breast cancer cells, which suggests that this particular cell line has undergone gross structural losses of chromosome 3p. Together these findings demonstrate that physical deletions of chromosome 3p occur as an early and frequent event during the immortal transformation of normal HMECs and are specifically associated with clonal bypass of the crisis barrier and de-repression of *hTERT* expression (Figure 6.3C). These findings are therefore also consistent with the notion that critical *hTERT* regulatory sequences may exist on chromosome 3p (Cuthbert *et al.,* 1999, Ducrest *et al.,* 2001, Szutorisz *et al.,* 2003, Ohmura *et al.,* 1995, Tanaka *et al.,* 1998, Abe *et al.,* 2010).

Physical Mapping of Chromosome 3p Structure at Different Stages of Human Mammary Epithelial Cell Immortalization

A. Genomic Positions of CNV Mapping Primers



Chromosome 3p Ideogram

B.

		Pre-Stasis HMEC	Post-Stasis HMEC	Stable Immortal HMEC 184D-Nip53sh Clones						Immortal HMEC		Breast Cancer CL
Gene	Genomic Position	184D	184D-Ni	2.1	2.3	3.3	6.1	6.2	6.3	184D-p16sh	184D-hT1	21NT
SUMF1	3p26.1	+/+	+/+	+/-	+/-	+/+/+	+/+	+/+	+/+	+/+	+/+	+/-
RAR-β	3p24.2	+/+	+/+	+/-	+/-	+/+	+/-	+/+	+/+	+/+	+/+	+/-
SETD2	3p21.31	+/+	+/+	+/-	+/-	+/-	+/+	+/+	+/+	+/+	+/+	+/-
PBRM1	3p21	+/+	+/+	+/-	+/-	+/-	+/+	+/+	+/+	+/+	+/+	+/-
FHIT	3p14.2	+/+	+/+	+/-	+/-	+/-	+/+	+/+	+/+	+/+	+/+	+/-
CADM2	3p12.1	+/+	+/+	+/-	+/+/+/+	+/-	+/+	+/+	+/+	+/+	+/+	+/-

+/+	+/-	+/+/+/(+)
Normal	Loss	Gain

Figure 6.4 - *Copy number variation (CNV) analysis of six genes spanning the length of human chromosome 3p (3p12.1-p26.1) within pre- and post-stasis HMEC 184D strains, stable immortal 184D-Nip53sh clones, immortal 184D-p16sh and 184D-hT1 cell line derivatives and the 21NT breast cancer cell line.* ***(A.)*** *An ideogram of chromosome 3p shows the relative positions of each gene used to map chromosome 3p structure.* ***(B.)*** *A table showing the number of alleles present within cell lines/strains at each gene loci examined, relative to the pre-stasis HMEC 184D strain.*

Recently, a high-density single nucleotide polymorphism (SNP) array (CytoSNP12, Illumina) was commissioned by my colleague Dr Yasaei to investigate genome-wide alterations in chromosome structure within the same set of finite lifespan HMEC 184D cell strains and immortal derivatives (Dr. Yasaei, unpublished data). As shown in Figure 6.5, the frequency and location of genomic losses and gains on chromosome 3p are largely consistent with those observed from the above CNV analysis of specific genes/regions spanning the length of chromosome 3p (Figure 6.4). SNP array analysis verified the incidence of genomic losses on

chromosome 3p within the same four immortal HMEC 184D-Ni*p53*sh clones (Figure 6.5). These data also confirmed extensive allele loss of chromosome 3p within HMEC 184D-Ni*p53*sh clones 2.1 and 2.3 and 3.3. However, CNV analysis of 184D-Ni*p53*sh clone 6.1 identified allele loss within the *RAR-β* locus, which is located within 3p24.2 (Figure 6.4). SNP array analysis of the same sample revealed the presence of two discrete regions of deletion; 3p14.2-p22.2 and 3p25.1-p26.2. The underlying cause of this inconsistent observation is unclear. However, as described in Table 6.2, gene expression and CNV analysis of this particular clone was carried out at passage number 34 (P34) whereas SNP array analysis was carried out at P36 (Dr. Yasaei, unpublished data). The identification of different structural alterations on chromosome 3p within later passage cells could indicate high levels genomic instability and evidence of clonal selection.

SNP array analysis demonstrated the absence of non-random structural alterations on chromosome 3 within post-stasis HMEC 184D-Ni cells, which confirmed that nickel chloride treatment and clonal emergence from stasis was not associated with any structural alterations on chromosome 3p. On the other hand, all 184D-Ni*p53*sh clones were found to have undergone copy number gains and/or losses of multiple regions on chromosome 3. This indicates that genomic changes on chromosome 3 are specifically associated with clonal emergence from crisis and de-repression of *hTERT* expression (Figure 6.3C). Four (56%) 184D-Ni*p53*sh clones displayed genomic losses on the long-arm (q-arm) of chromosome 3, the majority of which involved loss of a region localized to 3q29. In addition, copy number gain of multiple discrete regions on chromosome 3q was observed within 50% of immortal *p53*sh clones (Figure 6.5A). Similarly, the 21NT breast cancer cell line was found to have undergone copy number gain of the majority of chromosome 3q, which suggests that oncogenic sequences may exist within this region that promote malignant progression. However, the most common structural alteration that was found to occur on chromosome 3 within stable 184D-Ni*p53*sh clones, was whole or partial loss of chromosome 3p (Figure 6.5A). Alignment of chromosome 3 within 184D-Ni*p53*sh clones 2.1, 2.3, 3.3 and 6.1 revealed a common region of deletion involving 3p14.2-p22, which overlaps with commonly deleted regions that were thought to harbour a putative telomerase repressor sequence by Cuthbert *et al.,* (1999) (Figure 6.5B). These findings could signify the presence of critical sequences within this region that prevent bypass of telomere length-dependent barriers to proliferation, such as *hTERT* regulatory sequences. However, SNP array analysis confirmed the absence of chromosome 3p losses within the two remaining immortal 184D-Ni*p53*sh clones (6.2 and 6.3, Figure 6.5A), which demonstrates that large physical genomic deletions within this region are not always associated with *hTERT* repression and immortal transformation of normal HMECs.

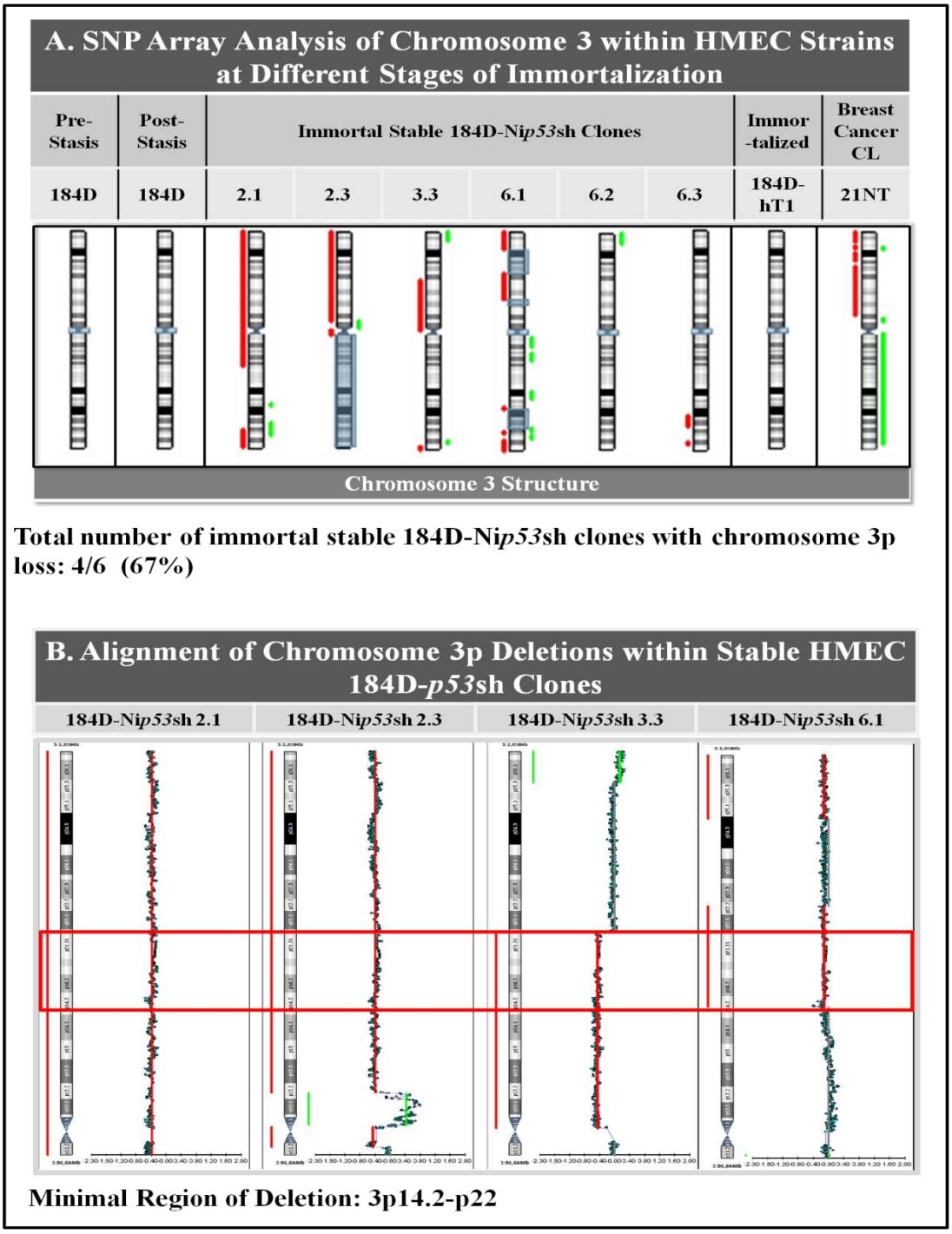


Figure 6.5 - *Single nucleotide polymorphism (SNP) array analysis of chromosome 3 within* ***(A)*** *pre- and post-stasis HMEC 184D strains, six immortal 184D-Nip53sh, the immortalized 184D-hT1 cell line and the 21NT breast cancer cell line relative to the pre-stasis HMEC 184D strain.* ***(B)*** *Alignment of chromosome 3p deletions within four immortal 184D-Nip53sh clones (courtesy of Dr. Yasaei, unpublished data). Green and red boxes/lines indicate the positions of chromosome 3 allele gains and losses respectively. Grey highlighted regions indicate the absence of heterozygosity. SNP array analysis was carried out using the Illumina iScan, Infinium HD Bead Chip at Barts and the London School of Medicine and Dentistry. Data analysis was carried out by Dr. Yasaei (Brunel University).*

6.4 DISCUSSION

Around 70% of ductal carcinoma *in situ* (DCIS) and 100% of invasive breast lesions have been found to express telomerase activity, indicating that cellular immortalization is an early event that precedes the acquisition of malignant features (Umbricht *et al.,* 1999). Normal human mammary epithelial cells (HMEC) have been found to encounter two distinct barriers to cellular immortalization *in vitro*; the stress-associated stasis barrier and the telomere dysfunction-associated replicative senescence barrier (Stampfer and Yaswen., 2002). Stasis bypass has been achieved *in vitro* through mutational or epigenetic silencing of *p16* (Stampfer and Bartley., 1985, Romanov *et al.,* 2001). Post-stasis or extended lifespan HMECs have been found to exhibit high levels of genomic instability due to progressive telomere shortening until cells enter replicative senescence (Romanov *et al.,* 2001). Overcoming the second growth plateau is dependent on the immortal transformation of cells through de-repression of *hTERT* and reactivation of telomerase activity (Newbold., 2002). It is thought that high levels of genomic instability, as cells approach replicative senescence, give rise to rare errors that are responsible for telomerase reactivation. However, the mechanisms that mediate *hTERT* repression within normal HMECs and the specific genomic errors required for *hTERT* de-repression during cellular immortalization remain obscure.

A common genomic alteration observed within up to 87% primary breast carcinomas is loss of heterozygosity (LOH) of the short arm of chromosome 3 (3p) (Matsumoto *et al.,* 1997, Martinez *et al.,* 2001, Maitra *et al.,* 2001, Chen *et al.,* 1994, De Oliveira *et al.,* 2012). 45% of pre-neoplastic breast lesions have been found exhibit genomic losses on chromosome 3p, which indicates that this region harbours important tumour suppressor genes that are lost as an early event in breast carcinogenesis (Maitra *et al.,* 2001). Using microcell-mediated monochromosome transfer (MMCT) techniques, Cuthbert *et al.,* (1999) demonstrated that sequences present on the short arm of chromosome 3 (3p) may function to repress telomerase activity within breast cancer cells. These repressor sequences are thought reside within 3p21.3-p22 and/or 3p12-p21.1. Subsequent studies showed that chromosome 3p-encoded sequences mediate strong telomerase repression and cell growth arrest through transcriptional silencing of *hTERT* (Ducrest *et al.,* 2001, Szutorisz *et al.,* 2003). In collaboration with my colleague Dr. Yasaei, an *in vitro* model of HMEC immortalization was used to investigate the timing, frequency and location of genomic losses on chromosome 3p. This particular model involved the generation of immortal transformants through abrogation of two relevant breast tumour suppressor genes; *p16* and *p53,* that are transcriptionally silenced and mutated in approximately 50% and 23% of breast cancers respectively (Geradts and Wilson., 1996, COSMIC database; Forbes *et al.,* 2015). Together, our findings demonstrate that genomic losses on chromosome 3p are a frequent clonal event that coincides with de-repression of *hTERT* and emergence from crisis. Alignment of chromosome 3p deletions revealed a common region of deletion

encompassing 3p14.2-p22. Interestingly, this region partially overlaps both commonly deleted regions, 3p21.3-p22 and 3p12-p21.1, observed within segregant, telomerase positive 21NT-chromosome 3 hybrids in the Cuthbert *et al.,* (1999) study. Furthermore, this identified region also encompasses the 490kb region of deletion located within 3p21.31, which was previously thought to harbour a putative telomerase repressor sequence (Dr. T. Roberts, unpublished data). These findings therefore provide additional evidence to show that critical *hTERT* repressor sequences may be located on chromosome 3p that are lost during the immortal transformation of breast epithelial cells. Consistent with these observations, the presence of *p53* mutations has been strongly associated with genomic deletions on chromosome 3p in breast cancer, which suggests that the co-incidence of these events may drive breast carcinogenesis (Deng *et al.,* 1996). A strong relationship between *p53* mutations and chromosome 3p loss has also been observed in head and neck squamous cell carcinoma (HNSCC) and the combination of these events is associated with worse patient survival (Gross *et al.,* 2014). Chromosome 3p LOH has been identified within pre-cancerous breast lesions *in vivo* (Deng *et al.,* 1994, Moinfar *et al.,* 2000). Around 41% of mammary ductal intraepithelial neoplasia (DIN) lesions have been shown to undergo LOH within chromosome 3p14.2 (Moinfar *et al.,* 2000). Furthermore, 60% of morphologically normal breast tissue samples obtained from regions adjacent to breast carcinomas have been found to harbour genomic losses within 3p22-p25 (Deng *et al.,* 1996). These findings indicate that critical tumour suppressor genes located within chromosome 3p may be lost as an early event during breast carcinogenesis.

Functional inactivation of *p53* within post-stasis extended lifespan HMEC cultures is associated with spontaneous emergence from crisis and has been shown to accelerate the acquisition of an immortal phenotype (Stampfer *et al.,* 2003, Gao *et al.,* 1996, Gollahan and Shay., 1996, Van Der Haegen and Shay., 1993). Continued proliferation of HMECs beyond the stasis barrier is associated with progressive telomere shortening resulting in gross chromosomal abnormalities including rearrangements, translocations, deletions and telomeric associations as cells approach replicative senescence (Romanov *et al.,* 2001). Telomere shortening has been observed within histologically normal breast epithelium and within almost 80% of DCIS lesions, which provides evidence that telomere dysfunction may occur *in vivo* and early in breast carcinogenesis (Meeker *et al.,* 2004). In addition, DCIS lesions have been found to exhibit a greater number of cytogenetic abnormalities than pre-cancerous hyperplastic lesions (O'Connell *et al.,* 1998). Higher levels of genomic instability have been observed within post-stasis HMECs lacking functional *p53* compared with those harbouring functional *p53* (Stampfer *et al.,* 2003). This has also been observed in primary breast cancers whereby abnormal *p53* protein expression is strongly associated with higher levels of genomic instability (Eyfjörd *et al.,* 1995). For this reason, it is thought that inactivation of *p53* may accelerate the acquisition of specific genomic errors required for *hTERT* de-repression and immortal transformation

(Stampfer *et al.,* 2003). However, due to the presence of multiple genomic aberrations in early breast lesions *in vivo* and post-stasis HMECs *in vitro,* the specific errors responsible for immortal transformation are difficult to elucidate.

In order to narrow the search for genomic errors specific to cellular immortalization, Severson *et al.,* (2014) carried out exome sequencing and copy number variation (CNV) analysis of benzo(a)pyrene (BaP)-treated post-stasis HMECs and two *p53*-functional immortal derivatives, which have not been found to exhibit high levels of initial and sustained genomic instability over continuing passages (Stampfer *et al.,* 2003). These immortal derivatives emerged from replicative senescence and were found to acquire sufficient levels of telomerase activity to maintain telomere lengths through a gradual process termed conversion (Stampfer *et al.,* 1997). Severson *et al.,* (2014) found that post-stasis BaP-treated cells were found to harbour mutations within known cancer driver genes. Intriguingly, over 95% of mutations observed within immortal BaP-clones were also identified within post-stasis BaP-precursor cells, indicating that only a few additional mutations were associated with immortal transformation (Severson *et al.,* 2014). Genes harbouring mutations that were predicted to influence protein function included *NKRF* (NFKB repressing factor), *SORBS2* (sorbin and SH3 domain containing 2) and *GLTSCR1L* (Glioma tumour suppressor candidate region gene 1-like). However, none of the additional mutated genes were common to both immortal clones. Interestingly, a clear difference in the number of CNVs between post-stasis BaP-precursors and immortal derivatives were identified. Aside from genomic losses on chromosomes 6 and 12, one immortal BaP-clone was found to have undergone single copy deletions on chromosome 3p. These findings are consistent with those presented in this Chapter demonstrating that chromosome 3p loss is an early event in the immortal transformation of HMECs and is associated with telomerase reactivation.

In a recent study, Garbe *et al.,* (2014) found that non-clonal immortal transformation of HMECs can be achieved by targeting both stasis and replicative senescence barriers directly through treatment with *p16*-shRNA and transduction of the *c-MYC* oncogene. Immortal derivatives were found to exhibit a normal karyotype and with none or few genomic abnormalities or CNVs. This demonstrates that gross genomic instability and specific losses on chromosome 3p are not absolute requirements for the immortal transformation of HMECs. Consistent with this findings, we observed no CNVs on chromosome 3p within the immortal 184D-*16*sh cell line derivative during this study. Novak *et al.,* (2009) observed an association between genome-wide DNA methylation changes and non-clonal bypass of senescence barriers, therefore epigenetic alterations are thought to be essential for immortal transformation (Garbe *et al.,* 2014). Collectively, these observations do not eliminate the possibility that critical telomerase repressor sequences may exist on chromosome 3p within normal HMECs. Aside from genomic deletions and rearrangements, genes residing within the 3p14-3p21 region,

including *FHIT* and *RASSF1A,* have been found to undergo frequent hypermethylation in breast cancer (Buchagen *et al.,* 1994, Zöchbauer-Müller *et al.,* 2001, Damman *et al.,* 2001). Therefore, investigating both genetic and epigenetic changes on chromosome 3p during the immortal transformation of HMECs, may narrow the search for critical telomerase regulatory sequences.

CHAPTER 7

7 GENERAL DISCUSSION AND FUTURE WORK

Cellular immortality is one of the ten hallmarks of human cancer (Hanahan and Weinberg., 2011). Normal human somatic cells proliferate for a finite number of population doublings before entering a telomere length-dependent growth barrier termed replicative senescence (Hayflick and Moorhead., 1961, Hayflick., 1965). Overcoming this barrier is associated with the acquisition of telomere maintenance mechanisms, which permit indefinite growth (Newbold., 2002, Stampfer and Yaswen, 2002). There is strong evidence to show that cellular immortalization is an essential pre-requisite for malignant transformation of mammalian cells and therefore represents a critical step in human carcinogenesis (Newbold *et al.,* 1982, Newbold and Overell., 1983) The predominant mechanism of telomere maintenance in human cancer is reactivation of telomerase activity, which is thought to be due to transcriptional de-repression of the gene encoding the reverse transcriptase subunit of telomerase, *hTERT* (Newbold., 2002). The immortal transformation of normal human somatic cells *in vitro* has only been found to occur as a rare event following bypass of senescence barriers through treatment with chemical carcinogens, transduction of viral oncoproteins or direct functional inactivation of senescence effector proteins (Newbold., 2002, Stampfer and Yaswen., 2002). Therefore, stringent mechanisms of *hTERT* repression must exist within normal human somatic cells that are lost as a rare event during immortal transformation.

Telomerase repression and restoration of limited growth potential in human cancer cells has been achieved through somatic whole cell fusion with normal human cells or following the introduction of single normal human chromosomes by microcell-mediated monochromosome transfer (MMCT) (Pereira-Smith and Smith., 1983, Cuthbert et al. 1999, Ducrest *et al.,* 2002). Monochromosomal hybrid studies have shown that human chromosomes 3, 4, 6, 7, 10 and 17 harbour regulatory sequences that repress telomerase activity in different cancer types (see Chapter 1.3.5.2.2.3, reviewed by Ducrest *et al.,* 2002, Tanaka *et al.,* 2005). The available data indicate that no single chromosome represses telomerase activity within all cancer cell lines. Furthermore, there is some evidence to suggest that multiple sequences may be present on the same chromosome, which function to repress telomerase activity in different cancer types (Tanaka *et al.,* 2005). This has led to the notion that telomerase regulation is tissue-specific and different telomerase regulatory sequences can be inactivated in different cancer types. However, it is unclear whether more than one mechanism of telomerase regulation may be inactivated within the same cancer type. To investigate this further, I carried out MMCT-mediated transfer of chromosome 3 and 17 into the 21NT primary breast cancer cell line, which had been

previously shown to undergo telomerase repression and growth arrest following introduction of chromosome 3 (Cuthbert *et al.,* 1999). Chromosome 17 harbours the *p53* tumour suppressor gene, which is mutated in around 23% of breast cancers and has also been shown to repress *hTERT* transcription within breast cancer cells (Shats *et al.,* 2004, COSMIC database; Forbes *et al.,* 2014). The majority of 21NT-chromosome 3 and 21NT-chromosome 17 hybrids were found to exhibit telomerase repression and were found to either enter growth arrest or display a significant reduction in cell growth rate. Consistent with these findings, Backsch *et al.,* (2001) found that introduction of human chromosomes 3 and 4 into the HeLa cervical carcinoma cell line generated telomerase-repressed hybrids. This demonstrates that multiple telomerase regulatory sequences and pathways may function to repress telomerase activity within cells of the same cancer type. Importantly, this also raises the possibility that reconstitution of a single pathway may be sufficient to confer telomerase repression.

Aside from breast and cervical carcinoma cell lines, MMCT-mediated transfer of chromosome 3 into a head and neck carcinoma and two renal cell carcinoma cell lines, was associated with telomerase repression (Cuthbert *et al.,*1999, Backsch *et al.,* 2001, Horikawa *et al.,* Tanaka *et al.,* 1998, Newbold., 1997). This suggests that chromosome 3 may harbour sequences that function to repress telomerase activity within multiple tissue types. Telomerase repression within 21NT-chromosome 3 hybrids was associated with loss of *hTERT* expression, but no changes in *hTERT* promoter activity (Ducrest *et al.,* 2001). Similarly, introduction of chromosome 3 into the RCC23 renal cell carcinoma cell line by MMCT was also associated with strong *hTERT* repression. However, *hTERT* promoter activity was significantly reduced within RCC23-chromosome 3 hybrids compared to parental cells. The binding of an unknown repressor molecule to an E-box binding element positioned +22-27bp downstream of the *hTERT* transcription start site was found to be responsible for mediating *hTERT* repression within these hybrids. On the other hand, Szutorisz *et al.,* (2003) found that introduction of chromosome 3 into the 21NT breast cancer cell line was associated with targeted chromatin remodelling of two sites within intron 2 of *hTERT*. Therefore sequences present on chromosome 3 encode or regulate the expression of a direct E-box binding repressor molecule and an epigenetic regulator of *hTERT*, which may function as critical repressors of *hTERT* in different tissue types.

Deletion mapping of telomerase positive, segregant 21NT-chromosome 3 hybrids within the Cuthbert *et al.,* (1999) study, identified two regions including 3p21.3-p22 and 3p12.21.1, that may harbour telomerase repressor sequences. Subsequent studies involving the transfer of radiation-induced chromosome 3 fragments into 21NT breast cancer cells by MMCT identified a single fragment, which was found to repress *hTERT* expression (Dr. T. Roberts, personal communication of unpublished data). Following MMCT-mediated retrotransfer of this fragment back into mouse A9 fibroblast cells, sequence-tagged-site (STS) mapping of hybrid

clones demonstrated that it was a chimeric fragment containing regions of chromosome 3 short and long arm material (Chapter 4.1). A single A9-chromosome 3 fragment hybrid was found to have undergone deletion of a 490kb sequence, which maps to 3p21.3 approximately 47Mb from the telomere. Unfortunately, both intact and 490kb-deleted fragments could not be successfully re-introduced into the 21NT cell line to confirm that this region harboured the putative telomerase repressor sequence (Dr. T. Roberts, unpublished data). Nevertheless, this 490kb sequence is located within the 3p21.3-p22 region, which is thought to harbour a putative telomerase repressor sequence (Cuthbert *et al.,* 1999). Furthermore, studies have shown that the 3p21-p22 region is a frequent site of loss is heterozygosity (LOH) in breast cancer, which suggests the presence of important tumour suppressor genes (Maitra *et al.,* 2001, Martinez *et al.,* 2001). Within the 490kb sequence, the SET domain-containing 2 (*SETD2*) gene and an adjacent long non-coding RNA sequence *FLJ/KIF9,* cloned by Dr. T. Roberts in our laboratory, were selected for further investigation. *SETD2* has been shown to function as a genome-wide epigenetic regulator of gene transcription (Sun *et al.,* 2005). In addition, *SETD2* has been found to be expressed at significantly lower levels within breast cancer tissue relative to normal breast tissue samples (Sarakbi *et al.,* 2009, Newbold and Mokbel., 2010).

In this project, I carried out gene copy number variation analysis (CNV) of six intronic/exonic regions within the *SETD2-FLJ* genomic locus and found that this region had undergone single copy deletion within the 21NT breast cancer cell line. Furthermore, a significant reduction in *SETD2* and *FLJ* mRNA levels were observed within 21NT cells relative to normal human mammary epithelial (HMEC) strains, which is consistent with the notion that that the *SETD2-FLJ* locus may have undergone LOH within this cell line. CNV analysis of this locus within a single *hTERT*-repressed 21NT-chromosome 3 hybrid clone (H3.5E) and a telomerase-positive segregant derivative (H3.5L), that emerged following a crisis event, demonstrated that single copy deletions within the *SETD2-FLJ* genomic locus were associated with emergence from crisis and re-activation of *hTERT* expression. These findings provide some evidence to suggest that *SETD2* and/or *FLJ* may play a functional role in *hTERT* repression within the 21NT breast cancer cell line. To investigate the direct effect of *SETD2* on *hTERT* transcriptional activity, I transfected mammalian expression vectors containing the *SETD2* coding region into 21NT cells and examined the effect of transient and stable *SETD2* expression on *hTERT* expression and telomerase activity. I found that a 3-245 fold increase in *SETD2* expression was not associated with consistent repression of *hTERT* transcription or telomerase activity within 21NT cells. Furthermore, siRNA-mediated knockdown of *SETD2* within the *hTERT* repressed H3.5E hybrid clone was also not associated with an observable change in *hTERT* expression. These findings suggest that *SETD2* is unlikely to function as a negative regulator of *hTERT* transcription within the 21NT breast cancer cell line. On the other hand, stable over-expression of *FLJ* within 21NT cells was associated with consistent repression

of *hTERT* expression and telomerase activity. However, curiously, stable empty-vector controls were also found to exhibit the same degree of *hTERT* and telomerase repression, which suggests that *FLJ* may not have been directly responsible for this phenotypic effect. These findings provide some evidence to show that *SETD2* and *FLJ* may not function as critical regulators of *hTERT* within breast cancer cells, but clearly further work is needed to explain the unexpected result with the empty vector controls in the *FLJ* gene transfer experiment.

Continuing the search for telomerase repressor sequences localized to the 3p21-p22 region, a review of the literature identified a set of four tumour suppressor genes that have been found to suppress breast cancer growth *in vitro,* and in some cases *in vivo,* and which have been functionally linked to the epigenetic regulation of target gene expression. These included the BRCA1 associated protein-1 (*BAP1*), Ras association domain family 1, transcript variant A (*RASSF1A*), polybromo-1 (*Pb1/PBRM1/BAF180*) and poly(ADP-ribose) polymerase family member 3 (*PARP-3*). These genes are located within the 3p21.1-p21.3 region, which is within or proximal to two discreet regions thought to harbour telomerase repressor sequences (Cuthbert *et al.,* 1999). In order to investigate the functional status and tumour suppressive role of candidate genes within the 21NT breast cancer cell line and a panel of eight other telomerase positive breast cancer cell lines, I examined candidate gene copy number and mRNA levels. The majority of breast cancer cell lines, as well as 21NT, were found to have undergone allele loss at all candidate gene loci examined including the *SETD2-FLJ* locus. This suggests that single copy deletions of a minimal 5.5Mb region, localized to 3p21.1-p21.3 and encompassing known tumour suppressor genes, may be a frequent event in breast cancer cells. Apart from *BAP1,* the expression levels of *RASSF1, PBRM1* and *PARP-3* candidate genes were found to be substantially reduced within a large proportion of the selected breast cancer cell lines, relative to normal HMEC strains, which implies that tumour suppressor genes located within this region may frequently undergo LOH in breast cancer cells. Studies have shown that forced over-expression of multiple genes localized to the 3p14.2-p21.3 region including *BAP1, RASSF1A, PBRM1* and *RAR-β2* is associated with significant inhibition of tumour cell growth *in vitro* through induction of cell growth arrest and senescence pathways (Burbee *et al.,* 2001, Ventii *et al.,* 2008, Jensen *et al.,*1998, Xia *et al.,* 2008, Seewaldt *et al.,* 1995). Consistent with these observations, I found that over-expression of *RASSF1A* and *PBRM1* was associated with a significant decrease in colony size and clonogenic survival of 21NT breast cancer cells respectively. Furthermore, Ji *et al.,* (2002) demonstrated that over-expression of wild-type *FUS1, 101F6, NPRL2* and *RASSF1A* genes, which were found to be part of a homozygous region of deletion within 3p21.3 in lung and breast cancer cells, was associated with inhibition of lung tumour cell growth *in vitro* and *in vivo* through induction of apoptosis. Therefore, several genes localized to chromosome 3p appear to function to suppress tumour growth through a variety of mechanisms.

In order to investigate the functional role of individual candidate genes in the transcriptional regulation of *hTERT* and telomerase activity, I transfected mammalian expression vectors containing cloned *BAP1, RASSF1A* and *PBRM1* coding regions and associated empty vector controls into 21NT cells. *hTERT* expression and telomerase activity levels were then examined within individual 21NT clones that were stably over-expressing each candidate gene. Stable over-expression of *PARP-3* within 21NT cells was carried out by Dr. T. Roberts in our lab. Interestingly, over-expression of both *BAP1* and *PARP-3* was associated with consistent repression of *hTERT* transcription within 21NT cells. However, telomerase activity levels within independent 21NT-*BAP1* transfection clones were found to increase to a similar level as stable empty vector controls over progressive passages, indicating that over-expression of *BAP1* alone was insufficient to confer stable repression of telomerase or induction of cell growth arrest. The telomerase activity and replicative potential of stable 21NT-*PARP-3* transfection clones is currently under investigation in our lab by Dr. T. Roberts. These findings provide evidence to suggest that at least two sequences present on chromosome 3p may be required for complete repression of *hTERT* transcriptional activity within breast cancer cells. Consistent with these findings, Cuthbert *et al.,* (1999) observed two discrete regions of deletion on chromosome 3p within segregant 21NT-chromosome 3 hybrids, which demonstrates that two repressor sequences may function to repress telomerase activity and loss of one repressor sequence may be sufficient to confer a degree of telomerase re-activation. Interestingly, primary breast tumours have been found to undergo discontinuous allele loss of multiple regions of chromosome 3p (Maitra *et al.,* 2001). Other cytogenetic studies have shown that there may be at least two independent regions of deletion on chromosome 3p within breast tumours involving 3p13-p14 and 3p24-26 as well as 3p21.3, which indicates that chromosome 3p harbours multiple tumour suppressor sequences that may be lost during breast carcinogenesis (Matsumoto *et al.,* 1997, Chen *et al.,* 1994). Reverse selection of 21NT-chromosome 3 hybrids that had lost the introduced chromosome 3 copy by ganciclovir (GCV) selection within the Szutorisz *et al.,* (2003) study, was associated with restoration of an 'open' chromatin conformation around intron 2 of *hTERT,* but not associated with consistent re-activation of *hTERT* expression when compared to parental 21NT cells. Despite ensuring the absence of residual, exogenous chromosome 3p14.1-21.2 material within the majority of revertant clones, the presence of another repressor sequence outside of this region (perhaps within 3p21.2-p22) may have been responsible for the sustained repression of *hTERT* transcription. Therefore, it is possible that one chromosome 3p-encoded sequence functions to regulate the chromatin conformation around intron 2 of *hTERT,* while another sequence(s) may function to prevent binding of transcriptional activators of *hTERT* within or around this region. It would be interesting to explore the mechanisms by which *BAP1* and *PARP-3* function to repress *hTERT* transcription within 21NT cells and whether they function collectively to confer stable telomerase repression.

Considering these observations, approaches to identify multiple sequences localized to chromosome 3p that function as transcriptional repressors of *hTERT* within the 21NT cell line, may improve our understanding of the mechanisms by which chromosome 3 mediates telomerase repression. Furthermore, in addition to protein-coding sequences, multiple non-coding microRNA (miRNA) sequences on chromosome 3p have been found to undergo frequent copy number alterations and aberrant expression in breast cancer (Qian *et al.,* 2012). In 2009, Miura *et al.,* demonstrated that over-expression of the RGM249 precursor miRNA sequence localized to chromosome 10p15.3, was associated with strong transcriptional repression of *hTERT* within A172 glioblastoma and HMc-Li7 hepatocellular carcinoma cell lines, which demonstrates that non-coding sequences may also function as critical regulators of *hTERT*. Therefore, approaches to identify regions on chromosome 3p associated with strong *hTERT* repression together with current genome mapping data, may facilitate the identification of a series of candidate regulatory sequences.

In 2010, Abe *et al.,* used chromosome engineering to generate a variety of chromosome 3 fragments lacking defined regions, to narrow the search for sequences on chromosome 3p that function to repress *hTERT* within the RCC23 renal cell carcinoma cell line. To achieve this, an intact chromosome 3 copy was first transferred by MMCT into chicken DT40 cells, which exhibit high homologous recombination frequencies. Together with a puromycin resistance gene, vector constructs containing sequences homologous to certain chromosome 3 regions were then transfected into DT40-chromosome 3 cells by electroporation to generate three selectable truncated fragments by homologous recombination; #3delp24-ter, #3delp22-pter and #3delp21.3-pter. Fragments were transferred by MMCT into RCC23 cells transfected with exogenous *hTERT* (RCC23-*exohTERT*) to permit ongoing proliferation. Both #3delp24-ter and #3delp22-pter fragments were found to repress endogenous *hTERT* expression, whereas RCC-*exohTERT* clones containing #3delp21.3-pter fragment were found to exhibit the same level of endogenous *hTERT* expression as parental cells. Through STS-mapping of chromosome 3 fragments, a 7Mb region within 3p21.3 was found to be responsible for the observed repression of endogenous *hTERT*. Therefore, by exploiting the high homologous recombination efficiency of chicken DT40 cells to generate various truncated chromosome 3 fragments lacking defined regions, Abe *et al.,* (2010) has demonstrated a novel approach for mapping chromosomal regions responsible for *hTERT* repression. According to Abe *et al.,* (2010), future studies aim to use this method to enable positional cloning of telomerase repressor sequences, which will then be inserted into a human artificial chromosome (HAC) vector. Unlike conventional gene delivery vectors, HACs have the capacity to incorporate large genomic loci together with associated regulatory elements to permit physiological expression of target genes within recipient cells (Reviewed by Oshimura *et al.,* 2015). Furthermore, HAC vectors are maintained episomally, which prevents insertional mutagenesis of host genomic material. In 2009, Hoshiya

et al., demonstrated the potential therapeutic applications of HAC vectors by cloning the 2.4Mb human dystrophin gene into a HAC vector, which was stably maintained and expressed within chimeric mice. Therefore, chromosome engineering could be an effective approach for identifying and reconstituting critical *hTERT* repressor sequences within human cancer cells.

Some researchers argue that the identification of rare molecular events that occur to permit *hTERT* de-repression and telomerase re-activation within human carcinomas is difficult due to the high level of genomic instability exhibited by tumour cells (Garbe *et al.,* 2014). It is thought that genomic instability caused by ongoing telomere shortening as cells approach replicative senescence, is responsible for generating errors required for telomerase re-activation as well as 'passenger' errors that are frequently observed within carcinomas (Garbe *et al.,* 2007, Garbe *et al.,* 2014). Therefore, the development of *in vitro* models of human epithelial cell immortalization have provided an alternative approach to understand the mechanisms by which *hTERT* becomes deregulated during human carcinogenesis. According to the current model, normal finite lifespan human mammary epithelial cells (HMEC) encounter two senescence barriers to indefinite proliferation; the stress-associated stasis barrier, and the telomere dysfunction-associated replicative senescence or crisis barrier (Stampfer and Yaswen., 2002). Overcoming the stasis barrier is associated with inactivation of the $p16^{INK4A}$*/pRb* pathway and has been found to occur spontaneously *in vitro* (Huschtscha *et al.,* 1998, Brenner *et al.,* 1998, Romanov *et al.,* 2001). On the other hand, spontaneous emergence from replicative senescence has never been found to occur *in vitro* (Stampfer and Yaswen., 2002). However, abrogation of *p53* function within post-stasis HMECs is associated with the rare emergence of immortal clones from crisis (Gao *et al.,* 1996, Gollahan and Shay., 1996, Van Der Haegen and Shay., 1993).

In order to study the molecular events that occur during the early stages of HMEC immortal transformation, my Brunel colleague Dr. H. Yasaei, generated post-stasis and immortal variants of a single HMEC strain derived from reduction mammoplasty tissue of a healthy female patient. This was achieved by abrogating the function of critical effectors of stasis and replicative senescence barriers, *p16* and *p53*. Immortal clones that emerged from crisis, were found to express *hTERT* transcripts and telomerase activity. Taking into account the findings presented by Cuthbert *et al.,* (1999), demonstrating the presence of *hTERT* repressor sequences on chromosome 3p, I investigated structural alterations within this region at each stage of immortal transformation. By comparing the copy number variation (CNV) of six genomic loci/regions spanning 3p12.1-p26.1 within finite lifespan pre-stasis and post-stasis HMEC cell strains and immortal variants, I demonstrated that single copy allele loss on chromosome 3p is a frequent, clonal event that is specifically associated with emergence from crisis and *hTERT* de-repression. Subsequent high density, genome-wide single nucleotide

polymorphism (SNP) array analysis of the same samples confirmed the presence of single copy deletions on chromosome 3p and identified a minimal common region of deletion within 3p14.2-p22 (Dr. H. Yasaei, personal communication of unpublished data). These findings demonstrate that allele loss on chromosome 3p may be an early event in breast tumorigenesis that is associated with immortal transformation. Consistent with the findings presented by Cuthbert *et al.,* (1999), these results also provide additional evidence to suggest that critical *hTERT* regulatory sequences may be present on chromosome 3p.

The recent development of pathologically relevant models of HMEC immortal transformation that do not involve clonal bypass of senescence barriers, have demonstrated that high levels of genomic instability, including gene copy number losses on chromosome 3p, are not absolutely required for complete immortal transformation (Garbe *et al.,* 2014). It is thought that in some instances, epigenetic changes may be responsible for de-regulation of *hTERT* expression during immortalization. Hypermethylation and silencing of tumour suppressor genes localized to chromosome 3p have been observed within pre-neoplastic breast lesions, non-tumorigenic breast cancer cell lines and primary breast carcinomas, which demonstrates that epigenetic alterations within this region may occur early in breast carcinogenesis (Damman *et al.,* 2001, Zöchbauer-Müller *et al.,* 2001, Dumont *et al.,* 2009). Therefore, it would certainly be of interest to investigate both global and chromosome 3-specific DNA methylation changes and altered histone modification patterns within *in vitro* models of HMEC immortalization.

7.1 SUMMARY AND CONCLUSION

De-repression of *hTERT* and telomerase re-activation is thought to be a critical event in human cancer development and is the predominant mechanism by which human tumour cells maintain their indefinite growth capacity. The functional inactivation of critical telomerase repressor sequences during the early stages of cancer development is thought to be responsible for telomerase re-activation and cellular immortalization. Gene transfer studies involving the introduction of single intact human chromosomes by microcell-mediated monochromosome transfer (MMCT) into human tumour cells demonstrate that multiple regulatory sequences exist throughout the genome, which may function to repress telomerase in a tissue-specific manner. Previously, our group has shown that transfer of normal chromosome 3 by MMCT into the 21NT primary breast cancer cell line is associated with strong telomerase repression and cell growth arrest of the majority of hybrid clones (Cuthbert *et al.,* 1999). Regulatory sequences responsible for the observed telomerase repression are thought to reside within 3p21.3-p22 and 3p12-21.1. Subsequent studies have shown that chromosome 3p-encoded telomerase repressor sequences mediate their function through transcriptional repression of *hTERT* (Ducrest *et al.,* 2001). This was found to be due, in part, to chromatin remodelling at two sites within intron 2 of *hTERT* (Szutorisz *et al.,* 2003). In this project, I have shown that transfer of an intact copy of human chromosome 17, as well as chromosome 3, by MMCT into the 21NT breast cancer cell line is also associated with consistent repression of telomerase activity, which provides some evidence to suggest that multiple independent pathways of *hTERT* regulation may be inactivated within the same cancer type.

Attempts to achieve positional cloning of *hTERT* repressor sequences on chromosome 3p, identified a single candidate gene *SETD2* and a neighbouring long non-coding RNA sequence *FLJ* localized to 3p21.3 (Dr. Roberts, unpublished data). By examining the effect of forced *SETD2* and *FLJ* expression within 21NT cells and the consequence of siRNA-mediated knockdown of *SETD2* within a single *hTERT*-repressed 21NT-chromosome 3 hybrid, I have provided some evidence that neither candidate gene functions as a regulator of *hTERT* transcription. Interrogation of relevant literature identified a set four candidate telomerase regulatory genes including *BAP1, RASSF1A, PBRM1* and *PARP-3,* located within the 3p21.1-p21.3 region that have been shown to play a functional role in the epigenetic regulation of target gene transcription. Using mammalian expression vectors containing candidate gene cDNA, my colleague Dr. Roberts and I have demonstrated that forced over-expression of *BAP1* and *PARP-3* within 21NT cells is associated with consistent, but not necessarily sustained, repression of *hTERT* transcription. Therefore, at least two sequences may be present on chromosome 3p that function together to regulate *hTERT* within breast cancer cells.

Using an *in vitro* model of HMEC immortalization developed by my colleague Dr. Yasaei, I have shown that single copy deletions on chromosome 3p are a frequent clonal event that is specifically associated with *hTERT* de-repression and immortal transformation. Subsequent single nucleotide polymorphism (SNP) array analysis of immortal HMEC variants identified a minimal common region of deletion encompassing 3p14.2-p22 (Dr. Yasaei, unpublished data). These findings provide additional evidence to show that critical *hTERT* repressor sequences may be present on chromosome 3p, which may be lost as an early event during breast carcinogenesis.

Despite efforts to achieve positional cloning of critical *hTERT* repressor sequences in different cancer types, the key molecular players and mechanisms by which they function to repress *hTERT* transcription remain unclear. The development of innovative chromosome engineering techniques, together with pathologically relevant *in vitro* models of human epithelial cell immortalization, should advance our understanding of this fundamental aspect of human carcinogenesis and identify novel therapeutic targets for cancer prevention or treatment.

7.2 FUTURE WORK

7.2.1 INVESTIGATING THE ROLE OF SETD2 IN BREAST CANCER

7.2.1.1 Background

The histone 3 lysine 36 (H3K36) trimethyltransferase enzyme, SET domain containing 2 (SETD2), has recently been shown to play a functional role in maintaining genome integrity in human cells by regulating homologous recombination (HR)-mediated repair of DNA double strand breaks (DSB) and DNA mismatch repair pathways (Pfister *et al.,*2014, Li *et al.,* 2013). In addition, SETD2 has been found to interact with p53 in breast cancer cells and regulate the expression of genes involved in the DNA damage response pathway through nucleosome positioning and chromatin accessibility in clear cell renal carcinomas (ccRCC) (Xie *et al.,* 2008, Simon *et al.,* 2013). In 2009, Sarakbi and colleagues demonstrated that *SETD2* mRNA levels are significantly lower within breast tumours than normal breast tissue samples and low levels of *SETD2* expression are associated with more progressive disease (Sarakbi *et al.,* 2009). Furthermore, *SETD2* has been found to be mutated within a significant proportion (12%-22%) of multiple cancer types including acute mixed lineage-rearranged leukaemia (MLL) and clear cell renal carcinoma (ccRCC) (Zhu *et al.,* 2014, Fontebasso *et al.,* 2013, Mar *et al.,* 2014, Hakimi *et al.,* 2013). Loss of SETD2 function is associated with RNA processing defects, increased spontaneous mutation frequency, microsatellite instability and reduced HR-mediated repair of DSB, which may contribute to genetic instability and drive tumorigenesis (Pfister *et al.,* 2014, Li *et al.,*2014, Simon *et al.,*2013). These studies provide evidence to suggest that SETD2 plays an important tumour suppressive role in human cells.

Taking the above observations into account, it was therefore surprising to find in my project work that forced overexpression of SETD2 within the oestrogen (ER) and progesterone receptor (PgR)-negative, HER2-positive breast cancer cell line 21NT, was associated with a significant increase in clonogenic survival when compared with empty vector controls (Chapter 4.3.2.3). Furthermore, according to the Kaplan Meier Plotter (KPM) online survival analysis program, breast cancer patients with ER-negative, PgR-negative and HER2-positive tumours or ER-negative and *p53*-mutant tumours that express high levels of *SETD2* mRNA, have significantly worse relapse free survival rates compared with those that display low levels of *SETD2* expression (Chapter 4.4, Gyorffy *et al.,* 2010). These findings are the first to suggest that SETD2 may play an oncogenic role in certain molecular subtypes of breast cancer.

Multiple anticancer strategies, such as ionizing radiation and chemotherapeutic agents, rely on the generation of toxic DNA lesions or DSBs within the genome of cancer cells in order to activate cell death signalling pathways (reviewed by Helleday., 2010). During S and G2 phases of the cell cycle, these lesions can be substrates for HR-mediated repair (Saleh-Gohari and Helleday., 2004). HR-defective cancer cells can be sensitive to chemotherapeutic agents

that cause DNA replication stress, such as topoisomerase I/II inhibitors and antimetabolites, which are used for the treatment of HER2-positive, ER-negative breast tumours (Chapter 1.2.5.3, Helleday, 2010). Conversely, genetic reversion of mutations and upregulation of genes involved in HR-mediated repair pathways, have been associated with resistance to replication stress-inducing anticancer agents. For instance, demethylation of the gene that encodes Fanconi anemia complementation group F (FANCF), which is involved in the FANC-BRCA DNA repair pathway, is associated with resistance to cisplatin in ovarian tumours (Taniguchi *et al.,* 2003). Independent studies have shown that SETD2 plays an important role in maintaining genome stability through the regulation of DNA repair pathways (Pfister *et al.,* 2014, Li *et al.,*2014, Simon *et al.,*2013). It is therefore possible that the observed decrease in relapse-free survival rates within breast cancer patients with HER2-positive and hormone receptor-negative tumours that express high levels of *SETD2* mRNA, could be linked to the more efficient repair of DNA lesions introduced by genotoxic anticancer agents. Future work would aim to determine whether *SETD2* expression within HER2-positive, hormone receptor-negative breast tumours is a predictive marker of patient survival and response to chemotherapy and/or radiotherapy and investigate the molecular mechanism(s) by which SETD2 functions to confer decreased relapse-free survival in breast cancer patients with this molecular subtype.

7.2.1.2 Future Experimental Approaches

As shown in Chapter 4, stable overexpression of *SETD2* within the tumorigenic, HER2-positive and hormone receptor-negative 21NT breast cancer cell line is associated with a significant increase in clonogenic survival compared with empty vector controls. These preliminary findings suggest a putative oncogenic role of SETD2 in breast cancer cells. However they provide no evidence to show that higher *SETD2* expression is associated with an increase in the tumorigenic potential of 21NT cells. One approach to investigate this further would be to generate mouse xenograft models of 21NT cell-derived tumours (as well as other HER2-positive, hormone receptor-negative breast tumours). By assessing tumour growth within immune-deficient, athymic nude mice following injection of stable 21NT-*SETD2* and 21NT-empty vector control transfection clones, it should be possible to examine the role of SETD2 on the tumorigenic potential of 21NT cells *in vivo.* In addition, the same mouse xenograft models could be employed to test the hypothesis that high *SETD2* expression is associated with resistance to radiotherapy or chemotherapy and decreased survival, by examining the effect of *SETD2* expression on tumour growth and host survival in response to treatment with genotoxic agents used currently in the treatment of HER2-positive, hormone receptor-negative breast tumours (e.g. radiation, cyclophosphamide and antimetabolites).

As demonstrated by Pfister *et al.,* (2014), siRNA-mediated depletion of *SETD2* within U2OS osteosarcoma cells is associated with a reduction in HR-mediated DNA damage repair

following irradiation compared with non-targeting controls. This was demonstrated by a reduction in replication protein A (RPA) and RAD51 foci and delayed removal of γ-H2AX foci within *SETD2*-depleted cells. Therefore, in order to investigate whether the functional role of SETD2 as a regulator of HR-mediated DNA repair is associated with resistance to radiotherapy and/or chemotherapeutic agents for HER2-positive, hormone receptor-negative breast cancer cells, the effect of forced *SETD2* expression on cell growth and the number of RAD51 and RPA foci formed following treatment of 21NT cells with irradiation and antimetabolites (e.g. methotrexate and fluorouracil) could be examined.

7.2.2 Narrowing the Search for hTERT Repressor Sequences on Chromosome 3p in Breast Cancer Cells

7.2.2.1 Background

During the search for a sequence localised to human chromosome 3p that functions to regulate *hTERT* in breast cancer cells, I found that stable overexpression of *BAP1* (3p21.1) within the 21NT breast cancer cell line was associated with a consistent but transient repression of telomerase activity (Chapter 5.3.2.1). In addition, stable overexpression of *PARP-3* (3p21.2) has recently been associated with repression of *hTERT* transcription within the same breast cancer cell line (Dr. T. Roberts, unpublished data). These findings provide some evidence to suggest that multiple sequences localised to chromosome 3p may function to repress *hTERT* transcription and telomerase activity in breast cancer cells. In addition to the 21NT breast cancer cell line, a human head and neck carcinoma cell line, RCC23 and KC12 renal cell carcinoma (RCC) and the HeLa cervical carcinoma cell lines, introduction of a normal copy of human chromosome 3 into the HSC3 human oral squamous cell carcinoma cell line by microcell-mediated monochromosome transfer (MMCT), has recently been found to produce *hTERT*-repressed hybrids (Nishio *et al.,* 2015, Cuthbert *et al.,* 1999, Tanaka *et al.,* 1998, Horikawa *et al.,* 1999, Backsch *et al.,* 2001). Consistent with the notion that multiple sequences regulate *hTERT* expression, whole cell fusion of HSC3 and RCC23 cell lines generated a series of *hTERT*-repressed hybrids, indicating that multiple telomerase regulatory sequences may be present on human chromosome 3.

7.2.2.2 Future Experimental Approaches

In order to narrow the search for sequences on chromosome 3 that function to regulate *hTERT* in breast cancer cells, high resolution genome mapping techniques such as genotyping arrays or targeted genome sequencing technologies could be employed to identify, map and align common regions of deletion on chromosome 3 within a series of telomerase-positive, segregant 21NT-chromosome 3 hybrid clones and early-passage, immortal human mammary

epithelial cell (HMEC) clones that have emerged from crisis. As demonstrated in Chapter 6, single nucleotide polymorphism (SNP) array analysis of individual, immortal HMEC clones that had emerged from crisis following targeted abrogation of the critical effectors of stasis and replicative senescence (*p16* and *p53* respectively), revealed the presence of a common 33Mb region of deletion encompassing 3p14.2-p22 within four out of six clones (Chapter 6.3.2). Interestingly, this region is thought to harbour putative telomerase repressor sequences in breast and renal cell carcinoma cell lines (Cuthbert *et al.,* 1999, Szutorisz *et al.,* 2003, Horikawa *et al.,* 1998, Tanaka *et al.,* 1998, Tanaka *et al.,* 2005, Abe *et al.,*2010). The remaining two immortal clones were not found to have undergone genomic deletions on chromosome 3p. However, it is possible that these clones underwent small, discreet deletions, insertions or point mutations, that were not identified by the SNP array used in this study (Chapter 6.3.2). Therefore, in order to obtain a detailed map of structural alterations on chromosome 3p within these HMEC clones as well as prospective immortal, post-crisis HMEC clones or segregant 21NT-chromosome 3 hybrids, the use of next generation DNA sequencing (NGS) technology or genotyping arrays that detect a greater number of markers (SNP, copy number variants and indels) should be considered.

An alternative approach to identify critical regulators of *hTERT* localised to human chromosome 3p in breast cancer cells would be to compare the expression levels of chromosome 3p-encoded genes within telomerase-repressed 21NT-chromosome 3 hybrids and telomerase-positive, segregant 21NT-chromosome 3 hybrids using transcriptome profiling technology. In 2011, Qi and colleagues compared the expression of around 22,000 transcripts by microarray analysis within telomerase-repressed and segregant, telomerase-positive B16F10-human chromosome 5 microcell hybrids and parental mouse melanoma B16F10 cells (Qi *et al.,* 2011). This led to the identification of *PITX1* as a repressor of *hTERT* promoter activity in mouse B16F10 and human A2058 melanoma cell lines. Using a similar approach, it may be possible to identify candidate *hTERT* repressor sequences in breast cancer cells by comparing the expression of genes localised to chromosome 3p in telomerase-positive segregant 21NT-chromosome 3 hybrids and immortal, post-crisis HMEC clones, with telomerase-repressed 21NT-chromosome 3 hybrids and pre-crisis HMECs. The recent identification of microRNA (miRNA) sequences that regulate *hTERT* within colorectal cancer (miR-21), head and neck squamous cell carcinoma (miR-512-5p) and gastric cancer (miR-1182, miR-1207-5p, miR-1266) cells, would suggest that future comparative gene expression analyses should also incorporate known, non-protein coding sequences present on chromosome 3 (Li *et al.,* 2015, Yang *et al.,* 2015, Zhang *et al.,* 2015, Chen *et al.,* 2014).

To investigate the functional role of candidate regions or sequences localised to human chromosome 3p in the transcriptional regulation of *hTERT* in breast cancer cells, the DNA sequence of the region of interest could be cloned into a human artificial chromosome (HAC)

vector and introduced into telomerase-positive breast cancer cell lines and/or post-crisis HMEC clones by MMCT. Changes in *hTERT* transcription and telomerase activity within breast cancer cell lines or post-crisis HMECs following introduction of the candidate region, could then be examined relative to parental cell lines. HACs are exogenous, linear mini-chromosomes that possess functional centrosomes and artificial telomeres, and have the ability to replicate and segregate autonomously within the cell (reviewed by Oshimura *et al.,* 2015). These vectors can be engineered using a telomere-associated chromosome fragmentation approach (top-down) or a *de novo* approach (bottom-up) using cloned chromosomal fragments. HAC vectors containing up to 10Mb of human DNA have so far been produced using both methods (Oshimura *et al.,* 2015).

With the aim of investigating the role of candidate sequences in the transcriptional de-repression of *hTERT* during HMEC immortalization, targeted genome editing systems, such as the CRISPR/Cas9 system, could be utilised to inactivate specific candidate sequences on chromosome 3p within post-stasis, telomerase-negative HMEC cell strains. Recently, Weber and colleagues used the CRISPR/Cas9 system to carry out targeted mutagenesis of multiple sequences within adult mouse liver cells, in order to identify the genetic drivers of hepatocellular carcinoma and intrahepatic cholangiocarcinoma (Weber *et al.,* 2015). The use of genome editing approaches to carry out targeted deletion of candidate sequences on chromosome 3 within telomerase-repressed, post-stasis HMECs, would confirm whether loss of these sequences is a critical event that confers transcriptional de-repression of *hTERT* and bypass of replicative senescence during the immortal transformation of normal HMECs.

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