Expression of Shelterin and Shelterin-associated Genes in Breast Cancer Cell lines

A thesis submitted for the degree of Doctor of Philosophy

By

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Declaration

I hereby declare that the research presented in this thesis is my own work, except where otherwise specified, and has not been submitted for any other degree.

Azadeh Motevalli
ABSTRACT

Mammalian telomeric DNA consists of tandem repeats of the sequence TTAGGG associated with a specialized set of proteins, known collectively as Shelterin. These telosomal proteins protect the ends of chromosomes against end-to-end fusion and degradation. The objective of this project was to investigate whether expression of Shelterin and Shelterin-associated proteins are altered, and influence the protection and maintenance of telomeres, in breast cancer cells. Initial findings showed that most of the Shelterin and Shelterin-associated genes were significantly down-regulated (at the mRNA expression level) in a panel of ten breast cancer cell lines. Epigenetic alterations to DNA (methylation at CpG Islands) and histones can result in altered expression of genes. Further investigations showed that the promoter region of POT1 was partially methylated in the breast cancer cell line, 21NT. To support these observations, a DNA methylation inhibitor, 5-aza-CdR, and a histone deacetylation inhibitor, TSA, were used in an attempt to reactivate the expression of silenced genes. This work generated novel findings. Treatment with 5-aza-CdR and TSA resulted in the highest recovery of TIN2 and POT1 mRNA levels at both short-term (48 and 72 hours) and long-term (3 weeks) treatment of the breast cancer cell line, 21NT cells. In addition, POT1 promoter methylation was analysed before and after treatment of 21NT cells. Bisulphite sequencing data were consistent with the mRNA expression results, showing up-regulation of POT1, as all methylation sites were demethylated after the treatment of 21NT cells with 5-aza-CdR. These studies also showed for the first time that both the short-term (72 hours) and 3 weeks treatment of 21NT cells with 5-aza-CdR was able to increase telomere lengths (using four measurement methods, i.e. TRF, q-PCR, flow-FISH and iQFISH).
Breast cancer cell lines expressed low levels of several telosomal mRNAs and that this down-regulation was found to be due in part to promoter methylation. Methylation was shown to be relieved through treatment of the cells with 5-aza-CdR and TSA; specifically, POT1 was shown to be up-regulated to a higher extent compared with other Shelterin genes. Given that previous studies involved over-expression of POT1 in telomerase-positive cells to demonstrate telomere length elongation, we addressed the possibility that over-expression of POT1 may affect telomere length in 21NT breast cancer cells. The results showed that the average telomere length of the POT1 over-expressing clones was increased by 2 to 3 kb compared with 21NT non-transfected and empty vector controls. The study also demonstrated that increased telomere length (by ectopic over-expression of POT1) is not due to a direct effect of telomerase enzyme activity. One explanation for this could be that POT1 may induce a negative regulator of telomerase activity to maintain telomere length. Taken together, the results generated in this project suggest that POT1 may control a localised activation of telomerase enzyme at the telomere end, and regulate stability of the Shelterin complex.
ACKNOWLEDGEMENTS

This thesis is the end of my journey in obtaining my PhD and has been kept on track and been seen through to completion with the support and encouragement of numerous people including my friends and colleagues. At the end of my thesis I would like to thank all those people who made this thesis possible and an unforgettable experience for me.

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<tr>
<td>ALT</td>
<td>Alternative Lengthening of Telomere</td>
</tr>
<tr>
<td>ANK</td>
<td>Ankarin</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline phosphate</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia Telangiectasia Mutated</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BCAC</td>
<td>Breast cancer association consortium</td>
</tr>
<tr>
<td>BFB</td>
<td>Breakage-Fusion-Bridge</td>
</tr>
<tr>
<td>B-ME</td>
<td>β-Mercaptoethanol</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>Bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast cancer susceptibility genes 1</td>
</tr>
<tr>
<td>BRCA2</td>
<td>Breast cancer susceptibility genes2</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BRIP1</td>
<td>BRCA1 interacting protein C-terminal helicase 1</td>
</tr>
<tr>
<td>C</td>
<td>Cytosine</td>
</tr>
<tr>
<td>CBP</td>
<td>CRB-binding protein</td>
</tr>
<tr>
<td>CDP-Star</td>
<td>Substrate solution</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic lymphocytic leukaemia</td>
</tr>
<tr>
<td>COSMIC</td>
<td>Catalogue of somatic mutations in cancer</td>
</tr>
<tr>
<td>CRUK</td>
<td>Cancer research UK</td>
</tr>
<tr>
<td>Ct</td>
<td>Threshold cycle</td>
</tr>
<tr>
<td>C5</td>
<td>Fifth carbon</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA-binding domain</td>
</tr>
<tr>
<td>DCIS</td>
<td>Ductal carcinoma in situ</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>DDR</td>
<td>DNA damage response</td>
</tr>
<tr>
<td>DIG</td>
<td>Digoxigenin</td>
</tr>
<tr>
<td>DKC</td>
<td>Dyskeratosis congenita</td>
</tr>
<tr>
<td>D loop</td>
<td>Displacement loop</td>
</tr>
<tr>
<td>D loop</td>
<td>Displacement loop</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DNMTs</td>
<td>DNA methyltransferases</td>
</tr>
<tr>
<td>DNMT1</td>
<td>DNA methyltransferase 1</td>
</tr>
<tr>
<td>DNMT2</td>
<td>TRDMT1</td>
</tr>
<tr>
<td>DNMT3L</td>
<td>DNA cytosine-like 5-methyltransferase</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>DSBs</td>
<td>Double-strand breaks</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>ECTR</td>
<td>Extra-chromosomal linear and circular telomeric DNA</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetra-acetic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epithelial growth factor</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>ES</td>
<td>Stem cells</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>EST1A</td>
<td>Ever shorter telomere 1</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FXLXP</td>
<td>TRF-binding motif</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GAR domain</td>
<td>Gly/Arg-rich domain</td>
</tr>
<tr>
<td>G</td>
<td>Guanine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
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<tr>
<td>HATs</td>
<td>Acetyl transferase</td>
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<td>HCC</td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td>HCI</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HMECs</td>
<td>Normal mammary epithelial cell strains</td>
</tr>
<tr>
<td>HR</td>
<td>Homologous Recombination</td>
</tr>
<tr>
<td>HRT</td>
<td>Hormone replacement therapy</td>
</tr>
<tr>
<td>HPA</td>
<td>Hybridisation protection assay</td>
</tr>
<tr>
<td>HPS</td>
<td>Histidine, proline and serine</td>
</tr>
<tr>
<td>IARC</td>
<td>The international agency for research on cancer</td>
</tr>
<tr>
<td>IDC</td>
<td>Infiltrating ductal carcinoma</td>
</tr>
<tr>
<td>ILC</td>
<td>Infiltrating lobular carcinoma</td>
</tr>
<tr>
<td>IPA</td>
<td>Isopropyl alcohol</td>
</tr>
<tr>
<td>IQ-FISH</td>
<td>Interphase Q-FISH</td>
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<tr>
<td>Kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>KCL</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo dalton</td>
</tr>
<tr>
<td>LCIS</td>
<td>Lobular carcinoma in situ</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of heterozigosity</td>
</tr>
<tr>
<td>LY-R</td>
<td>Radio-resistant mouse lymphoma cells</td>
</tr>
<tr>
<td>LY-S</td>
<td>Radio-sensitive mouse lymphoma cells</td>
</tr>
<tr>
<td>M1</td>
<td>Mortality stage 1</td>
</tr>
<tr>
<td>M2</td>
<td>Mortality stage 2</td>
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<tr>
<td>MeCP1</td>
<td>Methyl-CpG binding protein 1</td>
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<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
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<td>MEM</td>
<td>Modified eagle medium alpha</td>
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<td>MgCl2</td>
<td>Magnesium chloride</td>
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<td>MI</td>
<td>Millilitre</td>
</tr>
<tr>
<td>MI</td>
<td>Microlitre</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance Imaging</td>
</tr>
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<td>MSP</td>
<td>Methylation specific PCR</td>
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<td>Sodium chloride</td>
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<td>Sodium hydroxide</td>
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<tr>
<td>NHEJ</td>
<td>Non-homologous end Joining</td>
</tr>
<tr>
<td>NTC</td>
<td>No template control</td>
</tr>
<tr>
<td>Nt</td>
<td>Nucleotides</td>
</tr>
<tr>
<td>OB-fold</td>
<td>Oligonucleotide/oligosaccharide binding domain</td>
</tr>
<tr>
<td>OD</td>
<td>Optical densitometry</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly (ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer solution</td>
</tr>
<tr>
<td>PC3</td>
<td>Prostate cancer cell line</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PinX1</td>
<td>PIN2/TRF1-interacting, telomerase inhibitor 1</td>
</tr>
<tr>
<td>PNA</td>
<td>Peptide nucleic acid</td>
</tr>
<tr>
<td>POT1</td>
<td>Human protection of telomeres 1</td>
</tr>
<tr>
<td>Q-FISH</td>
<td>Quantitative in situ hybridization</td>
</tr>
<tr>
<td>RAP1</td>
<td>Repressor activator protein 1</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RNP</td>
<td>Ribonucleoprotein</td>
</tr>
<tr>
<td>RPA</td>
<td>Replication protein A</td>
</tr>
<tr>
<td>Rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell park memorial institute</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>RUNX3</td>
<td>Runt-related transcription factor 3</td>
</tr>
<tr>
<td>RQ</td>
<td>Relative quantification</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosyl-L-methionine</td>
</tr>
<tr>
<td>SCG</td>
<td>Single copy gene</td>
</tr>
<tr>
<td>Sec</td>
<td>Seconds</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SNPs</td>
<td>Single nucleotide polymorphisms</td>
</tr>
<tr>
<td>Ss</td>
<td>Single-stranded</td>
</tr>
<tr>
<td>SSC</td>
<td>Saline-sodium citrate</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single strand DNA</td>
</tr>
<tr>
<td>STELA</td>
<td>Single telomere length analysis</td>
</tr>
<tr>
<td>SV</td>
<td>Splice variants</td>
</tr>
<tr>
<td>T</td>
<td>Thymidine</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-Borate EDTA</td>
</tr>
<tr>
<td>TBP</td>
<td>Telomere binding proteins</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TEP1</td>
<td>Telomerase-associated protein 1</td>
</tr>
<tr>
<td>TERT</td>
<td>Telomerase reverse transcriptase</td>
</tr>
<tr>
<td>TFI</td>
<td>Telomeric fluorescence intensity</td>
</tr>
<tr>
<td>Telomere loop</td>
<td>T-loop</td>
</tr>
<tr>
<td>TIN2</td>
<td>TRF1-interacting nuclear protein2</td>
</tr>
<tr>
<td>TNGS1/2</td>
<td>Tankyrase 1 and 2</td>
</tr>
<tr>
<td>TR</td>
<td>Telomerase RNA</td>
</tr>
<tr>
<td>TRAP</td>
<td>Telomere-repeat amplification</td>
</tr>
<tr>
<td>TRDMT1</td>
<td>tRNA aspartic acid methyltransferase 1</td>
</tr>
<tr>
<td>TRF</td>
<td>Terminal restriction fragment</td>
</tr>
<tr>
<td>TRF1</td>
<td>Telomeric repeat binding Factor 1</td>
</tr>
<tr>
<td>TRF2</td>
<td>Telomeric repeat binding Factor 2</td>
</tr>
<tr>
<td>TSA</td>
<td>Trichostatin A</td>
</tr>
<tr>
<td>T-SCE</td>
<td>Telomere-sister chromatid exchanges</td>
</tr>
<tr>
<td>TSG</td>
<td>Tumour suppressor gene</td>
</tr>
<tr>
<td>5-aza-CdR</td>
<td>5-aza-2′-deoxycytidine</td>
</tr>
</tbody>
</table>
CHAPTER I

GENERAL INTRODUCTION
1.1-Structure and function of the breast

The female breast is an assemblage of mammary glands and fatty tissue in conjunction with nerves, veins, arteries and connective tissues known as the fascia (American cancer Society 2013, Cancer research UK). The glands are placed on the subcutaneous layer of the anterior and a portion of the lateral thoracic wall. The breast is made up of the secretory glandular tissue which is located in the upper portion of the breast and surrounding adipose tissues (Figure 1.1). The glandular tissues are separated by septa of connective tissues and organized into 15 to 20 sections which are called lobules. Each lobule contains its own duct systems and interacts with a lactiferous duct to course via the breast towards the nipple/areolar part. Additionally, during lactation, lobules produce milk which is transported to the nipple by the ducts (Rakha, El-Sayed et al. 2008) (Figure 1.1). Before puberty, functional and structural differences between the male and female breast cannot be distinguished. In women, however the production of hormones such as estrogen and progesterone results in the proliferation of ductal cells and the development of a network of lobes and lobules in preparation for lactation. In male, there are a small number of ducts just behind the nipple. However because men do not produce any milk there is no lobule development (Rakha, El-Sayed et al. 2008) (Figure 1.1).
1.2-Breast cancer

1.2.1-Epidemiology, incidence and mortality rates

Breast cancer is the most common type of cancer in women in developed countries (Cancer Research UK, 2008) (Figure 1.2). It is estimated that in the 1990s, breast cancer accounted for approximately 1 in 3 of all female cancers in the UK and Ireland. Indeed, current trends have shown a rise in incidence such that 1 in 8 women will develop breast cancer in the UK at some time in their lives (Cancer Research UK, 2010). Based on current estimated data in 2010, 49,564 females and 397 males were diagnosed with breast cancer, which also caused 11,556 women and 77 male deaths in the UK (Cancer research UK 2010).

Universally, the incidence and mortality rates of breast cancer vary around the world. As shown in Figure 1.2, in less-developed countries, the incidence and mortality rates
were lower in 2008 in comparison with those in Europe (Cancer Research UK, 2008). In the USA, newly estimated data obtained in 2013 showed that over 232,340 females and 2,240 males were diagnosed with breast cancer and it caused 39,620 female and 410 male deaths (National Statistics, 2013). In contrast, the mortality rates in the US have decreased since 1980, due to earlier detection methods and improved treatments (Chu, Tarone et al. 1996).

Figure 1.2-Standardized mortality for breast cancer in different countries. The estimated data show that female breast cancer incidence is highest in Western European countries with the lowest rate observed in Middle and Eastern Africa (Cancer Research UK, 2008 http://www.cancerresearchuk.org/cancer-info/cancerstats/types/breast/incidence/).
The International Agency for Research on Cancer (IARC) revealed that the highest male breast cancer incidence rates occurred in Israel with an age-adjusted rate of 1.24 per 100,000. Interestingly, the lowest incidence rates for both males (0.16/100,000) and females (18.0/100,000) were reported in Thailand. In 2013, so far, breast cancer caused 88,886 deaths in females in European countries representing a 7% decrease since 2009. In Southern and Eastern European countries, the highest incidence rates (among women and men) have been reported in Italy (Ly, Forman et al. 2013). However, the lowest breast cancer incidence rates were reported in the Russian Federation and Ukraine in comparison with the rest of European countries (Ly, Forman et al. 2013).

Like most epithelial cancers, breast cancer is strongly related to age. As shown in Figure 1.3, breast cancer accounted for approximately 17% of cancer-related deaths in females in European countries (Ferlay, Steliarova-Foucher et al. 2013). The variation of incidence and mortality rate in industrial countries indicated that many factors such as ethnic and lifestyle risk factors (diet, alcohol, smoking, and obesity), use of hormone replacement therapy (HRT) in post-menopausal women, and socio-economic status are implicated in increasing breast cancer development (Li, Weiss et al. 2000; Chen, Weiss et al. 2002; Rosenberg, Magnusson et al. 2006).

According to Cancer Research UK, in 1989, 15,625 women died from breast cancer in comparison with 12,417 in 2005. The estimated 5-year survival rates of women diagnosed with breast cancer was 50% in the UK and Wales in the 1970s, but since that time the survival rates have increased to more than 80% (National Statistics, 2007). This reduction in the number of deaths is thought to be attributed to various factors such as earlier detection of the disease due to raised awareness among females, widespread mammography
screening programs and development of more effective treatments (e.g. mastectomy or removal of lymph nodes, lumpectomy, radiotherapy, chemotherapy and hormone therapy) (Botha, Bray et al. 2003; Weigelt, Horlings et al. 2008).

![Figure 1.3-Incidences (A) and mortalities (B) of female cancers in Europe. The area of the pie chart indicates the proportion of incidence and death. Breast cancer in comparison with other cancers had the highest incidence and mortality rates (Ly, Forman et al. 2013).](image)

**1.2.2-Development of breast cancer**

Breast cancer is characterized by uncontrolled growth of mammary epithelial cells (e.g. luminal or ductal) when they are in proliferative state. Breast cancer is classified into different stages based on the tumour phenotype i.e.; normal to hyperplasia, carcinoma in situ, invasive carcinoma and metastatic cancer. Many breast cancers most commonly start
to develop within the ducts or lobules and are known in the early stages as ductal or lobular carcinoma in situ (DCIS/LCIS) or, subsequently, as an invasive carcinoma that infiltrates connective and fatty tissues (Weidner, Semple et al. 1991; Simpson, Gale et al. 2003). Cancer Research UK reported that DCIS accounts for approximately 25% of all breast cancers. In contrast, lobular carcinoma in situ is a rare form of cancer which is not classified as breast cancer (Table 1.1). Ductal carcinoma in situ (DCIS) or intraductal carcinoma of the breast is the most common type of non-invasive breast cancer as the abnormal epithelial cells have not grown beyond adjacent fatty and connective tissues and grow slowly (Figure 1.1) (Allred 2010). Such cancers would initially not overtly affect the woman’s health but can be detected by mammography. A positive mammogram would be normally confirmed by biopsy to identify the presence of malignant cells (American Cancer Society, 2013), which can then also be classified as low, intermediate or high grade. The estimated incidence rate indicates that DCIS is more common in females at age 60 and over. However, it is uncommon under the age of 35 years. The risk factors for DCIS include high body mass index (BMI), increased breast density and nullparity (Virnig, Tuttle et al. 2010). Lobular carcinoma in situ, also known as lobular neoplasia (LCIS) is a rare pre-cancerous type of cancer and is implicated as a risk factor in increasing the development of invasive breast cancer (Table 1.1) (Simpson, Gale et al. 2003; Arpino, Bardou et al. 2004). The predominant form of invasive ductal breast carcinoma is known as infiltrating ductal carcinoma (IDC) and is responsible for approximately 85% of cases. IDC originates in the milk ducts, invades into the fatty tissues and, from there, may metastasize to other parts of the body (Wiechmann and Kuerer 2008). A high Body Mass Index (BMI), nullparity or having late first child, increased breast density, early menstruation and drinking more than three alcoholic drinks
per day are all risk factors correlated with the development of IDC (Virnig, Tuttle et al. 2010). The lowest incidence rate of IDC is observed in females under 30 and the highest rates occur at age 75 and over (Virnig, Tuttle et al. 2010). Yet another form of invasive breast cancer is infiltrating lobular carcinoma (ILC) and, in comparison with LCIS, is more aggressive and accounts for 8-14% of breast cancer diagnosis (Table 1.1). ILC expands in the milk-producing glands (lobules) and often spreads to other regions of the body (Arpino, Bardou et al. 2004; Wiechmann and Kuerer 2008).

**Table 1.1**-Different stages and classification of breast cancer. The T classification of the primary tumour is based on clinical or pathological criteria or both. Tis = in situ disease (not fully invasive). Table obtained from National Cancer Institute, 2013: (http://www.cancer.gov/cancertopics/pdq/treatment/breast/healthprofessional/Table1)

<table>
<thead>
<tr>
<th>Stages</th>
<th>Tumour size</th>
<th>Characteristic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 0</td>
<td>T0</td>
<td>No evidence of primary tumour</td>
</tr>
<tr>
<td></td>
<td>Tis</td>
<td>Carcinoma in situ</td>
</tr>
<tr>
<td></td>
<td>Tis (DCIS)</td>
<td>Ductal Carcinoma in situ (DCIS)</td>
</tr>
<tr>
<td></td>
<td>Tis (LCIS)</td>
<td>Lobular Carcinoma in situ (LCIS)</td>
</tr>
<tr>
<td></td>
<td>Tis (Paget)</td>
<td>Paget’s disease of the nipple with no associated tumour mass</td>
</tr>
<tr>
<td>Stage 1</td>
<td>T1, Tumour size ≤20mm</td>
<td>Node status; clear or negative nodes</td>
</tr>
<tr>
<td></td>
<td>T1a, Tumour size &gt; 1mm</td>
<td>Node status; clear or negative nodes, Cancerous, No spread of tumour</td>
</tr>
<tr>
<td></td>
<td>T1b, Tumour size &gt; 5mm</td>
<td>Node status; clear or negative nodes, Cancerous, No spread of tumour</td>
</tr>
<tr>
<td></td>
<td>T1c, Tumour size &gt; 10mm</td>
<td>Node status; clear or negative nodes, Cancerous, No spread of tumour</td>
</tr>
<tr>
<td></td>
<td>T2, Tumour size &gt; 20mm</td>
<td>Node status; clear or negative nodes, Cancerous, No spread of tumour</td>
</tr>
<tr>
<td></td>
<td>T3, Tumour size &gt; 50mm</td>
<td>Node status; clear or negative nodes, Cancerous, No spread of tumour</td>
</tr>
<tr>
<td>Stage 4</td>
<td>T4, any size</td>
<td>Spread to chest wall or to the skin</td>
</tr>
<tr>
<td></td>
<td>T4a, any size</td>
<td>Spread to chest wall, not includes only pectorallis muscle</td>
</tr>
<tr>
<td></td>
<td>T4b, any size</td>
<td>Tumour has spread</td>
</tr>
<tr>
<td></td>
<td>T4c, Both T4a and T4b</td>
<td>Both T4a and T4b</td>
</tr>
<tr>
<td></td>
<td>T4d</td>
<td>Inflammatory carcinoma</td>
</tr>
</tbody>
</table>
1.2.3-Breast cancer diagnosis and treatment

Breast cancer is diagnosed by a variety of different methods, including self-examination, clinical examination, mammography, ultrasound and Magnetic Resonance Imaging (MRI) (Saslow, Boetes et al. 2007; Yu, Liang et al. 2010). The clinical examination typically reveals a lump within the breast tissue and in some cases involves the nipple or skin around the affected region (College of American Pathologists-CAP, 'Breast Cancer' 2011). Mammography, as a non-invasive method, is the most common imaging tool which is used to detect suspected breast tumours, premalignant or begin lesions, with a good degree of accuracy. In addition, mammograms are able to increase significantly the efficacy of diagnosis by detecting the detailed abnormalities of an early stage tumour including irregular densities, micro-calcification and architectural distortion progression, even before they can be recognized by clinical examination (Gotzsche and Nielsen 2011). MRI and ultrasound techniques are often used to refine diagnosis providing additional imaging information (Schmitz, Gianfelice et al. 2008).

The treatment of breast cancer depends on the accurate determination of the stage of the disease. For instance, tumour cells at stage IV (metastatic cancer, IDC) are more likely to have spread into surrounding stromal tissue. Therefore, initial visualisation of the tumour mass by mammography is essential for a better prognosis and survival chances. Lumpectomy which involves removal of the tumour lesion together with a margin of surrounding normal tissue is the most common treatment of DCIS (Weigelt, Horlings et al. 2008). However, patients at this stage of disease can decide to have removed a wide area or, alternatively, a relatively small amount of surrounding tissue. Mastectomy, involving removal of all breast tissue including skin and nipple, may be required in order to prevent
reoccurrence of the invasive ductal carcinoma (Cancer Research UK, 2013). In the case of LCIS and ILC, malignant cells rarely form a palpable mass, and are therefore not easily detectable with mammography or by breast examination (Biglia, Mariani et al. 2007).

1.2.4-Molecular genetics of breast cancer

Two classical models of breast cancer exist; (i) sporadic cancer, which is caused by a combination of somatic genetic and environmental factors and (ii) familial cancer, which is a result of a predisposing mechanism of genetic alteration (Table 1.2) (Kenemans, Verstraeten et al. 2004). Epidemiological studies have classified familial breast cancer genes susceptible to germline mutations into high-risk and low-to moderate-risk classes (Mangia, Malfettone et al. 2011). $BRCA1$, $BRCA2$, $PTEN$, $TP53$, $LKB1/STK1$ and $CDH1$ are categorized as high risk genes, whereas $CHEK2$, $TGFβ1$, $CASP8$ and $ATM$ are classified as those with low-to moderate-risk (Mangia, Malfettone et al. 2011).

Table 1.2-Represents familial and sporadic breast cancer genes (Eisenhauer, Chaturvedi et al. 2001; Murata, Khattar et al. 2002; Mangia, Malfettone et al. 2011; Deb, Do et al. 2013).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Familial breast cancer</th>
<th>Sporadic breast cancer</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRCA1</td>
<td>*</td>
<td></td>
<td>DNA damage response</td>
</tr>
<tr>
<td>BRCA2</td>
<td>*</td>
<td></td>
<td>DNA damage response</td>
</tr>
<tr>
<td>PALB2</td>
<td>*</td>
<td></td>
<td>DNA damage response</td>
</tr>
<tr>
<td>BRIP1</td>
<td>*</td>
<td></td>
<td>DNA damage response</td>
</tr>
<tr>
<td>ATM</td>
<td>*</td>
<td></td>
<td>DNA damage response</td>
</tr>
<tr>
<td>NBS1</td>
<td>*</td>
<td></td>
<td>DNA damage response</td>
</tr>
<tr>
<td>RAD50</td>
<td>*</td>
<td></td>
<td>DNA damage response</td>
</tr>
<tr>
<td>CHEK2</td>
<td>*</td>
<td></td>
<td>DNA damage response</td>
</tr>
<tr>
<td>PS3</td>
<td>*</td>
<td></td>
<td>Tumor suppressor</td>
</tr>
<tr>
<td>PTEN</td>
<td>*</td>
<td></td>
<td>Tumor suppressor</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>*</td>
<td></td>
<td>Coordinate a diverse range of cell functions</td>
</tr>
<tr>
<td>hMSH2</td>
<td>*</td>
<td></td>
<td>Tumor suppressor</td>
</tr>
<tr>
<td>hMLH1</td>
<td>*</td>
<td></td>
<td>DNA repair pathway</td>
</tr>
<tr>
<td>COMT</td>
<td>*</td>
<td></td>
<td>Enzymatic interaction in the metabolism of estrogen</td>
</tr>
<tr>
<td>YP1A1</td>
<td>*</td>
<td></td>
<td>Enzymatic interaction in the metabolism of estrogen</td>
</tr>
<tr>
<td>CYP1B1</td>
<td>*</td>
<td></td>
<td>Enzymatic interaction in the metabolism of estrogen</td>
</tr>
<tr>
<td>GSTM1</td>
<td>*</td>
<td></td>
<td>Enzymatic interaction in the metabolism of estrogen</td>
</tr>
<tr>
<td>GSTT1</td>
<td>*</td>
<td></td>
<td>Enzymatic interaction in the metabolism of estrogen</td>
</tr>
</tbody>
</table>
It is estimated that approximately 5% of breast cancer cases are hereditary and linked to germ-line alterations in \(BRCA1\) and \(BRCA2\) tumor suppressor genes. These genes are known to be mutated in breast cancer (Couch, Farid \textit{et al.} 1996; Brohet, Velthuizen \textit{et al.} 2013). In addition, it has been shown that \(BRCA1\) is sometimes hypermethylated at its promoter region in breast cancer (Catteau, Harris \textit{et al.} 1999; Jacot, Thezenas \textit{et al.} 2013).

Human epidermal growth factor receptor 2 (\(HER2\)) is another tumor suppressor gene that plays a fundamental role in cell growth. Martin (2006) reported that over-expression of \(HER2\) (primarily via gene amplification) in 20% of breast cancer cases could lead to excessive cell growth and result in aggressive tumor cells (Lacey, Devesa \textit{et al.} 2002; Martin 2006).

\(TP53\) is a key tumor suppressor gene (TSG) that is found to be mutated in approximately 40% of sporadic breast cancers (Miller, Smeds \textit{et al.} 2005). A high frequency of loss of heterozygosity (LOH) involving the \(BRCA\) and \(p53\) genes has been identified in many sporadic breast cancers (Johnson, Shaw \textit{et al.} 2002). A study by Byrnes \textit{et al.} (2008) showed that there are other genes apart from the ‘key driver’ genes involved in breast cancer (Byrnes, Southey \textit{et al.} 2008). For instance, \(BRCA1\) interacting protein C-terminal helicase 1 (\(BRIP1\)) plays a role in \(BRCA1\)-dependent DNA repair and cell cycle checkpoint function, and encodes a helicase that interacts with the \(BRCA1\) C-terminal domain. Mutation of this gene is also linked with an increased risk of breast cancer (Levitus, Waisfisz \textit{et al.} 2005; Litman, Peng \textit{et al.} 2005). A partner of \(BRCA2\), the \(PALB2\) gene is involved in the double-strand DNA repair pathway, and interacts with the \(BRCA2\) protein which plays a role in homologous recombination (HR). In multiple populations, this gene has been screened for the presence of mutations in multiple small studies of familial and early-onset of breast cancer (Reid, Schindler \textit{et al.} 2007; Tischkowitz, Capanu \textit{et al.} 2012). Previous findings by Byrnes \textit{et al.}
(2008) showed that mutations in the \textit{BRCA2} are correlated with a high risk of breast cancer. \textit{RAD51} paralogs or \textit{RAD51} and the family of \textit{RAD51}-related genes are associated with repairing DNA damage through cooperation with various DNA repair proteins such as \textit{BRCA1} and \textit{BRCA2}. The \textit{RAD51} protein has a central role in single-strand annealing and is involved in responding to DNA damage (Suwaki, Klare et al. 2011). Six monoallelic mutations of \textit{RAD51C}, one of the subfamily of \textit{RAD51}-related genes, were found in breast cancer (Meindl, Hellebrand et al. 2010). In addition, \textit{RAD51D} and \textit{RAD51L1} genes were reported as having a possible association with ovarian and breast cancer risk (Loveday, Turnbull et al. 2011; Pelttari, Kiiski et al. 2012).

1.3-Telomeres

Telomeres are made up of G-rich nucleotide repeats of the sequence (TTAGGG)$_n$ bound by associated proteins at the ends of the chromosomes of eukaryotic and all mammalian cells (Griffith, Comeau et al. 1999; Smogorzewska and de Lange 2004). Telomeric DNA together with the associated telosomal proteins, collectively known as the Shelterin complex, are essential for the overall maintenance of genome integrity and prevent DNA degradation and chromosome end-to-end fusions (Palm and de Lange 2008). In normal mammalian chromosomes, telomeric DNA contains 5 to 15 kilobases of tandem TTAGGG repeat sequences that get critically shorter in telomerase-negative cells after each cell cycle division, due to the end replication problem (Harley, Futcher et al. 1990). When the terminal primer is degraded through the process of lagging strand synthesis, the 5’ gap appears at both ends of linear chromosome (Levy, Allsopp et al. 1992). The ends of chromosomes are synthesized in a 5’ to 3’ direction which degrades about 130 to 210
nucleotides during each cell cycle, resulting ultimately in the telomere reaching a critically short length. The end replication problem was first suggested by Nobel prize winner, James Watson (Watson 1972). At about the same time it was proposed that short telomere length is linked to the end replication problem at the 3’ end (Olovnikov 1973) (Figure 1.4).

Figure 1.4—Schematic representation of the “end replication problem” during DNA replication. a) A parental DNA strands. b) In each cycle of DNA replication, the leading strand is synthesized. c) However, in the lagging strand formation of an Okazaki fragment requires an RNA primer (d) which leaves a gap of about 8 to 12 base pair in the 5’ end of lagging strand. e) Thus, the end of the lagging strand cannot be replicated and is lost after several rounds of cell division; hence results in the shortening of 5’ end and 3’ overhang. This model described by Makarov et al. (1997) (Makarov, Hirose et al. 1997).
During the process of DNA replication, telomeric DNA does not replicate properly due to the inefficiency of DNA polymerase to complete replication of the 5´ end of a linear chromosome. To prevent incomplete replication of the 5´end of a linear telomeric DNA, chromosomes are “capped” as hypothesized by McClintock and Muller (Muller 1938; McClintock 1941; Elizabeth H Blackburn 2006). Thus, the terminal nucleotides of chromosomal DNA are capped by telomeres which have evolved to protect them from enzymatic degradation and thus add stability to the chromosomes (Shampay, Szostak et al. 1984).

1.3.1-Telomere structure and function

The ends of telomeres contain a single-stranded 3’-guanine-rich extension (the G-strand) called the 3´ overhang that is located between 50-500 nucleotide from the end of the telomere; the complementary strand is known as the cytosine-rich strand (the C-strand). The telomerase enzyme is responsible for maintaining the 3´ overhang by extending its length by 100-300 base pairs beyond the C-rich strand, per division (Palm and de Lange 2008). It has been previously shown by electron microscopy that telomeres consist of two loops (Griffith, Comeau et al. 1999) (Figure 1.5). The telomere loop (T-loop) is a large duplex lariat structure in mammalian telomeres. The telomeric DNA folds back into itself to form T-loop and the 3' overhang binds to the 5' end of duplex strand of telomeric repeats to form a displacement loop (known as the D-loop) (Griffith, Comeau et al. 1999; Wright and Shay 2005) (Figure1.5).

The six-protein Shelterin complex packages telomeric DNA and helps to hide the chromosome ends from being detected as a double stranded break during the DNA
The proteins belonging to the Shelterin complex are: Telomeric Repeat binding Factor 1 (TRF1), Telomeric Repeat binding Factor 2 (TRF2), Protection of Telomeres 1 (POT1), TRF1-interacting nuclear protein 2 (TIN2), TPP1 (known as ACD, adrenocortical dysplasia homolog) and Repressor activator protein 1 (RAP1) (Figure 1.5). TRF1 and TRF2 bind to the double stranded T-loop of telomeric DNA and are implicated in maintaining the formation of the T-loop structure, while POT1 interacts with single-stranded TTAGGG repeats at the 3’ overhang, as well as in the D-loop of the T-loop configuration. TRF1 and TRF2 recruit TIN2, RAP1, TPP1, and POT1 to telomeric DNA (van Leth, Andrews et al. 2005).

**Figure 1.5-T-loop structure of the mammalian telomeric ‘cap’.**

A) T-loop structure of human telomere imaged by electron microscopy (Griffith, Comeau et al. 1999). B) The single-stranded portion of the 3’overhang of telomeric DNA folds into the double stranded DNA and forms a larger T-loop and a smaller D-loop (de Lange T. 2006). C) Schematic of the Shelterin complex on telomeric DNA. POT1 is shown only at the binding site closest to the duplex telomeric DNA and binds directly to the 3’ single stranded over hang. TRF1 and TRF2 are the only Shelterin proteins that bind to double-stranded telomeric DNA (de Lange 2005).
Another vital function of telomeres is to prevent the loss of genetic information at the 5' end of a newly synthesized chromosome, by allowing the terminal ends to be replicated completely. This process can be accomplished in the presence of the telomerase enzyme. However, in the absence of telomerase, telomeres are gradually lost due to the “end replication problem” with each consecutive cell cycle (Figure 1.4). In normal cells, when the length of a telomere becomes critically short, the cells stop dividing and, having reached their Hayflick limit, undergo replicative senescence or apoptosis (Hayflick and Moorhead 1961; Makarov, Hirose et al. 1997). At the point when telomeres become dysfunctional, chromosomal end-to-end fusions can occur (Levy, Allsopp et al. 1992). Conversely, in the presence of telomerase enzyme activity (in unicellular organisms, in germline mammalian cells and in cancers) the shortening of telomeres that leads to senescence is avoided (Shippen-Lentz and Blackburn 1989).

1.3.2-Telomere shortening in breast cancer

Telomere dysfunction through telomere shortening and/or dysregulation of telomeric DNA-binding proteins (Shelterin), occurs in both the in situ and invasive stages of many cancers, such as breast cancer (Butler, Hines et al. 2012). Clinical observations have indicated that short telomere length increases a risk of developing epithelial cancers (Plentz, Wiemann et al. 2003).

Butler et al. (2012) showed that Shelterin genes play a fundamental role in regulating telomere length in breast tumours. They reported that the mRNA levels of TRF1, TRF2 and POT1 were inversely correlated with telomere length. Telomere shortening was observed in breast cancer tissues whereas the mRNA levels of aforementioned genes were up-regulated.
(Butler, Hines et al. 2012). In addition, a recent study by Ramsay et al. (2013) showed that mutation of POT1 in many carcinomas such as lung, ovarian and breast causes telomere dysfunction (Ramsay, Quesada et al. 2013). Therefore, dysregulation of telomere length through telomere-binding proteins suggests a common molecular mechanism that underlies clinical abnormalities. Hence, a better understanding the role of telomeres and telomere binding proteins may have a fundamental impact on breast cancer diagnosis and treatment.

1.3.3-Telomere dysfunction and senescence

Numerous recent advances in molecular and cell biology have shown that telomere dysregulation is involved in cellular senescence and cell death. Telomere dysfunction appears through diverse mechanisms such as Shelterin dysfunction and telomere shortening (Sfeir and de Lange 2012). Mutations within telomere-associated (telosomal) proteins (such as POT1) can alter the expression or function of these proteins, leading to telomere dysfunction (Ramsay, Quesada et al. 2013). Several studies have shown that the DNA damage response (DDR) is induced by telomere shortening via the activation of tumor suppressor proteins namely p53 and Rb proteins, and consequently p53-p21 and p16-pRb growth arrest pathways (Campisi 2005; Kuilman, Michaloglou et al. 2010; Rodier and Campisi 2011). Two significant blockades have been postulated to inhibit normal cells transforming into cancerous cells: Mortality stage 1 (M1) and Mortality stage 2 (M2) (Shay, Pereira-Smith et al. 1991). At the M1 stage, cells stop dividing via the DDR which leads to senescence. However, some cells are able to bypass the M1 stage via inactivation of p53 and pRB pathways and hence, they can continue to divide with further decreasing telomere length. When the lengths of telomeres are reduced to critical levels, cells enter into the M2 stage. At this stage, genetic abnormalities such as end-to-end chromosomal fusions,
anaphase bridges and uncapped chromosome ends occur (Newbold 2005). Chromosomal instability is triggered through increased telomeric end-to-end fusions, resulting in dicentric chromosomes via breakage-fusion-bridge (BFB) cycles. It is important to note that, during mitosis, chromosome instability can continue when the two centromeres of a dicentric chromosome are pulled to the opposite poles by spindles (so the called breakage-fusion-bridge cycle). Genomic instability is correlated with the frequency of cell death or senescence making M2 difficult to distinguish from M1 (Stewart and Weinberg 2006). However, some rare cells are able to escape from M2 through reactivation of telomerase expression and become immortal (Wright, Pereira-Smith et al. 1989).

It is known that the telomerase enzyme is active in germ line and stem cells, but inactive in most normal diploid somatic human cells, and it is thought that the latter has evolved as a protection against cancer. Approximately 85 to 90% of cancer cells display high telomerase activity (Kim, Piatyszek et al. 1994; Cong, Wright et al. 2002). However, a minority of cancers (~15%) and some immortalized cells can maintain telomere length via Alternative Lengthening of Telomere mechanism (ALT) (Feldser, Hackett et al. 2003; Newbold 2005; Shay and Wright 2005; Stewart and Weinberg 2006). ALT cell lines are characterised by several unique features including heterogeneous telomere length, presence of extra-chromosomal linear and circular telomeric DNA (ECTR), high frequency of telomere-sister chromatid exchanges (T-SCE) and ALT associated promyelocytic leukemia bodies (APBs) (Royle, Foxon et al. 2008; Conomos, Pickett et al. 2013).
1.4-Telomerase

Telomerase is a unique cellular ribonucleoprotein (RNP) complex that synthesizes TTAGGG sequence repeats onto the 3’ end of chromosome terminals (Griffith, Comeau et al. 1999; Wyatt, West et al. 2010). The core enzyme contains two subunits: telomerase reverse transcriptase (TERT) and telomerase RNA (TERC) (Figure 1.6) (Meyerson, Counter et al. 1997). The TR molecule, complementary to the telomeric repeats, is an important element
of the telomerase enzyme; it consists of an RNA template region that facilitates adding telomeric repeats through the action of the reverse transcriptase catalytic subunit of telomerase (TERT) (Autexier and Lue 2006). These two subunits can bind with additional proteins that together expedite synthesis and elongation of telomeric DNA (Wyatt, West et al. 2010).

1.5-Shelterin genes structures and functions

1.5.1-Telomeric Repeat binding Factor 1 (TRF1)

TRF1, the first member of the Shelterin complex, was discovered in HeLa cells and is a ubiquitously expressed protein of 439 amino acids (Zhong, Shiue et al. 1992; Chong, van Steensel et al. 1995; van Steensel and de Lange 1997) (Figure 1.7). TRF1 contains a 50 amino acid C-terminal Myb DNA-binding domain that directly binds to the double stranded telomeric DNA and is localized to the nucleus. The Myb domain is one of the three helical domains that are involved in specific-protein-DNA or protein-RNA interactions (Chong, van Steensel et al. 1995; Bianchi, Smith et al. 1997). TRF1 contains a dimerisation domain which has a ~ 200 amino acid TRF-specific domain. This mediates homodimerisation which is essential for binding to TTAGGG repeat sequences. The acidic N-terminus domain of TRF1 binds to the Shelterin-associated proteins tankyrase 1 and tankyrase 2 (Palm and de Lange 2008). These two proteins are able to modify TRF1 to hinder its DNA-binding activity or remove TRF1 from telomeres and promote its degradation. The C-terminal Myb domain of TRF1 is able to induce bending, looping, and pairing of telomeric DNA, binds to the hetronucleotide repeats of DNA on both the parallel and antiparallel strands. TRF1 may be able to facilitate the folding back of the telomeric DNA in T-loop formation via other
telomeric binding protein such as TIN2, TPP1, and POT1 (Bianchi, Smith et al. 1997; Broccoli, Smogorzewska et al. 1997; Griffith, Bianchi et al. 1998). Therefore, TRF1 acts as a tether through which other Shelterin components interact with the 3' overhang and facilitate the protection of telomeric DNA from degradation and end-to-end fusion (Ye, Hockemeyer et al. 2004). The TRFH domain of TRF1 consists of a versatile peptide docking site to recruit other Shelterin proteins to telomeres. Chen et al. (2008) showed that TRF1 recruits TIN2 via its TRFH domain which interacts with PinX1 (Chen, Yang et al. 2008). PinX1 (PIN2/TRF1-interacting, telomerase inhibitor 1) is a telomerase inhibitor which maps to human chromosome 8p23 and exhibits heterozygosity in different cancers. However, whether PinX1 is inactivated in tumorigenesis is yet to be defined (Soohoo, Shi et al. 2011).

It has been suggested previously that TRF1 can interact with DNA-dependent RNA polymerase II to transcribe the C-strand of telomeric DNA (Schoeftner and Blasco 2008). Hence, it is important to note that the phosphorylation of threonine 122 position of TRF1 by CK2 plays a fundamental role for TRF1 binding to the telomere, thus regulating telomere length (Kim, Davalos et al. 2008). Preliminary observations in telomerase-positive human cells showed that TRF1 is a negative regulator of telomere length. It is believed that long term over-expression of TRF1 even in the presence of telomerase, leads to a gradual decrease in the process of telomere shortening. However, the expression of a dominant negative TRF1 mutant result in the inhibition of binding of endogenous TRF1 to double stranded telomeric DNA and induces abnormal telomere length elongation (van Steensel and de Lange 1997; Smogorzewska, van Steensel et al. 2000). All these findings support the role of TRF1 as a negative regulator of telomere length (in cis) to maintain the access of
telomerase to the end of telomeres (van Steensel and de Lange 1997; Ancelin, Brunori et al. 2002).

Figure 1.7- Interaction between human telomere binding proteins (Shelterin) and telomeric DNA. Binding of individual Shelterin proteins through interaction with specific sequences. TRF1 and TRF2 bind double stranded DNA, whereas POT1 and TPP1 bind single stranded telomeric DNA, therefore aiding in formation of the T-loop. TIN2 binds TPP1 and TRF1, TRF2 and RAP1. The image was obtained from Palm et al. (2008) (Palm and de Lange 2008).

1.5.2-Telomeric Repeat binding Factor 2 (TRF2)

Similar to TRF1, TRF2 has a TRF homology (TRFH) domain close to its amino-terminus and a C-terminal Myb DNA-binding domain which are bound via a flexible hinge domain (Bianchi A 1997; Bilaud, Brun et al. 1997; Fairall L 2001; Hanaoka 2005). However, unlike TRF1, the amino terminus in TRF2 contains the Gly/Arg-rich domain (GAR domain) on its N-terminus. The TRFH domain function acts as a docking site for target proteins that consists of FXLXP motifs (binding for TRF1) and YXLXP motifs (binding for TRF2) (Figure 1.7) (Bhanot and Smith 2012). Moreover, TRF2 is twice as abundant as TRF1 and the two proteins do not interact directly with each other (Diotti and Loayza 2011). TRF1 and TRF2, are closely related within their carboxyl-terminal Myb domains and both proteins bind to the double stranded
telomeric DNA as homodimers / or oligomers via homotypic interactions in the TRFH domain. Additionally, TRF2 can bind to the interstitial telomeric DNA repeated-related sequences (Smogorzewska, van Steensel et al. 2000). The T-loop-like structures are shaped through TRF2 when provided with a model telomere substrate and within this structure, TRF2 preferentially localizes to the junction between the single stranded and double TTAGGGG repeats and prevent the ends of telomeric DNA from being detected as DNA damage (Stansel, de Lange et al. 2001). Over-expression of TRF2 in HeLa and HT1080 tumor cells causes telomeres to become uncapped, which leads to the formation of chromosomal end-to-end fusions (Karlseder, Broccoli et al. 1999). Moreover, TRF2 can protect chromosome ends and inhibit activation of DNA damage response pathways in tumors and normal epithelial cells (Assmus, Urbich et al. 2003; Spyridopoulos, Haendeler et al. 2004; Gensch, Clever et al. 2007). It is important to note that TRF1 and TRF2 are required to form a T-loop-based mechanism to maintain and protect telomere length. TRF2, like TRF1, may be a negative regulator of telomere length homeostasis. It was previously reported that over-expression of TRF2 triggers cells to lose their 3' overhang (Smogorzewska, van Steensel et al. 2000). Celli and de Lange (2005) showed that conditional deletion of Trf2 in p53 null mouse embryonic fibroblasts induces telomeric DNA damage (Celli and de Lange 2005). Therefore, in human cells, TRF1 and TRF2 seem to play an important role in the protection and maintenance of telomeres.

1.5.3-Human Protection of telomeres 1 (POT1)

Human POT1 was originally discovered through its homology to the alpha subunit of the TEBPα/β telomeric end-binding complex in Oxytricha nova (Smogorzewska and de Lange 2004). POT1 was also identified in mammals, Aspergillus, Arabidopsis, and Caenorhabditis
elegans and appears to play a critical role in telomere maintenance in eukaryotes (Baumann and Cech 2001; Wei and Price 2003; Raices, Verdun et al. 2008). Similar to TEBPα, POT1 contains an N-terminal oligonucleotide/oligosaccharide DNA-binding domain (DBD) and a C-terminal protein-protein interaction domain. The hPOT1-DBD has 340 residues and has been co-crystallized with single strand DNA. POT1 also has two OB folds which are able to recognise the single strand DNA decamer telomeric sequence 5'-TTAGGGTTAG-3' in vitro and may possibly have one OB fold at its C-terminus (Figure 1.7) (Diotti and Loayza 2011). The OB-fold is an oligonucleotide/oligosaccharide binding domain (about 110 residues) consists of a five-strand β-sheet, coiled to shape a blocked β-barrel and capped through an α helix located between the third and fourth β strands (Theobald, Mitton-Fry et al. 2003; Bochkarev and Bochkareva 2004). The OB2 of POT1 binds to and protects the 3’ overhang of single stranded telomeric DNA, whereas the first N-terminal OB folds connects to the first six nucleotides (Lewis and Wuttke 2012). The interaction between the carboxyl-terminal of POT1 with TPP1 plays a key role for POT1 loading onto telomeres, while the OB fold of POT1 is essential for telomere localization (Wang, Podell et al. 2007). The POT1/TPP1 complex is able to interact with single stranded DNA (ssDNA) at many positions along the 3’ overhang (Lei, Podell et al. 2004). In addition, the POT1/TPP1 3’ end can bind to the displaced G-strand in the D-loop (Loayza, Parsons et al. 2004). It has been reported that POT1 protects telomere termini via inhibition of the ATR-mediated DNA damage response that is induced by telomere dysregulation. Co-immunoprecipitation experiments have revealed that POT1 protein interacts with TRF1, TRF2 and RAP1 along the double stranded of telomeric DNA via POT1/TPP1/ TIN2 protein bridges (Kelleher, Kurth et al. 2005). Extensive evidence indicates that POT1 appears to modulate telomere length through inhibiting telomerase enzyme
activity (Lei, Zaug et al. 2005). On the other hand, the POT1/TPP1 complex has been implicated in the recovery of telomerase activity acting as part of a telomerase processivity factor via reducing the rate of primer dissociation (Wang, Podell et al. 2007; Latrick and Cech 2010; Zhong, Batista et al. 2012). Previous studies by Kelleher et al. (2005) showed that binding of POT1 to the 3' overhang is essential to negatively regulate telomerase activity in vivo. They reported that POT1 is implicated in modulating telomerase activity through the access of telomerase to the telomere but not during the extension process. It appears that POT1 protein inhibits telomerase via steric hindrance by preventing base pairing between the telomerase RNA and the DNA primer (Kelleher, Kurth et al. 2005). Previous work showed that the deletion of the DNA-binding domain of POT1 with over-expression of N-terminally truncated POT1 induces telomere length (Loayza 2003; Liu, Safari et al. 2004; Ye, Hockemeyer et al. 2004). Moreover, partial deletion of POT1 affects telomere length at the 3' and 5' ends of chromosomes (Hockemeyer, Sfeir et al. 2005; Yang, Zheng et al. 2005). Furthermore, POT1/TPP1 complex covers the 3' overhang of single-stranded of telomeric DNA and inhibits binding of the telomere to telomerase (Wojtyla, Gladych et al. 2011). All these findings support an emerging view that POT1 can both positively and negatively regulating telomerase enzyme activities via interacting with TPP1.

1.5.4-TRF1-interacting nuclear protein 2 (TIN2)

TIN2 acts as the central component in the Shelterin protein complex. This protein is able to interact directly with the double stranded TTAGGG sequences via binding TRF1 and TRF2, thus providing a bridge between the single strand 3' overhang through connecting TPP1 and POT1 and double stranded telomeric DNA (Kim, Kaminker et al. 1999; Houghtaling BR 2004; Kim, Beausejour et al. 2004; Ye, Donigian et al. 2004; Ye, Hockemeyer et al. 2004).
The C-terminal TRF-binding motif (FXLXP) of TIN2 binds to the TRFH domain of TRF1, while TIN2 interacts with its specific site in the hinge domain in TRF2 through its N-terminal domain (Figure 1.7) (Misra, Mahajan et al. 2008; Bhanot and Smith 2012). TIN2 occupies a central position to form a bridge between TRF1 and TRF2. However, TIN2 does not associate with TRF2 within its TRFH domain but stabilises TRF2 at the telomere end (Houghtaling, Cuttonaro et al. 2004; Chen, Yang et al. 2008). In addition, TIN2 also binds TPP1 and POT1, using a third protein interaction site situated on its N terminus. The interaction between TIN2 and TRF2 is enhanced by TPP1 (O’Connor, Safari et al. 2006). Takai et al. (2011) showed that TIN2 disruption leads to a considerable decrease in localization of Shelterin complex at telomeres; this induces replication protein A (RPA) binding to telomere ends and increases the ATR-mediated DNA damage responses which can also affect the phenotypes in Pot1a and Pot1b in double knockout mice (Takai, Kibe et al. 2011). Additionally, TIN2 depletion by RNA interference (RNAi) results in telomere elongation, indicating that TIN2 is a negative regulator of telomere length (Ye and de Lange 2004). Previous studies investigating the role of TIN2 and TRF showed that these two components play a role in telomere cohesion (Canudas, Houghtaling et al. 2007). In mice, deletion of Tin2 results in embryonic lethality; this finding further supports the role of TIN2 in telomerase recruitment and telomere length regulation and maintenance via facilitating TRF2-dependent prevention of the ATM-mediated DDR pathway (Takai, Kibe et al. 2011).

1.5.5- Repressor activator protein 1 (RAP1)

RAP1 is a 399 amino-acid protein which is highly conserved with a carboxyl-terminal RCT domain homologous to the carboxyl-terminus of budding yeast Rap1 protein. RAP1 has an amino-terminal BRCT domain, a central Myb domain-(s), followed by a predicted coiled
domain, and carboxyl-terminus localization signal (Figure 1.7) (Zhu, Kuster et al. 2000; Celli and de Lange 2005). RAP1 binds TRF2 through its C-terminal domain and its association with telomeres depends on its interaction with TRF2. Furthermore, TRF2 maintains the localization and stability of RAP1 and deletion of TRF2 causes RAP1 to be released from the telomere component (Martinez, Thanasoula et al. 2010). O’Connor et al. (2004) discovered that DNA repair proteins such as; Rad50, Mre11, PARP1, BTBD12 and Ku86/Ku70 are found in the RAP1-TRF2 complex (O’Connor, Safari et al. 2004). BTBD12 complexes with RAP1-TRF2 to facilitate Holliday junction processing and the DNA damage response indicating that these complexes play a fundamental role at inhibiting homologous recombination (HR) at telomere (O’Connor, Safari et al. 2004).

1.5.6-TPP1 (ACD, adrenocortical dysplasia homolog)

TPP1 emerged as the last member of the Shelterin complex and was originally known as TIN2-interacting factor. This protein was found to interact with both POT1 and TIN2 (Houghtaling, Cuttonaro et al. 2004; Liu, Safari et al. 2004; Ye, Hockemeyer et al. 2004). As within POT1, TPP1 contains an N-terminal OB fold and is structurally similar to the homolog of *Oxytricha nova* TEBPβ. This finding suggested that the TPP1/POT1 heterodimer is the homolog of TEBPα-β heterodimer (Figure 1.7) (Wang, Podell et al. 2007; Xin, Liu et al. 2007). TPP1 binds POT1 through its interaction domain and TIN2 via its C-terminal interaction domain (Ye, Hockemeyer et al. 2004). The N terminus of TPP1 in the OB-fold domain can interact with telomerase. Therefore, there is a possibility that telomerase may be regulated by TPP1 (Ye, Donigian et al. 2004; Xin H 2007). Telomere length may be maintained by recruiting POT1 via its interaction with TPP1 and TIN2. Moreover, the complex of TPP1/TIN2/POT1 binds to TRF1 and TRF2 and is probably the main way in which POT1 is recruited.
to telomeres (Hockemeyer, Palm et al. 2007; Loayza D 2003). Evidence has been obtained that over-expression of TPP1 defective in interacting with POT1, results in telomere de-protection (Houghtaling, Cuttonaro et al. 2004; Xin, Liu et al. 2007). POT1 deficient in TPP1 binding can localize to telomeres as well as form a weak interaction between POT1 and TRF2 causing telomere dysfunction (Colgin, Baran et al. 2003; Yang, Zheng et al. 2005; He, Multani et al. 2006; O'Connor, Safari et al. 2006). Additionally, the TPP1/TIN2 complex plays a fundamental role in sub-cellular localization of TPP1 and POT1. It has been reported that deletion of the Tpp1 gene in mouse embryo fibroblasts results in chromosome end-to-end fusion and telomere dysregulation (Kibe, Osawa et al. 2010). Taken all of this information together, it is speculated that TPP1 plays an important role in regulating and protecting telomere length.

1.6-Shelterin-associated genes structures and functions

1.6.1-Tankyrase 1 and Tankyrase 2 (TNKS1/2)

Tankyrases are human telomere-associated poly (ADP-ribose) polymerases. Tankyrase 1 (TRF1-interacting ankyrin-related ADP-ribose polymerase 1) was first identified as a TRF1-associated factor via a yeast two-hybrid screen (Smith, Giriat et al. 1998). Tankyrase 1 consists of several domains such as: the N-terminal HPS domain, which comprises multiple runs of histidine, proline and serine (HPS) repeats. The large ankyrin (ANK) domain near to the N-terminus of TNKS1, contains 24 repeated ANK, and includes five functional sub-domains including the sterile α module (SAM) domain which is implicated in takyrase multimerization, and a poly (ADP-ribose) polymerase (PARP) domain which is located in the C-terminal region of tankyrase 1 (Figure 1.8) (Seimiya, Muramatsu et al. 2006; O'Connor, Safari et al. 2006).
The PARP domain of TNKS1 belongs to the super family of PARPs proteins that are involved in numerous cellular processing, particularly DNA repair and programmed cell death (Isabelle, Moreel et al. 2010). *In vitro*, TNKS1 is similar to other PARPs that utilize NAD$^+$ as a cofactor to synthesize long linear or branched poly ADP-ribose onto protein acceptors (Kaminker, Kim et al. 2001; Sbodio, Lodish et al. 2002; Gelmini, Quattrone et al. 2007; Hsiao and Smith 2008). Polymers of ADP-ribose can be added to protein by post-translational modification which then changes protein function. Tankyrase 1 is a homolog of tankyrase 2 and these two homologues have similar structures and domains (ankyrin, SAM and PARP). The only structural difference between these two molecules is that TNKS2 lacks the N-terminal His-Pro-Ser rich domain (Lehtio, Collins et al. 2008).

TRF1 interacts with the N-terminal acidic domain of TNKS1 and TNKS2 proteins. It has been reported that poly ADP-ribosylation of TRF1, *in vitro*, inhibits its binding to telomeric DNA via tankyrase 1. Poly ADP-ribosylation is a process by which multiple groups of ADP-ribose moieties can also transferred to proteins to form long branched chains. This protein modification is carried out by PARPs. The structure of PARPs is involved in the regulation of several cellular events such as maintenance of genomic instability, DNA repair and telomere maintenance (Diefenbach and Burkle 2005). Over-expression of *TNKS1* and *TNKS2* in human cells results to release TRF1 from telomeres and TNKS1 acts as a positive regulator of telomere length (Chiang, Nguyen et al. 2006). Tankyrases may be involved in the removal of telomerase-inhibiting complexes from telomeric DNA to maintain telomeric ends. Therefore, it has been suggested that TNKS1 presumably regulates access of telomerase to the telomeric end (Ha, Kim et al. 2012). Additionally, knockdown of *TNKS1* by
interfering RNA (RNAi) in human cells showed misaligned chromosomes and aberrant spindle structure (Kim and Smith 2013).

Figure 1.8-A Schematic of Tankyrase 1 and Tankyrase 2. These Shelterin-associated genes are positioned at the telomere. The ankyrin domain of tankyrase 1 and tankyrase 2 interact with TRF1 (Cook, Dynek et al. 2002). However, the sequences associated with binding of EST1A and TEP1 have not been elucidated thus far.

1.6.2-Ever shorter telomere 1 (EST1A)

EST1A (sometimes known as SMG6) is also considered to be a Shelterin-associated protein. Telomerase is activated by EST1A at the 3' end of the telomere. Therefore, EST1A is a positive regulator of telomerase (Salhab, Jiang et al. 2008). Over-expression of EST1A induces anaphase bridges due to chromosomal end-to-end fusion, and may affect telomere capping (Reichenbach, Hoss et al. 2003). It has been reported that TEP1 associates with telomerase components but the role of TEP1 in telomerase function is poorly understood (Liu, Snow et al. 2000).

1.7-DNA methylation status and cancer

Aberrant DNA methylation in various malignancies has become the subject of intense investigation. Malignant cells in comparison with their normal counterparts undergo major disruptions in their DNA methylation patterns. In particular, methylation of DNA
within promoter regions serves to suppress the expression of genes that could play a critical role in inhibiting tumorigenesis (Das and Singal 2004). Several tumour suppressor genes, such as the MLH1 mismatch-repair gene are implicated in colorectal and other cancers as well as the p16/CDKN2A cell-cycle control gene involved in various malignancies, and BRCA1 in early breast cancer, are all found to be hypermethylated (Esteller, Corn et al. 2001). In addition, Ottaviano et al. (1994) and Graff et al. (1995) showed that a variety of genes, including cell adhesion and steroid receptor genes play a fundamental role in the development of breast cancer, and are hypermethylated in these tumours (Ottaviano, Issa et al. 1994; Graff, Herman et al. 1995). Furthermore, Zinn et al. (2007) reported that the promoter region of hTERT was hypermethylated in lung, colon and breast cancer cell lines (Zinn, Pruitt et al. 2007). Since the expression of hypermethylated genes (e.g. those mentioned above) can be actively restored after treatment with DNA methylation inhibitors, such as 5-aza-2'-deoxycytidine (5-aza-CdR), therapeutic strategies have been developed to reverse critical methylation regions of DNA in breast cancer and other malignancies.

1.8-DNA Methylation and gene expression

DNA methylation patterns in normal cellular process and abnormal events associated with disease are becoming a very interesting and important field of research (Sharma, Kelly et al. 2010). Many studies now recognize alterations to the epigenome lie at the heart of many complex diseases such as cancer, autoimmune disease, psychiatric and behavioral disorders (Jirtle and Skinner 2007; Jones and Baylin 2007; Ballestar 2011).

In vertebrates, epigenetic alterations such as DNA methylation and histone modifications by specific enzymes play a fundamental role in regulating gene expression of
normal and disease cellular processes (Kim, Samaranayake et al. 2009). It has been known for many years that chromatin packages DNA in a condensed state to preserve its integrity. Epigenetic mechanisms change chromatin structure to produce different categories of epigenetic modifications such as: incorporation of histone variants, histone modifications and in mammals, cytosine-5 DNA methylation at CpG dinucleotides (Sharma, Kelly et al. 2010). In eukaryotic and prokaryotic cells, one of the most important epigenomic phenomena is defined by DNA methylation that plays a significant role in regulating gene expression and chromatin architecture, in cooperation with histone alterations and other chromatin associated proteins (Singal and Ginder 1999; Jurkowski and Jeltsch 2011). DNA methylation is carried out by DNA (cytosine-5) methyltransferases (DNMT). The transfer of a covalent methyl group from a donor S-adenosylmethionine (SAM) to the the fifth carbon (C5) of cytosine, mainly within the CpG dinucleotide, is catalysed by methyltransferases enzymes (Girault, Tozlu et al. 2003; Turek-Plewa and Jagodzinski 2005). Research has demonstrated that the resulting precise DNA methylation patterns can be inherited when DNA replicates by the cooperative activity of DNMTs (Holliday 1991; Kim, Samaranayake et al. 2009).

1.8.1-DNA methyltransferases (DNMTs)

The eukaryotic DNMT family includes five members: DNMT2, DNMT3A, DNMT3B, DNMT3L and DNMT1. The most abundant enzyme is DNA (cytosine-5) methyltransferase 1 (DNMT1) which preferentially methylates hemi-methylated DNA during replication for maintenance of the DNA methylation patterns (Bestor 2000; Robertson 2001; Turek-Plewa and Jagodzinski 2005). This enzyme has an important function in imprinting and in X-chromosome inactivation during embryogenesis (Sado, Fenner et al. 2000). TRDMT1
(formally known as DNMT2) is the smallest mammalian DNA methyltransferase and does not show major *de novo* or maintenance methyltransferase activity in embryonic stem cells (ES) or adult somatic tissue (Okano, Xie *et al.* 1998; Yoder and Bestor 1998). However, the structure of TRD1M1 has shown that this enzyme methylates at position 38 in aspartic acid tRNA (tRNA aspartic acid methyltransferase 1) (Squires, Patel *et al.* 2012). DNMT2 is involved in recognition of DNA damage, DNA recombination and mutation repair (Turek-Plewa and Jagodzinski 2005). Other known functional methyltransferases are DNMT3A and DNMT3B, which mainly methylate CpG dinucleotides without preference for hemimethylated DNA, and so have been classified as *de novo* methyltransferases predominantly during embryogenesis (Okano, Xie *et al.* 1998; Turek-Plewa and Jagodzinski 2005). The DNA cytosine-like 5-methyltransferase (DNMT3L) protein does not have methyltransferase active site motifs and must assist other *de novo* DNMTs (Aapola, Kawasaki *et al.* 2000). It has been considered that it may antagonize functional methyltransferase activity (Robertson 2001).

**1.8.2-How does DNA methylation repress transcription?**

Gene expression can be prevented by DNA methylation through multiple mechanisms. DNA methylation can significantly inhibit transcription factors for some genes. However, it does not account for the general repression of gene expression usually associated with DNA methylation (Kass, Pruss *et al.* 1997). The methyl group of 5-methyl-cytosine protrudes out into the major groove of the DNA helix and it is here that the main contacts are formed with various DNA binding proteins. Consequently, the most direct mechanism of transcriptional inhibition by DNA methylation is by direct interference with transcription factor binding (Maldonado, Hampsey *et al.* 1999). Alternatively, the presence
of a methylation-specific binding protein may possibly act as a repressor (Mossman, Kim et al. 2010).

1.8.3-DNA demethylating agents

DNA methylation inhibitors are a class of substances that can demethylate DNA, resulting in re-expression of silenced genes. DNA demethylating agents can be used for cancer therapy. The CpG-rich regions of normal mammalian genomes are usually non-methylated, with the exception of imprinted genes. These regions, within which genes activity are transcribed, become heavily methylated and inactivated or silenced in the genome of tumour cells (Ghoshal, Datta et al. 2005). Among many agents with DNA methylation-modifying capability, 5-azacytidine (5-aza-CR) or its analogue 5-aza-2’-deoxycytidine (5-aza-CdR) are DNA methyltransferase inhibitors (Figure 1.9).

![Figure 1.9-Mechanism of action of nucleoside analogue inhibitors.](image)

Deoxynucleoside analogues, 5-aza-CdR (indicated by Z) is converted into the triphosphate inside S-phase cells and is incorporated place of cytosine into DNA (Egger, Liang et al. 2004).
These inhibitors were initially shown to have significant cytotoxic and antineoplastic activities in many experimental tumors. However, it was subsequently discovered that they are strong inducers of DNA demethylation (Lin, Shaw et al. 2011). 5-aza-CdR is an analogue of cytosine that has more potent therapeutic effects than 5-aza-CR in human leukaemia, myelodysplastic syndromes and hemoglobinopathies. Following cellular uptake and sequential phosphorylation, 5-aza-CdR is incorporated into DNA but not RNA or protein whereas 5-aza-CR is incorporated into both RNA and DNA (Zhu, Hileman et al. 2004). Once incorporated into DNA, both compounds as are would expect, have related mechanisms of action; they irreversibly bind the methyltransferase enzymes (DNMTs) while they attempt to methylate the cytosine analogue. This depletion of DNMTs in the cells results in hypomethylation of DNA, and induction of DNA damage (Zhu, Hileman et al. 2004; Stresemann, Brueckner et al. 2006; Flotho, Claus et al. 2009). In addition, other potential compounds have been recently developed that are able to reduce the level of DNA methylation, such as zebularine (Zhou, Cheng et al. 2002), hydralazine, procaine (Rubin 2005; Sarzi-Puttini, Atzeni et al. 2005), an anti-sense oligonucleotide MG98 (Goffin and Eisenhauer 2002) and procainamide (Lin, Shaw et al. 2011). However, the detailed mechanisms of these compounds need to be assessed where hypermethylation-induced gene silencing plays an important role in disease pathology, especially in breast cancer.

1.9-Mechanism of Histone modifications and gene expression

Many post-transcriptional modifications of histones, such as phosphorylation, ubiquitinations, acetylation, deacetylation and methylation, have been revealed as epigenetic tags (Kouzarides 2007).
Histone deacetylase (HDACs) enzymes are responsible for removing the acetyl groups from N-terminal lysine/arginine residues in the amino-terminal tails of core histones, specifically the core histones of H2A, H2B, H3 and H4. Histone acetylation is catalysed by histone acetyl transferases (HATs) enzymes (Bannister and Kouzarides 2011). Deacetylation of histones is carried out to maintain the balance between silent and transcriptionally active chromatin. Removal of acetyl groups by HDACs leads to chromosome compaction and prevents transcription (Zhang 2008). HDACs classified as Class I HDACs include 1, 2, 3, and 8. Class IIa HDACs include 4, 5, 7 and 9 and Class IIb consists of HDAC6 and 10. HDACs Class I and II are zinc-dependent enzymes whereas Class III, sirtuins (sir 1-7) need NAD+ for their enzyme activity. Class IV HDAC is exemplified by HDAC11 (Blander and Guarente 2004). Histone acetyl transferase (HATs) enzymes are classified into two types including A HATs and B HATs. The class A HATs enzymes are located in nucleus and are involved in regulating gene expression via acetylation of nucleosomal histones (Roth, Denu et al. 2001). Type B HATs are positioned in the cytoplasm and are required for acetylating new synthesized histones before their aggregation into nucleosomes (Roth, Denu et al. 2001). In addition, the transcriptional co-activators such as CRB-binding protein (CBP) are involved to catalyse acetylation of core histones, which activate the HAT enzyme. Therefore, the acetylation of histones functions to modulate gene expression. Importantly, transcription is regulated by the interaction of HATs with a large number of transcription factors (Abel and Zukin 2008).

Of all known histone modifications, it has been shown that disruption of HATs or HDACs activity can contribute several cancers (Zhang 2008). For example several studies have been reported that inhibition of HDACs by Trichostatin A (TSA) results in the expression of key tumour suppressor genes such as p53, RB1, EGFR in HeLa cells (Zhang
2008). Trichostatin A, a microbial metabolite, is a potent inhibitor of mammalian HDAC Class I and II enzymes. This drug can be utilized to modify gene expression by preventing histone deacetylases and the access of DNA transcription factors to DNA molecules inside chromatin. For this reason inhibition of histone acetylation by TSA has been used successfully as an anticancer drug for cancer treatment (Drummond, Noble et al. 2005; Meng, Dai et al. 2008).
1.10-Aims of the Project

In the last few years, several studies have indicated that maintenance of telomere length can be influenced by the regulation of Shelterin proteins. Therefore, the first aim of this project (Chapter III) was to determine if there are any changes in the expression patterns of Shelterin and Shelterin-associated genes in breast cancer cells. Ten independently derived breast cancer cell lines were investigated and Shelterin gene expression compared with that in normal diploid breast epithelial cell strains (HMECs). The underlying mechanisms behind any changes in expression patterns were then studied by means of epigenetic analysis and mutational studies (Chapter IV). Because it has been demonstrated that the expression of Shelterin and Shelterin-associated genes is correlated with reduced telomere lengths, the effect of up-regulation of Shelterin gene expression, using 5-aza-CdR and TSA, on telomere length was investigated (Chapter V). For this purpose, 21NT, a breast ductal carcinoma cell line was studied (using four telomere measurement methods, i.e. TRF, q-PCR, flow-FISH and iQFISH). The aforementioned techniques were compared in terms of reliability and accuracy. Finally, (Chapter VI) based on previous studies demonstrating that over-expression of one of the Shelterin component, POT1, induces telomere lengthening, the final aim of the project was to examine whether over-expression of POT1 could affect telomere length elongation in the 21NT breast cancer cell line.
CHAPTER II

GENERAL MATERIALS AND METHODS
2.1-Cell lines and cell culture methodology

2.1.1-Cell culture complete growth media

- **Modified Eagle’s medium alpha (MEM):** 1x MEM stock, 1µl/ml hydrocortisone (1:1000), 1µl/ml insulin (1:1000), 10% fetal calf serum (FCS), 1% glutamax, 1% HEPES and 1% NEAA and 1µl/ml Epithelial Growth Factor (EGF)

- **DMEM/F12:** 1x DMEM/F12, 10% FCS and 1% glutamax, 0.5 µg/ml hydrocortisone

- **RPMI-1640:** 1x RPMI/1640, 1% glutamax, 10% FCS, 0.1mM Na Pyruvate, 1% HEPES.

- **F12:** 1x F12, 1% glutamax, 7% FCS

- **RPMI 1640:** 1x RPMI/1640, 10% FCS

Table 2.1 shows a summary of the cell lines that were used during the project, along with details of growth media.
Table 2.1 - Description of different cell lines and normal mammary cell strains (HMECs)

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Patient Age</th>
<th>Histopathological Diagnosis</th>
<th>Tumour Stage</th>
<th>Primary Site</th>
<th>Growth Media</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>21NT</td>
<td>36</td>
<td>*PDC</td>
<td>Primary</td>
<td>Breast</td>
<td>MEM</td>
<td>(Band, Zajchowski et al. 1990; Cuthbert, Bond et al. 1999)</td>
</tr>
<tr>
<td>21MT-2</td>
<td>36</td>
<td>PDC</td>
<td>Metastatic</td>
<td>Breast</td>
<td>MEM</td>
<td>(Band, Zajchowski et al. 1990; Cuthbert, Bond et al. 1999)</td>
</tr>
<tr>
<td>GI101</td>
<td>57</td>
<td>*IDC</td>
<td>III</td>
<td>Breast</td>
<td>DMEM/F12 without hydrocortisone</td>
<td>---</td>
</tr>
<tr>
<td>BT-20</td>
<td>74</td>
<td>IDC</td>
<td>---</td>
<td>Breast</td>
<td>DMEM/F12 without hydrocortisone</td>
<td>(Lasfargues and Ozzello 1958)</td>
</tr>
<tr>
<td>HSS78-T</td>
<td>74</td>
<td>IDC</td>
<td>---</td>
<td>Breast</td>
<td>DMEM/F12 without hydrocortisone</td>
<td>(Hackett, Smith et al. 1977)</td>
</tr>
<tr>
<td>BT474</td>
<td>60</td>
<td>IDC</td>
<td>---</td>
<td>Breast</td>
<td>DMEM/F12 without hydrocortisone</td>
<td>(Lasfargues, Coutinho et al. 1978)</td>
</tr>
<tr>
<td>MCF-7</td>
<td>69</td>
<td>IDC</td>
<td>IV</td>
<td>Pleural effusion</td>
<td>DMEM/F12 without hydrocortisone</td>
<td>(Soule, Vazquez et al. 1973)</td>
</tr>
<tr>
<td>HCC1143</td>
<td>52</td>
<td>PDC</td>
<td>II</td>
<td>Breast</td>
<td>RPMI/1640</td>
<td>(Gazdar, Kurvari et al. 1998)</td>
</tr>
<tr>
<td>MTSV1-7</td>
<td>---</td>
<td>Normal immortalized mammary gland</td>
<td>---</td>
<td>Breast</td>
<td>DMEM/F12 With hydrocortisone</td>
<td>(D'Souza, Berdichevsky et al. 1993)</td>
</tr>
<tr>
<td>PB1</td>
<td>36</td>
<td>PDC</td>
<td>Primary</td>
<td>Breast</td>
<td>MEM</td>
<td>(Band, Zajchowski et al. 1990; Cuthbert, Bond et al. 1999)</td>
</tr>
<tr>
<td>PC3</td>
<td>62</td>
<td>Prostatic adenocarcinoma</td>
<td>IV</td>
<td>Prostate</td>
<td>F12</td>
<td>(Kaighn, Narayan et al. 1979)</td>
</tr>
<tr>
<td>LY-R</td>
<td>---</td>
<td>Mouse Lymphoma, Radiosensitive</td>
<td>---</td>
<td>Lymphoma</td>
<td>RPMI/1640</td>
<td>Dr Andrzej Wojcik University of Warszawa, Poland</td>
</tr>
<tr>
<td>LY-S</td>
<td>---</td>
<td>Mouse Lymphoma, Normal Radiosensitive</td>
<td>---</td>
<td>Lymphoma</td>
<td>RPMI/1640</td>
<td>Dr Andrzej Wojcik University of Warszawa, Poland</td>
</tr>
<tr>
<td>HMEC1</td>
<td>---</td>
<td>Normal human mammary epithelial cell strain</td>
<td>---</td>
<td>Breast</td>
<td>Grown by Dr. H. Yasaei</td>
<td>(Labarge, Garbe et al. 2013)</td>
</tr>
<tr>
<td>HMEC2</td>
<td>19</td>
<td>Normal human mammary epithelial cell strain</td>
<td>---</td>
<td>Breast</td>
<td>Grown by Dr. H. Yasaei</td>
<td>(Garbe, Bhattacharya et al. 2009)</td>
</tr>
<tr>
<td>*Normal commercial</td>
<td>78</td>
<td>FirstChoice® Human breast total RNA</td>
<td>---</td>
<td>Breast</td>
<td>---</td>
<td>AM6952 (Applied Biosystems)</td>
</tr>
<tr>
<td>PC3/hTERT</td>
<td>62</td>
<td>Prostatic adenocarcinoma</td>
<td>---</td>
<td>Derived from PC-3 telomerised</td>
<td>F12</td>
<td>Professor Newbold group (Brunel University)</td>
</tr>
</tbody>
</table>

*Infiltrating ductal carcinoma (IDC), *Primary ductal carcinoma (PDC), *Normal mammary epithelial tissue.
2.1.2- Cell culture procedure

All cell culture was carried out in a LaminAirHB2448 (Heraeus Instrument) cabinet (hood). The culture medium was pre-warmed at 37°C in a water-bath for 10 minutes. Then the cell lines were taken out of liquid nitrogen and thawed in a water-bath for 3 minutes. Cells were transferred into a p100 tissue culture dish containing 15ml of warm culture medium and transferred to fully humidified incubators (HeraCell, Heraeus) set at 5% CO₂ and 37°C. After 24 hours, the medium in p100 dish was aspirated to wash away residual DMSO and fresh media added. Healthy cells were fed with fresh culture medium every 2 days. An inverted phase contrast microscope (Olympus CK40) was utilized for visualizing the cells. Digital images of cells were captured using an Olympus IX71 microscope attached to a coolSNAP cf camera (Photometrics). Cells were monitored every day and deemed suitable for cryostorage once 80-90% confluence was reached. All cell lines were sub-cultured with trypsin-EDTA (Gibco/Invitrogen) 1:3 at 80% confluence. 15 minutes prior to trypsinization, the required culture medium, versene (0.04% EDTA in 1XPBS) and trypsin-EDTA were all pre-warmed to 37°C in a water-bath. The external container surfaces, cabinet hood and all equipments were sterilized using 70% IMS to avoid any fungus or bacterial infection. Cell culture medium was aspirated from plates and the adherent cell monolayer washed once with 10ml of versene. After gentle swirling and aspiration of versene, 3ml of warm trypsin-EDTA was added to p100 dish and incubated for 5 minutes. Next, the detached cells in trypsin-EDTA were neutralized with 10ml complete cultured medium and the cells spun down at 15000rcf for 5 minutes in a Sorvall Legend T bench centrifuge. Supernatants were aspirated and the cell pellets re-suspended by gentle flicking to disperse the cells before re-
suspending them into appropriate volume of complete medium. Finally, 1ml of suspended cells was then put in a new p100 dish with the fresh medium.

2.1.3-Cryopreservation of cells

Before freezing, the cells were monitored for growth state and contamination. All healthy cell lines growing in log-phase in p100 tissue culture dish were fed with fresh medium 24 hours before freezing. The culture medium was removed and the cells were trypsinized as described above. The cell suspension was then transferred to a falcon tube and the pellet centrifuged at 15000rcf for 5 minutes. The supernatant was aspirated off and the pellet gently flicked and re-suspended in 1ml freezing mixture containing 90% FCS and 10% DMSO (dimethylsulfoxide, Sigma). Cell suspensions were aliquoted into 1.5 or 2ml ampoules for storage in liquid nitrogen. Prior to transfer to liquid nitrogen, the vials were kept in Nalgene Nunc cooler at -80°C for 24 hours. This allows a controlled rate of cooling to prevent the formation of intra-cellular ice crystals that may rupture cell membranes. The plastic holder was filled with Isopropyl alcohol (IPA). The ampoules were finally transferred into liquid nitrogen for long-term storage. Cells were routinely frozen down at 3-5x10⁶ per ml from P-100 tissue culture dishes and 1-2x10⁶ from p60.

2.2-RNA extraction

2.2.1-RNA extraction using RNeasy Mini Kit (50)

RNA extraction was carried out utilizing an RNeasy Mini kit (QIAGEN), which provided fast processing and effective purification of RNA from cells. The procedure was performed according to the manufacturer’s instructions. Before starting RNA extraction, β-Mercaptoethanol (β-ME) must be added to Buffer RLT. The number of pelleted cells was
approximately 5x10^6 so 6μl β-ME was added to 600μl of Buffer RLT. The cells were washed with 10ml of sterile PBS (Phosphate Buffer Solution) twice. PBS was then removed and the appropriate amount of Buffer RLT and β-ME (600μl for a 10cm plate) was added to the plate. The surface of the plate was scraped using a scraper (Sarstedt). After transferring the cells to a tube, passed the lysate at least 5 times through 20-gauge needle (0.9mm diameter) fitted to an RNase-free syringe. Next, 600μl of 70% ethanol (1 volume) was added to the homogenized lysate, and mixed well by pipetting. 700μl of the samples were transferred to an RNeasy mini column placed in a 2ml collection tube. Samples were centrifuged for 60 seconds at ≥ 13,000rcf. After this, 700μl of Buffer RW1 was added to the RNeasy column. Tubes were centrifuged for 60 seconds at ≥ 13,000rcf to wash the column. The RNeasy column was transferred into a new 2ml collection tube and 500μl Buffer RPE added. Tubes were centrifuged for 2 minutes at ≥ 13,000rcf to dry the RNeasy silica-gel membrane. This step was repeated to eliminate any chance of possible Buffer RPE carryover. The RNeasy column was placed in a new 2ml collection tube, centrifuged at full speed for 1 minute, and the column then transferred to a new 1.5ml collection tube. Finally, 50μl RNase-free water was added directly onto the RNeasy silica-gel membrane. Samples were then centrifuged for 1 minute at ≥ 13,000rcf. RNA concentrations were measured at 260/280 nm to ensure a ratio of > 1.7, indicating the RNA is free of contaminants. The samples were stored at -80°C for further analysis.

2.2.2-RNA extraction using TRIZOL reagent

Briefly, cells at approximately 80% confluence, was trypsinized and washed twice with 5ml of cold PBS. 1ml of Trizol reagent (Sigma) was added to the cells and left for at least 2 minutes at room temperature. The cell lysate was gently pipetted two or three times
and immediately stored at -80°C for long-term storage. All collected samples were incubated at room temperature for 5 minutes. 200µl of molecular biology grade chloroform (Sigma) was added per 1ml of Trizol. Then the tubes were shaken vigorously by hand for at least 15 seconds and centrifuged at 13000rcf for 30 minutes at 4°C in a bench centrifuge (Eppendorf centrifuge 5415R). The clear upper-aqueous phase containing RNA was carefully pippetted into a fresh microcentrifuge tube. 750µl of isopropyl alcohol per ml of Trizol was added to each sample and mixed gently prior to incubation for 10 minutes at room temperature. The tubes were centrifuged at 12000rcf for 15 minutes at 4°C. RNA precipitate forms a gel-like pellet normally on the side of the tube. The isopropyl alcohol was poured off and the RNA pellets were washed once with 75% ethanol. The pellets were washed thoroughly by vortexing about 10 seconds and centrifuged at 75000rcf for 5 minutes at 4°C. The 75% ethanol was carefully removed and the RNA pellets left to air dry at room temperature for 10 to 15 minutes. The RNA pellets were dissolved in 20µl of DEPC-treated water and retropipetted several times. The samples were left on ice for at least one hour. The absorbance of RNA was read at 260 and 280 nm.

2.3-cDNA synthesis

2.3.1-DNase treatment of RNA

1µl of 10x DNase I Reaction Buffer and 1µl DNase I (Amp Grade) were added to 1µg of RNA and DEPC-treated water to make a total volume of 10µl. Tubes were incubated for 15 minutes at room temperature. Then DNase I was inactivated by the addition of 1µl of 25 mM EDTA solution to the reaction mixture. The samples were heated 10 minutes at 65°C.
2.3.2-Reverse transcriptase

250ng/µl random primers and 1µl 10mM dNTP Mix (10 mM each dATP, dGTP, dCTP, and dTTP at neutral pH) were added to the DNase-treated RNA. Samples were incubated at 65°C for 5 minutes and cooled on ice for at least 1 minute. 4µl 5x First-Strand Buffer, 1µl 0.1 M DTT, 1µl RNaseOUT Recombinant RNase Inhibitor, and 1µl of SuperScript III were added to the tubes. The samples were mixed by pipetting gently up and down. The tubes were incubated at 25°C for 5 minutes, 50°C for 60 minutes, and 70°C for 15 minutes. The cDNA was ready to use as a template for amplification in PCR.

2.4-Primer design

Primers were designed for PCR and RT-qPCR using three different primer design program. These were the primer-BLAST (primer3) at: www.ncbi.nlm.nih.gov, CLC main workbench 6.1 (CLC bio) and primer express version 2.0 (Applied Biosystems). Some genes had different isoforms, for instance: TRF1, TIN2 genes had two isoforms, POT1 had 5 isoforms and SMG6 had 4 isoforms. Therefore, CLC main workbench was used to design primers to these splice variants as you can download each individual splice variant and design primers form specific defined regions of the gene. Primer parameters were set as follows:

- Primer Tm (melting temperature): min (57°C) – max (63°C), optimum (60°C)
- Primer length: min (18bp) – max (22) bp, optimum (20)
- Primer GC% content: min (45%) – max (55%)
- Amplicon product size: min (50bp) – max (200bp)
2.4.1-Primer optimization study

The Shelterin and Shelterin-associated primers were designed using NCBI primer blast software to check for specificity of each primer (Table 2.2).

Table 2.2-This table below shows Shelterin and Shelterin-associated primer sequences for use in Real-Time PCR

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer Sequences (5’ -&gt; 3’)</th>
<th>Product length (bp)</th>
<th>Accession Number</th>
</tr>
</thead>
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<tr>
<td>TIN2, SV1-F</td>
<td>CAAGACTGAGAAATCCACATGCAACCATCCT</td>
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<tr>
<td>TPP1, F</td>
<td>TTAGCGCTGTTGTTGCTCCTT</td>
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<td>NM_001082486</td>
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<tr>
<td>TPP1, R</td>
<td>GCTGCATCCAACTCAGCACAT</td>
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<tr>
<td>POT1, SV2-F</td>
<td>GAGAACAAGCGACTATGCCCA</td>
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<td>NR_003102</td>
</tr>
<tr>
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<td></td>
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<tr>
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<tr>
<td>GAPDH-R</td>
<td>GAAGATGGTGATGGGATTTC</td>
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</table>

F: Forward, R: Reverse
2.5-Real Time Polymerase Chain Reaction (RT-PCR)

2.5.1-Optimizing primer concentration

Before carrying out qPCR, primers were tested using conventional PCR to make sure they gave the correct PCR product size. Primer concentrations were also optimised so that no primer dimmers or secondary products would be produced that could interfere with qPCR data. Three different concentrations (10μM, 5μM and 2.5μM) of forward and reverse primers were tested to find the optimized primer concentration. 10μl of 1.1x Reddy Mix (Thermofisher) (containing tracking dyes) was added to 1μl Forward primer, 1μl Reverse primer, 7μl sterile water, and 1μl cDNA. The reaction mixture volume was 20μl and was incubated in a thermo cycler at 94°C for 5 minutes, followed by 35 cycles of 94°C for 45 seconds, 60°C for 45 seconds, and 72°C for 10 minutes. After that, PCR products were ready for agarose gel electrophoresis.

2.5.2-Agarose gel electrophoresis

A 2% agarose gel was made in 1x TBE buffer solution (Sigma-Aldrich) (1.0M Tris, 0.9M Boric Acid, and 0.01M EDTA). 5μl ethidium bromide (10mg/ml) was added to the 100ml of molten gel and the gel poured into a tray (final concentration, 0.5μg/ml). Once the agarose gel was set, the gel was covered with 300ml of 1x TBE buffer and 20μl of PCR products were loaded into the wells. 7μl of 1kb ladder (Invitrogen) was used as a marker to estimate the size of PCR products. The gel was run at 70 V for approximately 1.5 hours. To visualise the PCR products, an Alphaimager under U.V. light was used. A single band should be seen in a positive lane, and no band should be seen in a negative control lane. The size of the PCR product must match the expected product size.
2.6-Real-Time quantitative RT-PCR (qRT-PCR)

SYBR® green master mix and the ABI Prism 7900HT (Applied Biosystems) was used to perform quantitative real-time reverse transcriptase PCR (qRT-PCR). qRT-PCR reactions were carried out in 96-well plates (Microamp, Applied Biosystems) and each sample was run in triplicate. A final volume of 10μl pre-mix was prepared containing 5μl of 2x SYBR® green master mix, 1μl of 5μM of forward and reverse primers (See Table 2.2 for primers used), 1μl of cDNA, and distilled water to make the final volume up to 10μl. Target and endogenous pre-mixes were prepared separately and 9μl of reaction pre-mix was aliquoted into each of 96-well plate; 1μl of cDNA was immediately added and the plate was sealed utilizing an optical adhesive film (MicroAmp® Optical Adhesive Film, Applied Biosystems). Samples were minimized to light exposure. After that, 96-well plate was centrifuged at 13000rcf for 1 minute at room temperature. All samples were analysed in triplicate and control reactions (where no cDNA was added in the 96-wells, only dH2O) were included in the study design. The default PCR conditions are as listed below:

50°C ..................................................2 min 1 cycle
95°C ..................................................10 min 1 cycle
95°C.............................................. 15 sec 40 cycles
60°C ..............................................1 min 40 cycles

Finally the dissociation curve was constructed immediately after the PCR run to check and verify results. Relative quantification values were determined by the $2^{-\Delta\Delta Ct}$ method using SDS 2.3 software (Applied Biosystems). Dissociation curves were useful to detect nonspecific amplification, and primer dimers that may affect the quality of the data.
In this way, the mRNA expression level of each gene was detected by using qRT-PCR normalized to GAPDH. Both β-actin and GAPDH was evaluated as endogenous controls and GAPDH was found to be more reliable endogenous control.

2.7- Genomic DNA extraction using Wizard ™Genomic DNA Kit protocol

Genomic DNA (gDNA) was extracted from cancer cell lines and normal mammary epithelial cell strains (See Table 2.1) using the Wizard ™Genomic DNA Kit protocol (Promega). Briefly, plates containing approximately 3x10^6 cells were trypsinized and transferred to a labelled 1.5ml Eppendorf tubes. The cell pellets were centrifuged at 13,000rcf for 20 seconds. The supernatant was removed and cells were lysed by adding 600µl Nuclei Lysis solution. After removing all clumps by pipetting up and down, 3µl RNase A solution was added to the nuclear lysate. The samples were incubated for 30 minutes at 37°C. After adding 200µl of Protein Precipitating Solution, the tubes were shaken vigorously for 20 seconds. After that, proteins were precipitated by centrifugation for 5 minutes at 13,000rcf. The supernatant was transferred to a 1.5ml clean Eppendorf tube containing 600µl of (room temperature) isopropanol (2-Propanol) and gently mixed by inverting tubes. Following the precipitation of genomic DNA (gDNA), the samples were centrifuged at 13,000rcf for 5 minutes. After removing the supernatant, the resulting pellet was washed with 600µl of ice cold 70% ethanol and centrifuged for 1 minute at 13,000rcf. The pellets were dried for 10-15 minutes at room temperature. Pellets were re-suspended in 50µl of Rehydration Solution by incubating at overnight at 4°C. The isolated gDNAs were stored at -20°C until required. The concentration purity of DNA was read at 260 and 280 nm.
2.8-Western blotting

2.8.1-Protein isolation

After culturing cell lines to 80% confluence, the medium was removed and the plate was rinsed six times with 5ml of sterile ice-cold PBS and trypsinized as described above. The cell suspension was then transferred to a Falcon tube and centrifuged at 15000rcf for 5 minutes. The supernatant was aspirated off and the pellet washed three times with sterile ice-cold PBS. All the excess liquid was removed and 500μl of RIPA buffer (Radioimmunoprecipitation assay buffer, Sigma) and 10μl of 25x protease inhibitor (Thermo) was added to the tube and left for 5 minutes. After transferring cells to the fresh tube, samples were sheared by passing it through a 1ml syringe and a 23g needle 10 times (0.9 mm diameter). Samples were then collected into fresh Eppendorf tubes and centrifuged at 16,000rcf for 15 minutes at 4°C. The supernatant was aliquoted and transferred to clean Eppendorf tubes and stored at -80°C.

2.8.2-Determination of protein concentration

The protein concentration of samples was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific). Pierce BCA Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. The assay was performed according to manufacturer's guidelines. A standard calibration curve was set up using bovine serum albumin (BSA) diluted in RIPA buffer, ranging from concentrations 0-5μg/ml (Figure 2.1). All unknown sample protein concentrations were measured against the standard curve (Table 2.3).
**Table 2.3-Preparation of diluted BSA standards for BCA analysis**

<table>
<thead>
<tr>
<th>Tube</th>
<th>Volume of dH₂O</th>
<th>Volume of BSA</th>
<th>Final BSA concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>90</td>
<td>10</td>
<td>0.25</td>
</tr>
<tr>
<td>C</td>
<td>80</td>
<td>20</td>
<td>0.5</td>
</tr>
<tr>
<td>D</td>
<td>60</td>
<td>40</td>
<td>1</td>
</tr>
<tr>
<td>E</td>
<td>40</td>
<td>60</td>
<td>1.5</td>
</tr>
<tr>
<td>F</td>
<td>25</td>
<td>75</td>
<td>3</td>
</tr>
<tr>
<td>G</td>
<td>0</td>
<td>100</td>
<td>5</td>
</tr>
</tbody>
</table>

200µl of working reagent (BCA protein assay reagent A/B diluted 50:1) was prepared for each aliquot of protein extract and BSA protein standard concentration. To each 5µl of protein lysate, 200µl of the Working Reagent was added and the samples were vortexed thoroughly on a shaker for 30 second. The tube was incubated at 37°C for 30 minutes in a water bath and then allowed to cool at room temperature. Subsequently, 100µl of each sample was added to the 96-well plate. The A₅₆₂ of the standards and protein lysates was then measured using a plate reader (BP800, BioHit). A standard curve was prepared by plotting the blank-corrected measurement for each BSA standard against its concentration. The standard curve was then used to determine the protein concentration of each study sample.
2.8.3-Protein gel electrophoresis

Protein lysates were prepared from breast cancer cell lines and a normal human mammary epithelial cell strain. The protein concentration was determined utilizing the BCA assay as previously described. For each sample, approximately 30μg of protein was prepared in 4x Laemmle buffer (1.5M Tris-Cl pH 6.8, glycerol, β-mercaptoethanol, SDS, 1% bromophenol blue) to a total volume of 30μl. The lid of the Eppendurf tube was pierced and samples were placed at 95°C for 10 minutes to denature the globular structure of the proteins and then centrifuged at high speed for 30 seconds and the tubes were left on ice until used. 30μl of samples were loaded carefully onto each well of a ready-made 12% precast acrylamide gel (Bio-Rad). The protein marker (Sigma) was loaded in the first and last wells of the gel. The interior and exterior of the tank was filled with 1x ready-made running buffer (Bio-Rad). After that, the samples were initially run at 100 volts until proteins entered
the gel and the power was switched to 250 volts for approximately 45 minutes. The samples were checked regularly to prevent running off of the protein samples.

2.8.4-Blotting and transfer

Acrylamide gels were blotted onto Mini PVDF Transfer ready-made membrane (Bio-Rad) using the Trans-Blot® Turbo™ apparatus (Bio-Rad) according to the manufacturer’s protocol.

2.8.5-Blocking and antibody incubation

Once the transfer of protein from gel onto the ready-membrane was completed the membranes were blocked with 5% blocking solution. The blocking reagent contains 5g (w/v) of semi-skimmed milk (Marvel) in 100ml of Tris buffer saline-Tween (TBST) made with 16g (w/v) of NaCl, 0.2g (w/v) KCl, 3g (w/v) of Tris base, 0.1% (v/v) Tween-20 added to 800ml of distilled water adjusted pH to 7.6, and distilled water added to 1 litre. The membrane was incubated in 20ml of blocking solution for one hour on a shaker at room temperature. The milk mixture blocks the non-specific binding of an antibody to the membrane. Following one hour of blocking, the membrane was rinsed with TSBT and the primary antibody was added. The primary antibody was diluted down according to the manufacturer’s recommendation and was further optimized by the user. Table 2.4 below shows all antibodies used in this experiment with optimized dilution ranges.
Table 2.4-Primary and secondary antibodies used in western blot experiments

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer</th>
<th>Source</th>
<th>Clonality</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>POT1 Primary</td>
<td>Abcam</td>
<td>Rabbit</td>
<td>Monoclonal</td>
<td>1:5000</td>
</tr>
<tr>
<td>POT1 Secondary</td>
<td>Abcam</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>1:10000</td>
</tr>
<tr>
<td>TPP1 Primary</td>
<td>Abcam</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>1:7500</td>
</tr>
<tr>
<td>TPP1 Secondary</td>
<td>Abcam</td>
<td>Rabbit</td>
<td>Monoclonal</td>
<td>1:10000</td>
</tr>
<tr>
<td>β-actin Primary</td>
<td>Sigma</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>1:10000</td>
</tr>
<tr>
<td>β-actin Secondary</td>
<td>Abcam</td>
<td>Goat Anti-rabbit</td>
<td>Polyclonal</td>
<td>1:20000</td>
</tr>
</tbody>
</table>

Primary antibodies were diluted in 5% blocking buffer in 1x TBST and added to the membrane overnight on a shaker set at medium pace (100rpm/minute) at 4°C. The following day the membrane was washed four times with 1x TBST for 15 minutes each and incubated with a secondary antibody diluted in 5% blocking buffer on a shaker at room temperature for one hour.

2.8.6-Protein detection with chemiluminescence

After 1 hour incubation with a secondary antibody the membrane was washed four times in 1x TBST for 15 minutes. The amount of ECL plus (Enhanced chemiluminescence) kit (GE Healthcare) required for detection was based on the size of the membrane and was recommended by the manufacturer. 1ml of reagent A was mixed with 1ml of reagent B. The ECL mixture was pipetted onto the membrane, ensuring that whole surface of the membrane was saturated with the reagent, and covered with Saran wrap and left for 5 minutes in a dark room. The excess of the ECL was tipped off onto a paper towel, wrapped with the protein containing side of the membrane facing down onto a piece of clean Saran wrap and placed in an x-ray cassette. Exposure to ECL plus hyperfilm (Amersham) was done
for between 30 seconds and 20 minutes. The x-ray films were developed using an automatic machine (CURIX 60, AGFA). The ECL chemiluminescence was active for at least one hour allowing multiple exposures.

2.9-Statistical Analysis

All statistical analysis was performed using Student’s t test and the level of significance used throughout was $P \leq 0.05$. 
CHAPTER III

ANALYSIS OF EXPRESSION OF SHELTERIN AND SHELTERIN-ASSOCIATED GENES IN BREAST CANCER CELL LINES
3.1- Introduction

Telomeres are made up of G-rich nucleotide repeats (TTAGGG)_n that protect chromosome ends in mammalian cells. A six-protein complex called Shelterin or the telosome, comprised of TRF1, TRF2, POT1, TIN2, TPP1 and RAP1, packages telomeric DNA and helps to hide the chromosome ends from being recognized as sites of DNA damage during DNA replication (Martinez and Blasco 2010). Shelterin proteins interact with a number of other factors known as Shelterin-associated proteins that can influence chromosome-end integrity and dynamics. These Shelterin-associated genes are Tankyrase 1, Tankyrase 2, SMG6 and TEP1 (Smith, Giriat et al. 1998; Liu, Snow et al. 2000; Salhab, Jiang et al. 2008).

In the absence of telomerase, i.e., in most normal adult somatic cells, the hexanucleotide repeats decrease after each cell division; therefore cells undergo senescence or apoptosis when the lengths of telomeres are reduced to a critical level. In addition, telomere loss causes genome instability, resulting in destruction of cell-cycle control, one of the hallmarks of cancer (Lu, Zhang et al. 2011). The telomerase enzyme regulates telomere length elongation (Greider 1996). In most cancer cells, telomerase has been reactivated and prevents cancer cells from entering senescence or apoptosis (Lu, Zhang et al. 2011). Consequently, the activation of telomerase is an important step in development of human cancers (Salhab, Jiang et al. 2008). Previous studies reported that Shelterin genes (TRF1, TRF2, and POT1) were up-regulated in gastric, breast, cervix and brain cancer cell lines (Matsutani, Yokozaki et al. 2001; Lee, Rha et al. 2008) whereas another group (Yamada, Tsuji et al. 2002) demonstrated that the expression of Shelterin genes (TRF1, TRF2 and TIN2) was decreased in gastric cancer cell lines. Moreover, Salhab et al. (2008)
quantified comprehensively the levels of mRNA expression of hTERT, hTR, Shelterin and Shelterin-associated genes in breast cancer tissue samples by real-time polymerase chain reaction. Their results showed that the expression levels of TNKS2, POT1 and TRF2 were significantly lower in malignant tissues compared with normal matched tissue samples. However, TEP1, TNKS1, and EST1 were up-regulated (Salhab, Jiang et al. 2008). Furthermore, subsequent studies have confirmed that POT1 is over-expressed in gastric cancer tissues (Gao, Zhang et al. 2011). Also, the transcriptional level of TRF2 was found to be correlated with tumour size; i.e, large tumours expressed higher levels of TRF2 (Gao, Zhang et al. 2011).

In order to obtain a better and clearer understanding of the exact role and function of Shelterin and Shelterin-associated proteins in protection and maintenance of telomeres in human breast cancer, the mRNA expression levels of Shelterin and Shelterin-associated genes in a panel of ten breast cancer cell lines and one prostate cancer cell line were compared with commercially (Applied Biosystems) available normal human mammary tissue (cDNA) and that from normal primary human mammary epithelial strains (controls).
3.2-Materials and methods

3.2.1- Analysis of cDNA quality

Total RNA from about 1x10^6 human epithelial cells was isolated using RNeasy Mini Kit (50) (QIAGEN Company). Total RNA (1µg) was reverse-transcribed into cDNA using superscript III (Invitrogen). The forward and reverse primer sequences used for all Shelterin and Shelterin-associated genes are detailed in Table 2.2. Analysis of the standard Shelterin and Shelterin-associated RT-PCR products on agarose gels showed a correct size for all human breast cancer cell lines, normal mammary epithelial cell strain (HMEC) and a prostate cancer cell line (PC-3). In all cases, observation of a specific fragment in the absence of primer-dimmer band was sufficient to confirm the quality and quantity of cDNA samples to utilize subsequently for qRT-PCR.

3.3-Quantification of Shelterin and Shelterin-associated mRNA in breast and prostate cancer cell lines

To determine the level of Shelterin and Shelterin-associated mRNA transcription in breast cancer cell lines, qRT-PCR was performed. To quantify Shelterin and Shelterin-associated expression levels, the C_t values obtained for each mRNA were normalised to those for human GAPDH mRNA (endogenous control). Each sample was run in triplicate (Figure 3.1) and each experiment was performed at least three times to ensure the reproducibility and accuracy of the results. The mean value of each individual triplicate sample was used in further calculations utilizing the 2^-ΔΔCt method to determine relative quantification (RQ) values using the SDS 2.3 software (Applied Biosystems). Relative transcription levels of all Shelterin and Shelterin-associated genes from the panel of cancer cell lines were calibrated by calculating the RQ mean values and human breast total RNA
(Normal) was used as a calibrator. Applying the qRT-PCR amplification program, the quality of the target and endogenous (Shelterin, Shelterin-associated and GAPDH) cDNA products, with no primer-dimer formation, was confirmed by constructing the dissociation curve analysis (Figure 3.1).

**Figure 3.1-qRT-PCR analyses.** The images show schematic example of (A) $C_T$ of each individual triplicate sample for target gene and GAPDH cDNAs, (B) dissociation curve for RT-GAPDH and RT-POT1 primers.
3.4-Results

3.4.1-Determination of Shelterin, Shelterin-associated and GAPDH mRNA expression levels using real-time quantitative RT-PCR

Expression levels of Shelterin and Shelterin-associated genes were quantified in normal breast tissue, a large panel of breast cancer epithelial cell lines (MCF-7, GI101, BT474, 21MT-2, MTSV1-7, HCC1143, BT20, PB1, HS578-T, and 21NT) and a prostate cancer cell line (P-C3) by quantitative RT-PCR to compare the expression level of each gene in normal commercial tissue and cancerous cells. Some of the Shelterin genes have different splice variants; for example, there are five splice variants (SV) of POT1 whereas TNKS1 and TNKS2 do not have splice variants (Table 3.1). To investigate whether there is a difference in mRNA expression level between POT1 isoforms in cancer cell lines, two sets of primers were designed for SV1 and SV2 (Table 3.1). In addition, TIN2 and TRF1 genes have two splice variants which were quantified by qRT-PCR while four isoforms have been observed in SMG6 (EST1) from which only one isoform was assessed (Table 3.1).
Table 3.1-Shelterin and Shelterin-associated genes that encode splice variants

<table>
<thead>
<tr>
<th>Gene</th>
<th>No of splice variants</th>
<th>Size of variants (bp)</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>POT1</td>
<td>5</td>
<td>V1: 4095</td>
<td>NM_015450</td>
</tr>
<tr>
<td></td>
<td></td>
<td>V2: 4215</td>
<td>NR_003102</td>
</tr>
<tr>
<td></td>
<td></td>
<td>V3: 4006</td>
<td>NR_003103</td>
</tr>
<tr>
<td></td>
<td></td>
<td>V4: 3964</td>
<td>NM_001042594</td>
</tr>
<tr>
<td></td>
<td></td>
<td>V5: 4192</td>
<td>NR_003104</td>
</tr>
<tr>
<td>SMG6</td>
<td>4</td>
<td>V1: 5936</td>
<td>NM_173156</td>
</tr>
<tr>
<td></td>
<td></td>
<td>V2: 5798</td>
<td>NM_201568</td>
</tr>
<tr>
<td></td>
<td></td>
<td>V4: 4789</td>
<td>NM_201569</td>
</tr>
<tr>
<td></td>
<td></td>
<td>V5: 6054</td>
<td>NM_001174061</td>
</tr>
<tr>
<td>TNKS1</td>
<td>----</td>
<td>9599</td>
<td>NM_003747</td>
</tr>
<tr>
<td>TNKS2</td>
<td>----</td>
<td>6274</td>
<td>NM_025235</td>
</tr>
<tr>
<td>TIN2</td>
<td>2</td>
<td>V1: 1869</td>
<td>NM_001099274</td>
</tr>
<tr>
<td></td>
<td></td>
<td>V2: 2196</td>
<td>NM_012461</td>
</tr>
<tr>
<td>TRF1</td>
<td>2</td>
<td>V1: 2960</td>
<td>NM_017489</td>
</tr>
<tr>
<td></td>
<td></td>
<td>V2: 2900</td>
<td>NM_003218</td>
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<tr>
<td>TRF2</td>
<td>----</td>
<td>2996</td>
<td>NM_005652</td>
</tr>
<tr>
<td>RAP1</td>
<td>----</td>
<td>2196</td>
<td>NM_018975</td>
</tr>
<tr>
<td>TEP1</td>
<td>----</td>
<td>10694</td>
<td>NM_007110</td>
</tr>
<tr>
<td>TPP1</td>
<td>----</td>
<td>3540</td>
<td>NM_000391</td>
</tr>
</tbody>
</table>

As shown in Figure 3.2, the expression of POT1, SV1, was lower in breast cancer cell lines than in normal breast tissue. This difference was statistically significant when comparing the levels in normal tissue with those in primary and advanced tumours \((P<0.5)\). Figure 3.2-B shows that POT1 SV2 was also down-regulated in tumour cell lines \((P<0.05\) and \(P<0.01\) respectively). However, no substantial differences in expression between POT1 SV1 and POT1 SV2 were observed (See also Figure 3.9).
Figure 3.2 - Expression of POT1 variants 1 and 2 in tumour cell lines. The level of POT1 normalised against GAPDH mRNA in breast cancer cell lines compared with prostate cancer and normal breast tissue, determined by a quantitative reverse transcription polymerase chain reaction. A) POT1, SV1 and B) POT1, SV2 expression in breast cancer samples, a normal breast tissue and a prostate cancer cell line (PC-3). The PC-3 cell line was included for comparison. Normal breast tissue (cDNA) was used as the calibrator. Error bars represent SEM, * P<0.05 and ** P<0.01.
The mRNA expression of RAP1, TNKS1 and TNKS2 were substantially decreased in all cancer cell lines compared with normal breast tissue ($P<0.05$, $P<0.01$, and $P<0.001$ respectively) (Figures 3.3 and 3.4). However, expression of RAP1 was at least 2-fold higher in MCF-7 and PC-3 compared with other cancer cell lines (Figure 3.3).

**Figure 3.3-Expression of RAP1 in tumour cell lines.** Quantification of RAP1, mRNA levels determined by qRT-PCR in PC-3 and breast cancer cell lines. Normal breast tissue (cDNA) was used as the calibrator. Error bars represent SEM, * $P<0.05$, **$P<0.01$, ***$P<0.001$. 
Figure 3.4-Expression of TNKS1 and TNKS2 in tumour cell lines. The histograms in (A) and (B) represent qRT-PCR analysis of TNKS1 and TNKS2 mRNA isolated from cancer cell lines. A) TNKS1 and B) TNKS2 expression in breast cancer, prostate cancer, and normal tissue. Normal breast tissue (cDNA) was used as the calibrator. Error bars represent SEM, *P<0.05, **P<0.01, ***P<0.001.
Figure 3.5 shows that with TIN2 (SV1 and SV2) levels in all breast tumour cell lines and the prostate cancer cell line (PC-3) were also considerably lower than that of normal breast tissue; the most significant reductions were observed in GI101, MTSV1-7, BT20, and HS578-T cells (P<0.05 and P<0.01 correspondingly). However, no major differences have been observed between the two TIN2 splice variants (P<0.05, and P<0.01 respectively).

**Figure 3.5-Expression pattern of TIN2, SV1 and SV2.** The graphs (A and B) represent analysis of mRNA from the indicated human cell lines by qRT-PCR to detect the TIN2 SV1 and TIN2 SV2. Error bars represent SEM, * P<0.05, ** P<0.01.
As shown in Figure 3.6-A, all the breast cancer cell lines examined expressed substantially lower levels of SMG6 ($P<0.05$, $P<0.01$, and $P<0.001$ respectively) in comparison with normal breast tissue control. In marked contrast to the other Shelterin genes, with TPP1, MCF-7, MTSV1-7, PB1, BT20, and PC-3 cell lines expressed high levels of TPP1 mRNA compared with normal tissue, whereas the remainder of the breast cancer cell lines expressed low levels of TPP1 (Figure 3.6-B) ($P<0.05$ and $P<0.01$ correspondingly).

Results are presented for TRF1 SV1 and SV2, and TRF2 in Figures 3.7-A, 3.7-B and 3.8-A. The mRNA expression of TRF1 SV1, its splice variant 2 and TRF2 was significantly lower ($P<0.05$ and $P<0.01$ correspondingly) in comparison with expression of TRF1 and TRF2 from normal breast tissue. However, the mRNA levels of TRF1 SV1 and SV2 were higher in the prostate cancer cell line (PC-3) in comparison with breast cancer cell lines. TRF2 and TEP1 showed a trend similar to that of TRF1 in all tumour cell lines (Figures 3.7 and 3.8). In this section, all the results indicated that, with the notable exception of TPP1, all Shelterin and Shelterin-associated genes were down-regulated in tumour cell lines.
Figure 3.6-Expression patterns of *SMG6* (or *EST1*) and *TPP1*. A) Represents analysis of mRNA from the indicated human cell lines by qRT-PCR to detect SMG6 mRNA levels. B) The mRNA levels of *TPP1* determined by qRT-PCR in breast, prostate cancer cell lines and normal breast tissue. Normal breast tissue (cDNA) was used as the calibrator. Error bars represent SEM, *P*<0.05, **P*<0.01, ***P*<0.001.
Figure 3.7 - The level of TRF1 V2 and V1 in breast and prostate cancer cell lines, determined by qRT-PCR. A) TRF1, SV2 and B) TRF1, SV1 expression in breast cancer, prostate cancer, and normal tissue. Normal breast tissue (cDNA) was used as the calibrator. Error bars represent SEM, *P<0.05, **P<0.01, ***P<0.001.
Figure 3.8 - TRF2 and TEP1 mRNA transcription levels in tumour cell lines and normal tissue. The image (A) and (B) illustrate qRT-PCR analysis of TRF2 and TEP1 mRNA isolated from cancer cell lines. A) TRF2 and B) TEP1 expression in breast cancer, prostate cancer, and normal tissue. Normal breast tissue (cDNA) was used as the calibrator. Error bars represent SEM, *P<0.05, **P<0.01, ***P<0.001.
3.4.2-POT1 and TPP1 mRNA and protein expression in normal breast epithelial (HMEC) cells and cancer cell lines in culture

3.4.2.1-Gene expression of POT1 and TPP1 in breast cancers and the human mammary epithelial strain (HMEC1)

As shown in previous section (3.4.1), all Shelterin and Shelterin-associated genes, except TPP1, were down-regulated in breast cancer cell lines in comparison with a normal breast tissue control. Previous work by Salhab et al. (2008), showed that levels of POT1 mRNA were significantly lower in malignant breast tissues in comparison with normal tissues ($P=0.0008$ and $P=0.038$ respectively). Moreover, with regard to the hypothesis of a critical role of POT1 and TPP1 in telomere length maintenance (Wang F 2007), reanalysis of these genes with a normal mammary epithelial culture cell strain (HMEC1) was warranted.

Human tissues are organized communities of several different cell types that work together to control the function of individual organs. Therefore, these cells may vary at the mRNA and/or protein level in order to carry out their specific functions. In this way, cellurally heterogeneous tissue samples taken for analysis may perhaps exhibit highly varied gene expression or protein levels, compared with individual pure cell strains (Bryant and Mostov 2008). In order to further validate the obtained results, a normal breast mammary epithelial cell strain (HMEC1) was analysed for mRNA levels of POT1 and TPP1.

Based on the results depicted in Figure 3.9-A, it is evident that, the transcription levels of POT1 in malignant cell lines was substantially lower ($P<0.05$ and $P<0.01$ respectively) compared with non-malignant breast tissue. In addition, the mRNA levels of POT1 in normal mammary cell strains were approximately 20-fold lower than normal breast
tissue control. Therefore, in order to validate obtained results, the graph was re-plotted. As shown in Figure 3.9-B, it is evident that all malignant cell lines expressed substantially lower levels of POT1 in comparison with HMEC1 (P<0.05 and P<0.01 respectively).

Figure 3.9-Expression patterns of POT1 SV2 in normal and cancer cell lines. The level of POT1 mRNA determined by qRT-PCR in breast, prostate cancer cell lines, and normal human epithelial cell (HMEC1) and normal tissue. A) Normal breast tissue (cDNA) was used as the calibrator. B) Normal mammary epithelial cell strain (HMEC1) was used as the calibrator. Error bars represent SEM, *P<0.05, **P<0.01.
As shown in Figure 3.10-A, the mRNA expression of *TPP1* in PC-3, MTSV1-7, MCF-7, BT-20 and PB1 was over-expressed compared with normal control tissue (*P*<0.05 and *P*<0.01 respectively). In fact, all tumour samples except HS578-T expressed high levels of *TPP1* in comparison with HMEC1 (*P*<0.05 and *P*<0.01 respectively) (Figure 3.10-B).

**Figure 3.10-Expression patterns of *TPP1* in all normal and cancer cell lines.** The mRNA levels of *TPP1* determined by qRT-PCR in breast, prostate cancer cell lines, HMEC1 and normal breast tissue (cDNA). **A)** Normal tissue (cDNA) was used as a calibrator. **B)** HMEC1 was used as the calibrator. Error bars represent SEM, *P*<0.05, **P**<0.01.
3.4.2.2-Protein analysis of POT1 and TPP1 in breast cancer cell lines and HMECs control

An attempt was made to corroborate qRT-PCR results with western blot analysis on a panel of eight human breast cancer cell lines and a normal primary mammary epithelial cell strains at different passage numbers. The western blot analysis was carried out using POT1 rabbit monoclonal antibody (Abcam) and TPP1 rabbit polyclonal antibody (Abcam) and the values were normalised using β-Actin rabbit antibody (Sigma). The imageQuant 5.0 densitometry was used for densitometry analysis. The protein expression of POT1 and TPP1 was normalised to total β-Actin as a housekeeping gene expression and optical density values are presented in all figures. A 71-KD band (Abcam) was evident in each samples corresponding to POT1 (Figure 3.11-A). Figure 3.11 indicates that the POT1 protein levels were similar in BT20, GI101, HCC1143, BT-474 and MCF-7 breast cancer cell lines in comparison with HMEC1. This data was not completely consistent with the qRT-PCR results. However, 21NT, 21MT-2 and HS578-T expressed considerably high levels of POT1 protein compared with HMEC1 which was not in line with the gene expression data (Figures 3.9-B and 3.11).
Figure 3.11-Western blot analysis of POT1 protein levels in normal mammary epithelial (HMEC1) and breast cancer cell lines. A) A 12% SDS-PAGE gel indicating POT1 protein expression and β-Actin ratio levels in western blots of all 8 breast cancers and normal mammary epithelial cell strain (HMEC1). B) Densitometric analysis of POT1 protein normalised to total β-Actin protein and reported as optical densitometry (OD) unites. Error bars represent SEM, **P<0.01.

In order to determine effect of passage number of normal mammary epithelial cell strains on protein level of POT1, different passage numbers of HMECs from two disease-free patients were analysed by western blot. As the protein expression level of 21NT cells was higher than expected, several passages of HMECs was used to compare with 21NT cells. In addition, all passage numbers were different due to the availability of the cell lines.
Western blot analysis showed higher levels of POT1 in patient 1 (HMEC1) at passage 6 in comparison with the other passages (HMEC1-p8 and HMEC1-p10). However, POT1 levels reduced significantly in all patients compared with 21NT cells ($P<0.05$, $P<0.01$, $P<0.001$ and correspondingly). Moreover, no substantial differences of POT1 protein levels have been observed between each passage numbers in patient two (HMEC 2) (Figure 3.12).

Figure 3.12-Western blot analysis of POT1 protein levels in normal mammary epithelial cell strain (HMECs) at different passage numbers and 21NT breast cancer cells. A) A 12% SDS-PAGE gel showing POT1 protein expression and β-Actin ratio levels of two different patients of normal mammary cell strains and 21NT. B) Densitometric analysis of POT1 protein was performed, normalised to total β-Actin protein and reported as optical densitometry (OD) unites. Error bars represent SEM, *$P<0.05$, **$P<0.01$ and ***$P<0.001$, compared with 21NT control.
Western blot analysis revealed a 58-KD band (Abcam) of TPP1 protein levels in normal and breast cancer cell lines (Figure 3.13-A). After imageQuant 5.0 densitometry analysis, the highest expression of TPP1 protein was detected in BT-474 in comparison with HMEC1 (P<0.05). The level of TPP1 protein was lower in 21NT and 21MT-2 cells than the HMEC1 strain control, which was not in line with the gene expression (mRNA) data (Figures 3.10-B and 3.13).

**Figure 3.13-Western blot analysis of TPP1 expression in normal mammary epithelial cell strain and breast cancer cell lines. A) A 12% SDS-PAGE gel indicating TPP1 protein expression and β-Actin ratio levels in western blots of all 8 breast cancers and normal mammary epithelial cell strain (HMEC1). B) Densitometric analysis of TPP1 protein normalised to total β-Actin protein and reported as optical densitometry (OD) unites. Error bars represent SEM, *P<0.05, **P<0.01.**
However, surprisingly, the TPP1 protein levels in HS578T cells were approximately 3-fold higher than HMEC1 ($P<0.01$) which was not consistent with the qRT-PCR results. Furthermore, BT20, GI101, HCC1143 and MCF-7 expressed high levels of TPP1 protein compared with HMEC1 which were also observed by qRT-PCR results ($P<0.05$) (Figures 3.10-B and 3.13).

As with POT1, to address increasing passage numbers possibly causing changes in the protein levels of TPP1, HMECs at different passage numbers from two patients were examined by western blot. The 21NT breast cancer cell line was also utilized as a control to compare with each different passage of HMECs. The results (Figure 3.14) showed little difference in TPP1 protein expression in HMEC1 from p10, to HMEC2 p13, p15 and p17 in comparison with 21NT cells. However, the protein levels of TPP1 in patient 1 (HMEC1) at passage 8 was approximately 2-fold higher than that 21NT cells ($P<0.01$) (Figure 3.14). Moreover, no substantial differences of TPP1 protein levels have been observed between HMEC1 at passage 6 and 21NT cells.
Figure 3.14-Western blot analysis of TPP1 expression in normal mammary epithelial cell strains (HMECs) at different passage numbers and 21NT breast cancer cell line. A) A 12% SDS-PAGE gel showing TPP1 protein expression and β-Actin ratio levels of two different patients of normal mammary epithelial cell strains and 21NT. B) For quantitative representation, densitometric analysis of TPP1 protein was performed, normalised to total β-Actin protein and reported as optical densitometry (OD) units. Error bars represent SEM, **P<0.01, compared with 21NT control.
3.5-Discussion

Previous work carried out by Salhab et al. (2008), Cookson et al. (2009) and Lu et al. (2011), reported different expression levels of Shelterin and Shelterin-associated genes in different human cancers (Salhab, Jiang et al. 2008; Cookson and Laughton 2009; Lu, Zhang et al. 2011). Salhab et al. (2008) indicated over-expression of TNKS1, hTERT, EST1, and TEP1 and down-regulation of TNKS2 and POT1 mRNA levels in breast cancer tissues compared with normal breast tissues. Moreover, the lower expression of TRF1 and TRF2 was found to be associated with the development and progression of breast cancer. However, findings by Hu et al. (2010) in other cancers appeared to be contradictory to this. They demonstrated significant over-expression of TRF1, TRF2, and TIN2 in precancerous lesions, gastric cancer tissues, and lymph node metastase in comparison with normal gastric mucosa tissues (Hu, Zhang et al. 2010). In addition, recently published data by Lu et al. (2011), showed over-expression of POT1 mRNA levels in gastric cancer tissues.

The aim of the work described in this chapter in relation to the published data was to determine the expression level of each Shelterin and Shelterin-associated genes in ten breast cancer cell lines, commercially available normal human breast tissue (cDNA), together with two different passage number of normal mammary epithelial cell strains (HMEC1 and 2) and the prostate cancer (PC-3) cell line by qRT-PCR. Additionally, based on the potential role of TPP1 and POT1 on telomere length maintenance (Hwang, Buncher et al. 2012) re-analysis of these genes using HMECs, as an additional normal controls, was further quantified at both gene expression (mRNA) and protein levels.
Human tissues are made up of several different cell types that work together to control the function of individual organs. Therefore, these cells may vary at the transcriptomic and/or proteomic level in order to carry out their specific functions (Bryant and Mostov 2008). In this way, cellularly heterogeneous tissue samples taken for analysis may exhibit highly varied gene expression or protein levels, in comparison with individual normal cell strains. In order to validate results obtained when using commercial breast tissue samples as a normal control, a normal mammary epithelial cell strain (HMECs) was therefore used.

As described in the Results section, in Figures 3.2 and 3.9, it was evident that POT1 SV1 and SV2 were significantly down-regulated at the mRNA level in breast cancer cell lines compared with normal breast tissue and the pure HMEC1 cell strains ($P<0.5$ and $P<0.01$ respectively). Among the other Shelterin and Shelterin-associated genes implicated in telomere maintenance, the expression of TRF1 (SV1 and SV2) and TRF2, along with SMG6, TIN2 (SV1 and SV2), TEP1, TNKS1, TNKS2, and RAP1 in normal tissue and breast cancer cell lines were quantified. The results indicated that these genes were down-regulated in breast tumor cell lines compared with breast normal tissue (Figures 3.3, 3.4, 3.5 3.6, 3.7 and 3.8). The marked contrast, the findings revealed that TPP1 mRNA levels were higher in most breast cancer cell lines compared with HMEC1 and normal tissue controls. The exception was, HSS78-T which expressed low levels of TPP1 compared with HMEC1 and normal tissue controls (Figure 3.10).

Alterations in the expression of telomere binding proteins (TBPs) in cancers may disrupt the capping complex, resulting in telomere degradation and shortening independently of the telomerase status (Yamada, Tsuji et al. 2002). The fact that short
telomeres are observed in the vast majority of breast and other epithelial cancers may at least in part be attributable to this.

In order to confirm the results obtained from qRT-PCR, western blot analysis was performed. The POT1 protein levels did not positively associate with mRNA levels in BT20, GI101, HCC1143, BT-474 and MCF-7 breast cancer cell lines in comparison with HMEC1. These tumour cells showed that the POT1 protein levels were similar in comparison with HMEC1 which were not observed by qRT-PCR results. However, 21NT, 21MT-2 and HS578-T expressed considerably high levels of POT1 protein compared with HMEC1 which was not also in line with the gene expression data ($P<0.01$) (Figures 3.9-B and 3.11).

The TPP1 protein levels showed a positive correlation with mRNA expression levels in BT-20, GI101, MCF7 and BT474 breast cancer cells. These tumour cell lines showed up-regulation of mRNA levels of TPP1 in comparison with the normal HMEC1 which was consistent with protein level (Figures 3.10-B and 3.13). As shown in Figure 3.13, the highest TPP1 protein level was detected in BT-474 in comparison with HMEC1. However, the protein level of TPP1 was lower in 21NT and 21MT-2 cells compared with HMEC1. In addition, high TPP1 protein levels and low mRNA expression were observed in HS578-T in comparison with HMEC1 which was not consistent with qRT-PCR (Figures 3.10-B and 3.13).

To determine whether different passage culture of the HMECs may impact on TPP1 and POT1 protein levels, different passage numbers of HMECs (HMEC1 and 2) from two patients were examined at low (p6) and high (p17) passage by western blot. The result showed a slight difference in TPP1 expression at different passages. The difference was relatively small. However, approximately 2-fold increase of TPP1 was observed in HMEC1 p8 compared with 21NT cells (Figure 3.14). Moreover, the effect of different passage numbers
on POT1 expression was also studied. As the protein expression level of 21NT cells was higher than expected, several passages of HMECs was used to compare with 21NT. Western blot analysis showed substantially lower levels of POT1 in all patients in comparison with 21NT cells ($P<0.05$, $P<0.01$ and $P<0.001$ respectively). As shown in Figure 3.12, HMEC1, cells from sixth passage had higher protein level of POT1 than cells of the tenth and eighth passage. Furthermore, no substantial difference in POT1 level was detected in HMEC2 p13, p15, and p17 (Figure 3.12).

The lack of correlation between protein and mRNA levels could be due to transcriptional splicing, post-transcriptional splicing, translational modifications, translational regulation, and protein complex formation, which might have an effect on the relative quantities of mRNA and protein (Hartwell, Hopfield et al. 1999; Brett, Pospisil et al. 2002; Brockmann, Beyer et al. 2007; Glisovic, Bachorik et al. 2008). Most research assumes that protein concentrations are generally proportional to mRNA concentration. However, since publication of the complete human genome sequence in 2004, many papers have proved this hypothesis wrong. For instance, Tian et al. (2004) mapped the abundance ratio of 425 proteins to their corresponding mRNA expression levels in multipotent mouse EML cells and their differentiated progeny, MPRO cells. Over all they identified 150 signature genes which showed significant alterations at their protein and/or mRNA level between the two cell types. In total 19% of genes showed reasonable correlation between mRNA and protein levels; 45% showed significant difference at the mRNA level but not at the protein level, 35% represented significant changes at the protein but not at the mRNA level. Surprisingly, the mRNA and protein levels were inversely correlated in two genes. Furthermore, the expression of the c-kit receptor kinase protein and its mRNA varied seven-
fold and nine-fold respectively between the two cell lines, while the c-kit ligand protein showed a five-fold higher expression level in the EML cell line with no change in the mRNA level. The expression levels of nine mitochondrial proteins were significantly lower in MPRO cell lines compared to EML cells, while the expression of their corresponding mRNA was higher or similar (Tian, Stepaniants et al. 2004). Going further, Schwanhausser et al. (2011) used NIH3T3 mouse fibroblast to analyse the correlation between expression levels of protein and mRNA (Schwanhausser, Busse et al. 2011). They quantified 5279 unique proteins and measured their half-lives along with their mRNAs. Consequently, they reported a median half-life of 46 hours for proteins and 9 hours for mRNA, hence showing proteins to be on average five times more stable than their mRNAs. Furthermore, they divided genes and proteins into three different groups based on their function and looked at their protein-mRNA stability. Looking at their results, the housekeeping genes (i.e., genes coding for ribosomal and glycolytic proteins) showed stable mRNA and protein. The chromatin modifying enzymes, cell-cycle associated genes, and transcription factors have unstable mRNA and proteins. The last group showed stable mRNA and unstable protein, comprising of RNA-processing proteins, genes encoding kinsases, proteases and integrin mediated pathways (Schwanhausser, Busse et al. 2011). Looking at these two papers and other published data, it is evident that only 40% of protein levels in cultured mammalian cells are correlated with mRNA levels. Also there is further evidence suggesting that the mRNA levels may be correlated with protein levels only in housekeeping genes with both stable mRNA and protein. On the other hand, the mRNA level may be a poor surrogate for protein levels in genes with stable mRNA and unstable protein e.g. transcription factors and genes
encoding proteases and kinases (Tian, Stepaniants et al. 2004; Lundberg, Fagerberg et al. 2010; Vogel, Abreu Rde et al. 2010; Schwanhausser, Busse et al. 2011).

The relationship described between mRNA and protein in these 4 papers is also evident in our results; the Shelterin protein encoding gene TPP1 had high mRNA levels and low protein levels in 21NT cells, suggesting more stable mRNA compared to protein. However, there is an expression pattern observed in our results which has not been observed and/or explained previously. Based on our results, the Shelterin protein encoding gene POT1 has low mRNA and high protein levels in 21NT cells (Figures 3.9 and 3.11). Looking at the control cell lines HMECs, the POT1 encoded protein level is less than that of the 21NT breast cancer cells. This suggests that POT1 encoded protein is more stable than its mRNA.

Previous work by Marks et al. (1991) found that a mutation in the p53 gene lead to high expression of the p53 protein in ovarian cancer cells (Marks, Davidoff et al. 1991). Therefore, it may be possible that the POT1 protein within 21NT cells is mutated and non-functional. This may explain the higher protein levels observed within 21NT breast cancer cells in comparison with HMECs. To test this hypothesis, further investigation were carried out to look at mutational and epigenetic changes involving the POT1 gene as described in the following chapter (V).
CHAPTER IV

EPIGENETIC REGULATION OF SHELTERIN AND SHELTERIN-ASSOCIATED GENES AS A MECHANISM FOR ALTERED EXPRESSION IN CANCER CELLS
4.1-Introduction

Down-regulation of some Shelterin genes and up-regulation of TPP1 in breast cancer cell lines could be due to a number of factors including mutation, DNA methylation, single allele deletions, chromatin remodelling, and haploinsufficiency of the genes. To address the question of whether mutations in Shelterin genes may cause down-regulation of their expression, the literature was consulted. Salhab et al. (2008) reported that POT1 was significantly down-regulated in malignant breast tissues in comparison with normal tissues. Moreover, according to our findings presented in the previous chapter (III), POT1 was one of the most down-regulated of the Shelterin genes in breast cancer cell lines. Therefore, this gene was selected for further investigation. Previous results demonstrated that human POT1 is most commonly mutated in a wide range of cancers, such as: papillary thyroid (Cantara, Capuano et al. 2012), breast (Shen, Gammon et al. 2010) and leukaemia (Poncet, Belleville et al. 2008). Further studies revealed single nucleotide polymorphisms (SNPs) of POT1 in breast cancer (Savage, Chanock et al. 2007). Moreover, mutation of POT1 was observed in approximately 5% chronic lymphocytic Leukaemia (CLL) (Ramsay, Quesada et al. 2013). In addition, mutations in POT1 exon12 were detected in human carcinoma cell strains (HeLa and HO8910-PM) (Hou, Huang et al. 2006). However, in 2009, according to the COSMIC database (Catalogue of Somatic Mutations in Cancer, Sanger centre UK http://www.sanger.ac.uk); no mutation was identified in the genomic sequence of the Shelterin genes, including the POT1 gene, in cancer. Therefore, based on the published data (Hou, Huang et al. 2006). It was important to screen the POT1 gene for existence of exon12 mutation in breast cancer cells.
Epigenetic modifications such as abnormal DNA methylation play an important role in cancer development, for example, by facilitating carcinogenesis and tumour apoptosis (Lund and van Lohuizen 2004). DNA hypermethylation is mediated by DNA methyltransferase enzymes (DNMTs) on cytosine residues in the 5'-CG-3' sequence. DNA hypermethylation within these regions inhibits gene expression via recruitment of repressive proteins such as: methyl-CpG binding protein 1 (MeCP1), MeCP2, and Methyl-binding domain proteins 1, 2, 3, and 4. Transcription is hindered by these proteins via the recruitment of the nucleosome remodelling complex (Mossman, Kim et al. 2010).

In chapter III we presented data that demonstrated Shelterin and Shelterin-associated genes were down-regulated in breast cancer cell lines compared with normal breast tissue and HMEC1 strain controls. A DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine (5-aza-CdR), and a histone deacetylation inhibitor, Trichostatin A (TSA), were used to demethylate DNA and deacetylate histones, respectively, and to induce the expression of silenced genes. It is well established that 5-aza-CdR can repress the growth of numerous tumours in vitro, including lung cancer, melanoma, and breast cancer (Mirza, Sharma et al. 2010) cells. Therefore, the effect of these drugs on Shelterin and Shelterin-associated genes was investigated in 21NT breast cancer cell line. Moreover, the effect of these drugs on cytosine methylation in the promoter region of POT1 was investigated.
4.2 - Materials and methods

4.2.1- Mutation at exon12 of POT1

DNA was isolated from breast cancer cell lines as previously described in Section 2.7. 50ng of double stranded DNA was utilised in experiments and a pair of primers was synthesized covering exon12 of POT1 gene (Table 4.1).

Table 4.1- Primer sequences of POT exon12

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’-3’)</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>POT1 Ex12 Forward</td>
<td>GCAAAAGGAGTATTCTAAACAAACAG</td>
<td>300</td>
<td>(Hou, Huang et al. 2006)</td>
</tr>
<tr>
<td>POT1 Ex12 Reveres</td>
<td>TCACGTCTACACAAATCG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PCR was performed using 1.1x Reddy Mix and 10μM of the Forward and Reverse primer, respectively. The reaction mixture volume was 25μl and was incubated in a thermo cycler at 94°C for 5 minutes, followed by 35 cycles of 94°C for 45s, 60°C for 45s, and 72°C for 45s and then a final extension at 72°C for 5 min.

4.2.1.1- PCR product purification using QIAquick™ PCR Purification Kit

Five volumes of PB buffer were added to one volume of PCR reaction and mixed thoroughly. The entire mixture was transferred to a QIAquick spin column placed in a 2ml collection tube and centrifuged for 1 minute at 16,000rcf. The flow-through was discarded and the QIAquick spin column was placed back in the same collection tube. About 750μl of PE wash buffer was added to the QIAquick column and centrifuged for 1 minute at 16,000rcf. The flow-through was also discarded and QIAquick column was placed back in the same collection tube. The column was centrifuged once more to remove residual ethanol. The QIAquick column was placed in a clean 1.5ml micro-centrifuge tube and 15μl of elution buffer EB (pH 7.0-8.5) was added to the centre of QIAquick column membrane and
centrifuged for 1 minute at 16,000rcf. All DNA sequencing reactions were carried out using 8µl DTCS Quick Start Master Mix (Beckman Coulter Kit), approximately 3µl of purified DNA, and 10µM of the Forward primer (Table 4.1). Amplification was carried out using a thermal cycler with the following conditions:

- 96°C – 20 seconds
- 50°C – 20 secs
- 60°C – 4 minutes

30 cycles

4.2.1.2-Precipitation of DNA from sequencing reactions

DNA was precipitated by adding, 2µl of Sodium Acetate (pH5.2), 2µl of 100mM Na2-EDTA (pH 8.0) and 1µl of 20mg/ml of glycogen to the sequencing reaction mix. Then 60µl of cold 95% ethanol was added to the samples. Samples were immediately centrifuged at 16000rcf for 15 minutes at 4°C. Then the DNA pellet was washed twice with 200µl of cold 70% ethanol. For each rinse, samples were centrifuged immediately at 16000rcf for 2 minutes at 4°C. After centrifugation, all of the supernatant was carefully removed and then allowed to completely evaporate at room temperature for 10 minutes. Samples were re-suspended in 40µl of sample loading solution (Beckman Coulter). The re-suspended samples were transferred to a 96-well plate and one drop of mineral oil (provided in the Kit) was added to each sample. The samples were loaded to the instrument to start sequencing.
4.2.2-Promoter methylation analysis of the POT1

In this technique, DNA is denatured and treated with sodium bisulphite. This causes unmethylated CpG dinucleotides in the promoter region of the genome to convert to uracils whereas methylated cytosines remain unchanged (Figure 4.1).

![Chemical schemes for the conversion of cytosine to uracil](http://www.methods.info/Methods/DNA_methylation/Bisulphite_sequencing.html)

**Figure 4.1-Chemical schemes for the conversion of cytosine to uracil.** Unmethylated cytosine residues converted to uracil after treatment with sodium bisulphite. After bisulphite PCR amplification, uracil residues converted to thymidine. Image obtained from http://www.methods.info/Methods/DNA_methylation/Bisulphite_sequencing.html website.

4.2.2.1-Bisulphite treatment of DNA samples

Genomic DNA was isolated from breast cancer cell lines, using the Wizard™ Genomic DNA Kit protocol (Promega) (see Section 2.7). Methylcode™ Bisulphite conversion Kit
(Invitrogen) was used for bisulphite treatment of DNA-samples. According to the manufacturer’s instructions, CT conversion reagent was prepared by adding 900µl of sterile water, 50µl of Resuspension Buffer, and 300µl of Dilution Buffer directly to one tube of CT Conversion Reagent. Then, the tube was mixed by vortexing for 10 minutes. A mixture of 2µg of genomic DNA samples (total volume 20µl) and CT conversion reagent mix (130µl) was added to the PCR tube. After thorough mixing, the bisulphite treatment was performed using a thermal cycler with the following conditions:

- 98°C for 10 minutes (DNA denaturing step)
- 64°C for 2 hours and 30 minutes (Bisulphite conversion step)
- 4°C storage for up to 20 hours

After completion of bisulphite treatment, the samples were cleaned up by placing them in collection tube and 600µl of Binding Buffer was added to each column. The mixture was inverted several times and centrifuged at full speed (≥9,000rcf) for 30 seconds. The flow-through was discarded and 100µl of wash buffer (prepared with ethanol) was added to the column followed by centrifugation at full speed for 30 seconds. After discarding the flow-through, desulphonation was performed by adding 200µl of desulphonation buffer and incubating columns for 15 minutes at room temperature. When the incubation was completed, the columns were centrifuged at full speed for 30 seconds. The flow-through was again discarded and the columns were washed twice with 200µl of wash buffer prepared with ethanol. To remove residual liquid, the columns were placed in new 2ml collection tubes after the second wash, and centrifuged at full speed for 30 seconds. The elution of bisulphite-treated DNA was performed by placing the columns in clean 1.5ml micro-centrifuge tubes and adding 16µl of Elution Buffer to the centre of the membrane.
The bisulphite-treated DNA was eluted by centrifugation at full speed for 30 seconds. The samples containing bisulphite-treated DNA were stored in -20°C until further processing.

4.2.2.2-Primer design for bisulphite sequencing

The bisulphite-treated DNA was amplified by PCR in which the primers were specifically designed for methylated and unmethylated DNA products. MethPrimer program was used to identify CpG islands within a given sequence and assist in designing methylated and unmethylated primers (Figures 4.2-A and 4.2-B).

![MethPrimer program for POT1 CpG Island](http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi)

**Figure 4.2-A** MethPrimer program for POT1 CpG Island. Thick horizontal “blue shaded area” indicates CpG Island with individual CpG dinucleotides (seventeen vertical red lines). To assist designing methylated and unmethylated primers, CpG Island Searcher program was used. Image obtained from [http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi](http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) website.
Figure 4.2.8) MethPrimer program for TIN2 CpG Island. Thick horizontal “blue shaded area” indicates CpG Islands with individual CpG dinucleotides (twenty vertical red lines). To assist designing methylated and unmethylated primers, CpG Island Searcher program was used for this purpose. Image obtained from http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi website.

The methPrimer program was used to identify 5' promoter regions of all six Shelterin genes. This website recognised the promoter regions of POT1, TIN2 and RAP1. To define the position of POT1 and TIN2 promoter region, approximately 700 base pair upstream of the first exon from http://www.ncbi.nlm.nih.gov/genome/ website was pasted into the http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi website. PCR-primers selected for each gene and its promoter region are presented in Table 4.2 below. The regions chosen for each gene were based upon the density of CpG-sites.
Table 4.2-MSP primer sequences for PCR-products

<table>
<thead>
<tr>
<th>Gene / Assay</th>
<th>Forward primer (5´→ 3´)</th>
<th>Reverse primer (5´→ 3´)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>POT1-M*</td>
<td>AGAAAGGTTTTTATAGGAGT</td>
<td>CCAATAACTTCCAACCTTCGTA</td>
<td>118</td>
</tr>
<tr>
<td>POT1-U*</td>
<td>AAGGTGTGTGTTAGAGTTTT</td>
<td>CCAATAACTTCCAACCTTCAT</td>
<td>116</td>
</tr>
<tr>
<td>TIN2-M*</td>
<td>AAAGTAGGTGGGAGGATTTAG</td>
<td>ACAAAAAAACGTAACGATACG</td>
<td>161</td>
</tr>
<tr>
<td>TIN2-U*</td>
<td>AAAGTAGGTGGGAGGATTTAG</td>
<td>ACCACACAAAAACACCATAACAT</td>
<td>164</td>
</tr>
</tbody>
</table>

*Methylated, *Unmethylated

4.2.2.3-Detection of DNA methylation in POT1 promoter region

In order to generate the 118 base pair and a 116 base pair methylated and unmethylated product, PCR was performed using 1.1x Reddy Mix, 1µg (2µl) of DNA (see Section 4.2.2.1), 25µM of the Forward and Reverse primer, respectively. A negative control was included in every PCR performed. The reaction mixture volume was 25µl and was incubated in a thermo cycler at 94°C for 5 min, followed by 50 cycles of 94°C (denaturation) for 45s, 56°C for 45s (annealing), and 72°C for 45s (extension) and then a final extension at 72°C for 5 min. Then, the PCR products were purified as mentioned in Section 4.2.1.1.

4.2.2.4-Sequencing and analysis of POT1 data

The concentration of DNA was adjusted and the methylated and unmethylated PCR products were sent for sequencing (Beckman Coulter Company).
4.2.3-Effects of 5-aza-CdR and TSA on 21NT breast cancer cell line

4.2.3.1-Drug optimization and cell viability assay (Trypan Blue Assay)

Careful preliminary experiments were performed to determine the optimal drug doses. Briefly, $3 \times 10^5$ cells were seeded into 6-well plate to a total volume of 3ml media per well. Each experiment was carried out in duplicate with different drug concentrations. Twenty-four hours after cell plating, the cells were randomly assigned into control, TSA (Sigma biochemical), 5-aza-CdR (Sigma, St. Louis, MO) and mixed 5-aza-CdR/TSA treatment groups. TSA and 5-aza-CdR were dissolved in dimethylsulphoxide (DMSO). Aliquot of stock solution of 5-aza-CdR (5µM) and TSA (5, 15, 25, 50, and 100ng/ml) were prepared and stored at -20°C. The culture medium was replaced with culture medium containing 5, 15, 25, 50, and 100ng/ml of TSA. Following optimization, cells were treated with the same concentrations of TSA, followed by the addition of 5µM of 5-aza-CdR (was optimized by Dr H Yasaei, personal communication). Cells that were treated with 5-aza-CdR and TSA at different concentrations were cultured and trypsinized with trypsin/EDTA and a cell suspension was made with Alpha modification MEM medium. Then the cell suspension was taken into a fresh Eppendorf tube and stained with Trypan blue (Invitrogen). Stained blue cells were scored as dead cells and unstained bright cells as viable cells. The stained cells were then counted immediately under a microscope by using a haemocytometer and their viability was determined by the following formula:
4.2.3.2-Cell culture, maintenance and treatment

The 21NT cell line was cultured at 37 °C, 5 % CO₂ as previously described. Cells were plated at a density of 1x10⁵ in p100 dish. Twenty-four hours after cell plating, the cells were randomly assigned into treatment and control groups. In treatment groups, the culture medium was replaced with fresh medium containing TSA (50ng/ml) or 5-aza-CdR (5µM) separately. Cells were also treated with both drugs concomitantly. Thereafter, 5µM 5-aza-CdR was added for 48 hours, TSA (50ng/ml) added only during the last 16 hours of treatment. For control groups, two plates were treated with and without 0.02% DMSO. The purpose of these treatments was based on the published data (Pryzbylkowski, Obajimi et al. 2008). Moreover, the short-term and long-term effects of the drugs on the mRNA levels of Shelterin and Shelterin associated genes were examined in order to compare the difference between each time points of the treatment. At the end of the experiment, four groups of cells were treated for: 24hrs, 48hrs, 72hrs, 96hrs, 120hrs, 144hrs, 7 days, 3 weeks, 6 weeks and 2 month plus 72 hours retreatment period. At each time point, samples were taken for further experiments. Twenty-four hours later, the medium containing drugs or DMSO-treated was removed and replaced with fresh media. After 2 months of treatment, the cells were retreated again with the same concentrations of 5-aza-CdR and TSA for 72 hours in
order to examine the effects of a double-dose treatment on 21NT cells. Then cells were harvested for RNA, DNA and protein extraction.

4.2.4-RNA isolation and quantitative RT-PCR analysis

RNA was extracted by Trizol reagent (Invitrogen) as described in Section 2.2.2 after different time point treatment of the 21NT cells. Invitrogen SuperScript III Reverse Transcriptase was used to synthesize cDNA. The forward and reverse primers sequences of all Shelterin genes and amplification length are detailed in Table 2.2. qRT-PCR was performed using SYBR green PCR Master Mix (Applied Biosystems). All of the PCR reactions were performed in triplicate and independently repeated three times depending on the experiment. Results are shown as SEM and significance was calculated by using paired t-test. A p value of less than 0.05 was considered significant.
4.3-Results

4.3.1-Mutation analysis of POT1 in breast cancer cell lines

Previous work by Hou, Huang et al. (2006) showed that POT1 gene within the exon12 region was mutated in human carcinoma cell lines. Therefore, mutation in the POT1 exon12 gene was screened in ten breast cancer cell lines. The PCR products on 2% agarose gel showed an intense band between 200bp and 300bp for all human breast cancer cell lines which was consistent with the product size. After checking the product size (Figure 4.3), the exon12 PCR product of POT1 was sequenced in Beckman Coulter (CEQ™8000 Genetic Analysis System) machine to detect the presence of mutations in all the lines. The results revealed no evidence of mutations in any of the breast cancer cell lines within the exon12 (data not shown, see also Figure S1).

Figure 4.3-The PCR Results of POT1 Exon12 in cancer and normal (HMEC1) cell lines. Representative agarose electrophoresis image of POT1 exon12 PCR. Each lane indicates breast cancer cell lines and HMEC1 strain.
4.3.2-Detection of DNA methylation of POT1 and TIN2 promoter regions in breast cancer cell lines

Based on the results presented in Chapter III showing down-regulation of Shelterin genes in certain breast cancer cell lines, it is possible that the expression of these genes has been modulated by DNA methylation. To confirm this hypothesis we set out to identify evidence of DNA methylation in the promoter of POT1 and TIN2 (in the normal mammary epithelial cell strains (HMEC1) and the breast tumour cell lines, 21NT, BT474, BT-20, MCF-7, GI101, 21MT2, PB1 and HS578-T). To this end the methPrimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) program was used to identify the 5' promoter regions of all six Shelterin genes. This website recognised the promoter regions of POT1, TIN2, and RAP1.

In this section, we only analysed the promoter region of POT1 and TIN2 as these two genes demonstrated a significant up-regulation of gene expression following 5-aza-CdR and 5-aza-CdR/TSA treatment in comparison with the other Shelterin genes (Section 4.3.5). The promoter regions of POT1 and TIN2 genes were analysed in all untreated (i.e., not exposed to 5-aza-CdR and TSA) breast cancer cell lines. As shown in Figure 4.4, partial methylation was present in POT1 and TIN2 promoter regions in breast cancer cell lines. The results indicated that the POT1 promoter contains more methylated DNA in breast cancer cells 21NT, BT474, 21MT-2 and PB1. These cell lines also have unmethylated DNA. This is based on the level of intensity of the PCR band on the agarose gel. Moreover, BT20, GI101, and HS578-T have more unmethylated DNA which is consistent with the control (HMEC1). More evidence of POT1 methylation in 21NT, BT474, 21MT-2, and PB1 cells in comparison with
HMEC1 was observed. However, no significant differences of TIN2 were observed in methylated and unmethylated lanes (Figure 4.4).

Figure 4.4-Methylation Specific PCR (MSP) analysis of POT1 (A) and TIN2 (B) genes in breast cancer cell lines and normal mammary epithelial cell strain (HMEC1). A) POT1 panel viewed from left to right in breast cancer cell lines. Lane 1 is the ladder, M specifies the presence of methylated DNA, and U demonstrates unmethylated. B) TIN2 panel viewed from left to right shows a ladder, the presence of methylated and unmethylated DNA in breast cancer cell lines.
4.3.3-Sequence analysis of POT1 promoter region in breast cancer cell lines

As described in the previous section (4.3.2), POT1 appeared to be more methylated in 21NT and 21MT-2 cell lines in comparison with HMEC1 control. In contrast, the GI101 showed a stronger unmethylated signal in the same promoter region of POT1. Therefore, these cell lines were examined more in detailed for POT1 promoter methylation. To identify precisely where in the promoter region of POT1 methylation occurs in breast cancer cell lines and normal epithelial cell strains. Genomic DNA from 21NT, 21MT-2, GI101, and HMEC1 were prepared for bisulphite sequencing.

A fragment with a length of 118 base pairs from the POT1 gene promoter region was utilized for the bisulphite direct sequencing. For this purpose, breast cancer cell lines (21NT, 21MT-2, and GI101) and the normal epithelial cell strains (HMEC1) were sequenced. Figure 4.5 indicates that 21MT-2 and GI101 breast cancer cell lines were unmethylated in the upstream promoter region of the POT1 CpG Island. Bisulphite sequencing also showed that the methylation ratio in 21NT was about 30%, which showed frequent methylation in the amplified region. However, no methylation in this region was observed in HMEC1 control (Figure 4.5). The sequencing chromatograms also showed that all of the CpG Island in 21MT-2 and GI101 was unmethylated, as about all cytosine was converted to thymidine (Figure 4.5) (Figure S2). As shown in Figure 4.6, 11 possible CpG sites upstream of POT1 exon1 were analysed. The degree of hypermethylation varied from 3/11 CpG sites in 21NT to 0/11 CpG sites in 21MT-2, GI101, and the HMEC1 control.
Figure 4.5- *POT1* methylation analysis of the normal mammary epithelial cell strain (HMEC1), 21NT, 21MT-2 and GI101. **A)** The top panel is upstream promoter region in 21NT cells. **B)** The second panel is 5’ CpG Island in HMEC1. **C)** The third panel is upstream promoter region in GI101 and **D)** is 5’ CpG Island in 21MT-2 using bisulphite sequencing directly from PCR products. The comparison is based on the percentage of CpG Islands. The chromatogram shows the location of CpG Islands which appears to be partially methylated in 21NT cell line whereas no CpG is methylated in the *POT1* promoter region of HMEC1 cell strain, 21MT-2 and GI101 breast cancer cells. Each colour represents nucleotides T: thymidine, C: cytosine, G: guanine. Arrows indicate CpG dinucleotide which is methylated and the rest remained unmethylated.
Figure 4.6-CpG methylation status of the POT1 promoter. About 118 base pair upstream of exon1 was analysed by bisulphite sequencing. The POT1 promoter region was identified and the rectangular area represents CpG regions. Three breast cancer cell lines and HMEC1 cell strains control were analysed. The black symbols show methylated CpGs and white symbols sites of unmethylated CpGs.
4.3.4-Attempts to reactivate full \textit{POT1} expression by treatment of 21NT breast cancer cells with TSA and 5-aza-CdR

4.3.4.1-Optimization of TSA and 5-aza-CdR concentrations on 21NT cells

The effect of 5-aza-CdR and TSA concentrations on \textit{POT1} expression in 21NT cells was investigated as it was found to be down-regulated in 21NT cancer cells (Chapter III). In this way, the optimization of 5-aza-CdR and TSA was carried out. Figure 4.7-B shows that there is seemingly a very gradual dose dependent increase up to a threshold of 50ng/ml followed by an abrupt step-change between 50 and 100ng/ml. Significantly increased expression levels of \textit{POT1} were observed at the highest concentration of TSA (100ng/ml). However, cell viability was reduced to 61% with single-agent treatment (TSA) at the highest dose (100ng/ml) when compared with untreated control (Figure 4.7-A). As shown in Figure 4.8-A, treatment with TSA at 50 and 100ng/ml with 5µM 5-aza-CdR increased the percentage of cell survival compared with TSA alone (Figure 4.7-A). The highest up-regulation levels of \textit{POT1} were observed at the concentration of 50 and 100ng/ml of TSA with 5µM 5-aza-CdR (Figure 4.8-B). Reduced cell survival after treatment with the highest concentration of TSA indicates cytotoxicity (an inhibition of cell growth /division and all death). The results showed an average cell viability and maximum expression of \textit{POT1} at TSA concentration of 50ng/ml along with 5µM 5-aza-CdR in 21NT cells. Therefore, this suggests that 50ng/ml was the best concentration of TSA to be used.
Figure 4.7-The changes in cell viability (A) and expression level of \textit{POT1} (B) after treatment with TSA. The 21NT cell line was treated with TSA for 48 hours at different concentrations. A) The cell viability assay was performed to determine cell survival. B) qRT-PCR results show changes in \textit{POT1} expression levels in 21NT cell after treatment with TSA at different concentrations. Untreated 21NT was used as the calibrator.
Figure 4.8-The changes in breast cancer cell viability (A) and expression level of POT1 (B) after treatment with TSA and 5-aza-CdR. 21NT cells was treated with TSA at different concentration and with a single concentration (5µM) of 5-aza-CdR. A) The cell viability assay was performed to determine cell survival. B) qRT-PCR results show changes in POT1 expression levels in 21NT cells after treatment with TSA at different concentrations and 5-aza-CdR.
4.3.4.2- Effect of TSA and 5-aza-CdR in 21NT cells at different time points

To determine the effect of TSA and 5-aza-CdR on the 21NT cell lines, cells were treated with both drugs individually or together at different time points (Table 4.3). Since the first replication takes 24 hours, it was decided to compare POT1 gene expression at 24 and 48 hours to examine the effect of each drug. Cells were treated with two different concentrations of TSA (50 and 100ng/ml) whereas the concentration of 5-aza-CdR was kept constant at 5µM throughout the experiment. In treatments 1-4 (Table 4.3), 21NT cells were treated twice with TSA and 5-aza-CdR with 8 hours gap between each treatment (Ghoshal, Datta et al. 2005). The cells were collected either at 24 (1 and 2) or 48 hours post treatment (3 and 4) for further analysis. In treatments 5-8, 21NT cells were treated twice with TSA and 5-aza-CdR with 4 hours gap between each treatment (Ghoshal, Datta et al. 2005). The cells were collected either at 24 (5 and 6) or 48 hours post treatment (7 and 8) for further analysis. In treatments 9 and 10 21NT cells were treated with 5µM 5-aza-CdR for 48 hours. TSA at concentrations of 50ng/ml and 100ng/ml, were added to the cell cultures (9 and 10 respectively) for the last 16 hours of treatment (Pryzbylkowski, Obajimi et al. 2008; Mirza, Sharma et al. 2010). As depicted in Figure 4.9, treatment 9 consisting of 5µM 5-aza-CdR and 50ng/ml of TSA (for the last 16 hours of treatment) had the highest effects on mRNA expression levels of POT1. Therefore, 21NT cells were treated with the same regimen for further analysis of effect on other Shelterin and Shelterin-associated genes.
Table 4.3 - Regimens for treating 21NT cells with different concentration of TSA (50 or 100ng/ml) and 5µM of 5-aza-CdR at different time points

<table>
<thead>
<tr>
<th>No of Experiment</th>
<th>TSA Concentration (ng/ml)</th>
<th>5-aza-CdR Concentration(µM)</th>
<th>Time in Culture treated with TSA/5-aza-CdR</th>
<th>Total Time in Culture</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>50</td>
<td>5</td>
<td>8h twice</td>
<td>24h</td>
</tr>
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<td>2</td>
<td>100</td>
<td>5</td>
<td>8h twice</td>
<td>24h</td>
</tr>
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<td>3</td>
<td>50</td>
<td>5</td>
<td>8h twice</td>
<td>48h</td>
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<tr>
<td>4</td>
<td>100</td>
<td>5</td>
<td>8h twice</td>
<td>48h</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>5</td>
<td>4h twice</td>
<td>24h</td>
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<td>5</td>
<td>4h twice</td>
<td>48h</td>
</tr>
<tr>
<td>9</td>
<td>50</td>
<td>5</td>
<td>48h with AZA*</td>
<td>48h</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>5</td>
<td>48h with AZA</td>
<td>48h</td>
</tr>
</tbody>
</table>

*AZA: 5-aza-CdR

Figure 4.9 - Effect of 5-aza-CdR and TSA on expression levels of POT1. The qRT-PCR results show changes in POT1 expression levels in 21NT cells after treatment with 50 and 100ng/ml of TSA with 5-aza-CdR at different time points. Untreated 21NT cells were used as the calibrator.
4.3.5-Up-regulation of Shelterin and Shelterin-associated genes by 5-aza-2’-deoxycytidine (5-aza-CdR) and Trichostatin A (TSA)

To further understand the mechanisms controlling epigenetic regulation of Shelterin genes in breast cancer cells, the effects of 5-aza-CdR and TSA individually and 5-aza-CdR/TSA together on all Shelterin and Shelterin-associated gene expression were investigated in 21NT cells after 48 hours treatment (Figure 4.10). These cells were chosen for analysis because they were available at an earlier passage compared to the other breast cancer cell lines included in this study.

Figure 4.10-Representative images showing an example of individual treatment of 21NT cells with 5-aza-CdR and TSA at x5 magnification. A) 21NT cells containing 0.02% DMSO-treated control and cultured for 48 hours, B) 21NT cells containing 5µM 5-aza-CdR and cultured for 48 hours, C) 21NT cells containing 5µM 5-aza-CdR and 50ng/ml TSA cultured for 48 hours, D) 21NT cells containing 50ng/ml TSA cultured for 48 hours. Each treatment of 21NT cells appears to have rounded cell shape and no difference between each treated cells shape have been observed.
To explore whether the down-regulation of genes encoding members of the Shelterin and Shelterin-associated genes observed in this study, is due to DNA or histone modifications, the 21NT cells was treated with 5-aza-CdR and TSA. Therefore, demethylation of a gene by exposure to 5-aza-CdR and or suppression of the activity of HDAC by TSA should result in removal of a mechanism which should in theory lead to restoration of normal expression the relevant Shelterin and Shelterin-associated genes.

The expression of Shelterin and Shelterin-associated genes was detected using qRT-PCR normalised with endogenous GAPDH. Treatment of 21NT cells in combination with 5-aza-CdR and TSA, lead to increased expression of POT1 and TIN2 (Figures 4.11-A and 4.13-A) as compared with the DMSO sample (p<0.05). In contrast, the up-regulation of TNKS2, TRF1, TRF2, and RAP1 did not reach the statistical significance (Figures 4.11-B, 4.12-A, 4.12-B, and 4.13-B). However, 21NT cells treated solely by TSA, did not show up-regulation of TNKS2, TRF2 and RAP1 (Figures 4.11-B, 4.12-A, 4.13-B). Treatment with 5-aza-CdR alone resulted in up-regulation of all the above genes (except TRF1) and there was a significant up-regulation of POT1, TIN2 and TPP1 mRNA in 5-aza-CdR/TSA and 5-aza-CdR treated samples (p<0.05) (Figures 4.11-A and 4.13-A, 4.14-A). In addition, in the previous results Chapter (III), it was evident that TPP1 was over-expressed in 21NT cells in comparison with the HMEC1 (Figure 3.10). However, these trends were not statistically significant. The result in this chapter showed that the expression of TPP1 after 48 hours of treatment with 5-aza-CdR increased by 4-fold when compared with DMSO and untreated controls (p<0.05) (Figure 4.14-A).
Figure 4.1-Eff ects of 5-aza-CdR and TSA on the expression of POT1 and TNKS2 in the 21NT breast cancer cell line. qRT-PCR analysis of POT1 (A) and TNKS2 (B) mRNA levels following 5-aza-CdR and TSA treatments in 21NT cells for 48 hours. Expression of each gene values were normalised to GAPDH mRNA level. DMSO control was used as the calibrator. RQ and error bars indicate relative quantification and SEM. Asterisk indicates significant difference between 21NT treated cells and DMSO-control (*P<0.05).
Figure 4.12-Effects of 5-aza-CdR and TSA on the expression of TRF1 and TRF2 in the 21NT breast cancer cell line. The image (A) and (B) represent qRT-PCR analysis of TRF1 and TRF2 mRNA isolated from 21NT treated with 5-aza-CdR and TSA for 48 hours. TRF1 and TRF2 expression were normalised to the expression of the GAPDH. DMSO control was used as the calibrator. RQ and error bars indicate relative quantification and SEM.
Figure 4.13-Effects of 5-aza-CdR and TSA on the expression of TIN2 and RAP1 in the 21NT breast cancer cell line. The graphs (A and B) represent analysis of mRNA from the 21NT treated cells with qRT-PCR to detect the TIN2, RAP1 and GAPDH mRNAs. DMSO control was used as the calibrator. Error bars represent SEM. Asterisk indicates significant difference between 21NT treated cells and DMSO-control (*P<0.05).
Previous results demonstrated a significant increase in the mRNA levels of *TPP1* in 5-aza-CdR treated samples (*p*<0.05) after 48 hours (Figure 4.14-A). Therefore, 21NT cells were also treated by 5-aza-CdR and TSA individually and in combination with 5-aza-CdR and TSA, for 72 hours or 3 weeks. The long-term (3 weeks) and short-term (72 hours) effect of drugs on levels of TPP1 protein in cultured cells were analysed by western blot. In all samples TPP1 was detectable as a 58 KDa (rabbit polyclonal antibody Abcam) protein (Figures 4.15-A and 4.15-B). The amount of TPP1 protein in 21NT cells treated with 5-aza-CdR/TSA for 72 hours was increased in comparison with the DMSO-control (Figures 4.15 A-B). Thus the protein level of TPP1 was consistent with the qRT-PCR results (Figures 4.14-A and 4.15 A-B). However, the protein level of TPP1 in 21NT cells treated with 5-aza-CdR alone (short-term)

![Figure 4.14](image)

**Figure 4.14-Expression of TPP1 in 21NT treated cell line determined by qRT-PCR. A)** The graph represents analysis of transcription level of *TPP1* from the indicated 21NT treated cells with 5-aza-CdR and TSA for 48 hours. All mRNA levels were normalized to the level of *GAPDH* mRNA. Error bars represents SEM. DMSO control was used as the calibrator. Asterisk indicates significant difference between 21NT treated cells and DMSO-control (*p*<0.05).
was inconsistent with mRNA results. Also, no considerable difference in TPP1 protein level was observed in long-term treated sample compared with short-term treatment (Figure 4.15 B-C).

**Figure 4.15-Western blot analysis of TPP1 in 21NT treated cells with 5-aza-CdR and TSA. B) A 12% SDS-PAGE gel indicating TPP1 protein expression and β-Actin ratio levels as a lane loading control in DMSO, 5-aza-CdR and 5-aza-CdR/TSA treatment of 21NT cells for 72 hours and 3 weeks. C) Densitometric analysis of TPP1 protein normalised to total β-Actin protein and reported as optical densitometry (OD) units.**
4.3.6-Effects of prolonged treatment of 21NT cells with 5-aza-2'-deoxycytidine and Trichostatin A on transcription level of Shelterin genes

As discussed in Section 4.3.5, 5-aza-CdR, TSA and 5-aza-CdR/TSA treatment after 48 hours had differential effects on Shelterin and Shelterin-associated gene expression levels. The preliminary results showed significantly increased expression of POT1 and TIN2 mRNA ($p<0.05$) following 5-aza-CdR and 5-aza-CdR/TSA treatment in the absence of consistent promoter DNA demethylation and histone deacetylation (Figures 4.11-A and 4.13-A). Therefore, we sought to investigate long-term treatment of 21NT cells to identify whether the expression of these genes may possibly change after longer period of exposure. We

![Figure 4.16.A-Expression of POT1 in 21NT treated cells at different time points.](image)

The graph represents analysis of mRNA from the indicated 21NT treated cells by qRT-PCR. 0.02% of DMSO-treated (control) was used as the calibrator. Error bars represent SEM. Asterisk indicates significant differences between 21NT treated cell lines and DMSO-control ($^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).
examined the expression of POT1 and TIN2 by 50 ng/ml TSA and 5 μM 5-aza-CdR treatments for 48h, 72h, 96h, 120h, 144h, 7 days, 3 weeks, 6 weeks and 2 months plus retreatment for 72 hours in 21NT cells. Analysis of each of these genes employed gene-specific primers see Table 2.2. The expression levels of the genes were assessed by qRT-PCR. qRT-PCR was performed in triplicate for each of the cDNA pools. The data showed that treatment of 21NT cells significantly increased transcription levels of POT1 and TIN2 upon on treatment with TSA, 5-aza-CdR, and 5-aza-CdR/TSA after different time points. Figure 4.16 shows the expression of POT1 after 72 hours of treatment with TSA and 5-aza-CdR/TSA was over four-fold higher in the 21NT cells treated than in 21NT untreated cells (P<0.05, P<0.01 and

![Figure 4.16.B-Expression of POT1 in 21NT treated cells determined by qRT-PCR.](image)

**Figure 4.16.B-Expression of POT1 in 21NT treated cells determined by qRT-PCR.** B) The graph represents analysis of transcription levels of POT1 from the indicated 21NT treated cells with 5-aza-CdR and TSA at different time points. All mRNA levels were normalized to the level of GAPDH mRNA. Error bars represent SEM.
In addition, treatment of 21NT cells with 5-aza-CdR and TSA alone, and in combination with 5-aza-CdR and TSA for 48 hours resulted in significant up-regulation of POT1 ($P<0.01$ and $P<0.001$ correspondingly). Nevertheless, no substantial differences in expression of POT1 has been observed in 96, 120, 144 hours and 7 days of treatment (Figures 4.16-A and 4.16-B). In addition, the biphasic response of POT1 and TIN2 gene expression was seen with an optimal peak at 72 hours, which then declined and the expression was increased significantly again at 3 weeks treatment (correlated with telomere length see Chapter V).

To observe the effects of 5-aza-CdR and TSA on protein levels of POT1 in cultured cells, POT1 protein expression in control (DMSO-treated) and treated cells (5-aza-CdR and TSA) were analysed by western blotting. In all samples, POT1 was detectable as a 71 KDa (rabbit polyclonal antibody, Abcam) protein (Figure 4.16-C). Interestingly, combined treatment with 5-aza-CdR and TSA for 72 hours increased the levels of POT1 protein compared with the single treatment with 5-aza-CdR which was consistent with the qRT-PCR results (Figure 4.16-D). Moreover, less POT1 protein was also expressed in 21NT cells after 3 weeks treatment relative to the DMSO control. The combined use of 5-aza-CdR and TSA for 72 hours treatment had more effect on POT1 protein expression than with 3 weeks of treatment.
Figure 4.16 C and D-Western blot analysis of POT1 in 21NT treated cell line with 5-aza-CdR and TSA for 7 days and 3 weeks. C) 5-aza-CdR and 5-aza-CdR/TSA treatment increased POT1 protein levels in 21NT cells. High levels of POT1 (71 KD) protein was detected by western blotting following 5-aza-CdR and 5-aza-CdR/TSA treatment of 21NT cells for 72 hours. β-Actin utilized as a lane loading control. D) Densitometric analysis of POT1 protein normalised to total β-Actin protein and reported as optical densitometry (OD) unites.

As shown in Figures 4.16-A and 4.16-B, POT1 mRNA levels up-regulated in 5-aza-CdR/TSA treated samples after 6 weeks and 2 month treatments. However, these trends did not reach a statistical significance. Following treatment of 21NT cells with 5-aza-CdR and TSA at different time points, the data suggested that 5-aza-CdR and the combination...
treatment of 5-aza-CdR and TSA induced up-regulation of Shelterin genes. Therefore, it is assumed that Shelterin genes are at least in part down-regulated by DNA methylation.

Treatment of 21NT cells also increased mRNA levels of *TIN2* after different time points of treatment. As shown in Figures 4.17-A and 4.17-B, the use of 5-aza-CdR for 120, 144 hours and combined treatment with 5-aza-CdR and TSA for 48, 72, 120, and 144 hours, significantly activated re-expression of *TIN2* in 21NT cells (*P*<0.05 and *P*<0.01 respectively).

![Graph](image)

**Figure 4.17.A-Expression of TIN2 in 21NT treated cells at different time points.** The graph indicates analysis of mRNA from the indicated 21NT treated cell line by qRT-PCR to detect the *TIN2* and *GAPDH* mRNAs. DMSO control was used as the calibrator. Error bars represent SEM. Asterisk indicates significant differences between 21NT treated cell lines and DMSO-control (*P*<0.05, **P*<0.01).
Similar findings were also detected with combined treatment of the drugs after 3 weeks (correlated with telomere length see Chapter V). As evident in Figures 4.17-A and 4.17-B, TIN2 mRNA levels were significantly up-regulated in 5-aza-CdR/TSA treated samples ($P<0.05$). Nevertheless, no considerable difference in expression of TIN2 has been observed in 6 weeks and 2 month retreated samples (Figures 4.17-A and 4.17-B).

**Figure 4.17.B-Expression of TIN2 in 21NT treated cells at different time points.** The graph indicates analysis of mRNA from the indicated 21NT treated cell line by qRT-PCR to detect the TIN2 and GAPDH mRNAs. DMSO control was used as the calibrator. Error bars represent SEM.
4.3.7-**POT1** methylation analysis on genomic DNA of 21NT treated cells

In an effort to investigate whether 5-aza-CdR effects on cytosine methylation levels in the promoter region of **POT1**, the genomic DNA of 21NT treated with DMSO and 5-aza-CdR for 72 hours was examined. As before, a 118 base pair fragment with the length of from the **POT1** promoter region was utilized for the bisulphite direct sequencing method. The 12 possible CpG regions in the promoter region of **POT1** were identified. The results showed that the **POT1** CpG Island in DMSO control was partially methylated whereas 5-aza-CdR treatment had reduced CpG Island methylation at the promoter region (Figure 4.18) (Figure S3). In three of the 12 CpG sites, there was methylation in the DMSO control whereas no methylation was observed after treatment with 5-aza-CdR treatment (Figure 4.19). These data again strongly suggest that **POT1** promoter region at least partly under the control of DNA methylation in 21NT cells.

**Figure 4.18**-Interpretation of methylation sequencing of **POT1** promoter region. A) The upstream promoter region of 21NT cells treated with DMSO B) 21NT cells treated with 5-aza-CdR for 72 hours using bisulphite sequencing directly from PCR products. Chromatograms show methylation regions of 21NT treated at different time points. Arrows indicate a CpG methylated dinucleotide and the rest remained unmethylated. The C-picks indicate cytosine which is unmethylated in 21NT treated cells, the T-peaks show thymidine, the A-picks show adenine and the G-picks show guanine.
Figure 4.19-CpG methylation status of *POT1* promoter in 21NT breast cancer cells before and after treatment with 5-aza-CdR. About 118 bp upstream of exon1 was analysed by bisulphite sequencing. One CpG promoter region was identified and the rectangular area represents CpG regions. 21NT cells treated with 5-aza-CdR and DMSO control for 72 hours. The black symbols show methylated CpGs and white symbol sites of unmethylated CpGs.
4.4-Discussion

The previous chapter (III) described results from this project providing evidence for altered expression of certain Shelterin genes in breast cancer cell lines. Two main ways in which gene expression can be altered is through mutation or epigenetic modifications. According to the 2009 COSMIC database (Catalogue of Somatic Mutations in Cancer, Sanger Centre UK http://www.sanger.ac.uk) no mutation was identified in the genomic sequence of any Shelterin genes, including POT1 in breast cancer cell lines. However, currently (2013), the COSMIC database shows over 127 somatic mutations in the POT1 gene in different cancers, such as breast, skin, and cervix. In addition, Ramsay et al. (2013) showed that POT1 is commonly mutated in multiple myeloma, breast, lung, squamous cells, and hepatocellular carcinomas. However, at the time this project started, only one somatic mutation in exon12 of POT1 had been discovered in HeLa and HO8910-PM cells (Hou, Huang et al. 2006). Based on these findings, genomic DNAs were isolated from the breast cancer cell lines and DNA sequencing of exon12 was carried out to identify a possible genetic mechanism that might lead to reduction of POT1 expression in these cell lines. Sequence analysis of exon12 showed that no mutation was present in the panel of 10 breast cancer cell lines studied in this project. However, it remains possible that mutations exist in other exons of POT1. It was not possible to sequence all exons of the POT1 gene in all breast cancer cell lines due to lack of time and resources.

Gene expression can be regulated both by DNA methylation and by histone modifications (such as acetylation, methylation, phosphorylation, and ubiquitination) through specific chromatin modifying enzymes (Jaenisch and Bird 2003; Bannister and Kouzarides 2011). In order to account for the observed reduction in Shelterin gene
expression, these genes may possibly be silenced by DNA methylation in some breast cancer cells. Abnormal DNA methylation and histone modification are thought to play an essential role in carcinogenesis and apoptosis (Kondo, Shen et al. 2003). It has been previously reported that the combined treatment of breast cancer cell lines with 5-aza-CdR and TSA resulted in alteration of the expression level of estrogen receptor alpha (ER) (Pryzbylkowski, Obajimi et al. 2008). In addition, findings by Meng et al. (2008) showed that treatment of an ovarian cancer cell line with 5-aza-CdR and TSA resulted in DNA demethylation of \textit{hMLH1} gene (Meng, Dai et al. 2008).

The work described in this chapter, a powerful DNA methylation inhibitor, 5-aza-CdR, and a histone deacetylase inhibitor, TSA, used to address the possible mechanism for down-regulation of Shelterin and Shelterin-associated genes in 21NT breast cancer cells. The qRT-PCR results revealed that Shelterin genes were transcriptionally repressed through methylation and/or deacetylation as their mRNA expression was up-regulated in 21NT cell lines after 48 hours treatment with the drugs (Figures 4.11, 4.12, 4.13 and 4.14-A). The mRNA expression of \textit{POT1}, \textit{TIN2}, and \textit{TPP1} was significantly increased in treated 21NT cells compared with untreated and DMSO-treated controls \((P<0.05)\). The transcription levels of \textit{TNKS2}, \textit{TRF1}, \textit{TRF2} and \textit{RAP1} appeared higher in 21NT cells after treatment (compared with untreated and DMSO controls). However, these trends did not reach a statistical significance. With TSA as a single agent approximately a 2-fold up-regulation in \textit{TRF1}, \textit{RAP1} and \textit{TPP1} was observed in comparison with untreated and DMSO-treated controls. Nevertheless, no substantial up-regulation of \textit{TNKS2} and \textit{TRF2} were observed after TSA treatment. This suggests that \textit{TRF2} and \textit{TNKS2} genes are marginally up-regulated by promoter demethylation rather than histone modifications (Figures 4.11, 4.12, 4.13 and
4.14-A). With the other Shelterin genes taken together, the results in this section indicated that Shelterin and Shelterin-associated genes were up-regulated due to the synergistic effects of 5-aza-CdR in combination with TSA treatment at 48 hours in 21NT cells. Based on the preliminary results, a significant increase in the expression of POT1 and TIN2 ($p<0.05$) could be observed following 5-aza-CdR and 5-aza-CdR/TSA treatment in 21NT cells. The effect of drugs at different time points was examined in order to investigate the expression of POT1 and TIN2 genes affected by 5-aza-CdR and 5-aza-CdR/TSA treatment of 21NT cells. To evaluate changes in these genes, the expression levels of POT1 and TIN2 were compared at each different time points ranging from 48 hours to 2 month treatment. The results showed that 5-aza-CdR and TSA only affected TIN2 and POT1 mRNA levels at short-term (48 and 72 hour) and 3 weeks exposure. In addition, the biphasic response of POT1 and TIN2 gene expression was seen with an optimal peak at 72 hours, which then declined and the expression was increased significantly again at 3 weeks treatment (correlated with telomere length see Chapter V). Nonetheless, no considerable differences in the expression of these genes were observed in 2 months cells retreated for 72 hours. Collectively the results indicated that 5-aza-CdR and TSA lose their effectiveness at the longest time points of treatment (6 weeks and 2 month retreatment) (Figures 4.16 and 4.17). Therefore, treatment is not permanent and reversible.

The effects of 5-aza-CdR and TSA treatment on 21NT cells at different time points on POT1 and TIN2 mRNA levels, had not been studied in the project to date. Enzymes known as DNA methyltransferases (DNMTs) carry out DNA methylation at the 5 position of CpG dinucleotides. These enzymes regulate DNA methylation by catalysing the transfer of a methyl group from S-adenosyl-L-methionin (SAM) to a cytosine (Bestor 2000). DNMT1 is the
main methylation maintenance enzyme that methylates hemi-methylated DNA during the process of DNA replication (Pradhan, Bacolla et al. 1999; Kim, Samaranayake et al. 2009). Since many tumour suppressor genes are known to be inhibited through the DNA methylation process during carcinogenesis, 5-aza-CdR has been used to reactive these genes through inhibiting DNMTs. 5-aza-CdR which is incorporated into gDNA during replication, and thereby inhibits DNA methylation via irreversible covalent binding to DNMT1 (Maslov, Lee et al. 2012). By this mechanism, it could be argued that the lowest mRNA levels of POT1 and TIN2 between 72 hours and 3 weeks may perhaps resulted from an increase in the expression of the DNA methyltransferase 1 enzyme, leading to hypermethylation of POT1 and TIN2 after several replications.

Recent work by Kang et al. (2013) demonstrated that Runt-related transcription factor 3 (RUNX3), a tumour suppressor gene, was hypermethylated in MCF-7, breast cancer cell line. They showed that 5-aza-CdR induces apoptosis and inhibits cell proliferation by demethylating the promoter region of RUNX3 and reactivating its expression (Kang, Dai et al. 2013). Therefore, based on previous investigations, it should be noted that 5-aza-CdR is a chemotherapeutic drug, which causes cell death via induction of apoptosis pathways. Consistent with this observation, approximately 72 hours after treatment with 5-aza-CdR, a significant reduction in 21NT cell number was observed. Therefore, by 7 days of treatment, cells which have become resistance to the treatment but retained the unmethylated status will start to grow and continue to grow up to 3 weeks accompanied by an increase in gene expression.

POT1 appears to bind directly to the 3' overhang of single stranded telomeric DNA. This protein interacts with TIN2 via TPP1 protein to become part of the Shelterin complex.
Hence, TPP1 is the only protein that is directly bound to POT1 and TIN2. qRT-PCR results showed significant up-regulation of *TPP1* after 48 hours treatment of 21NT cells with 5-aza-CdR treatment (*p*<0.05). We then investigated the protein levels of TPP1 and POT1 on short-term (72 hours) and long-term (3 weeks) treatment of the 21NT cells to examine whether the expression of these genes at the protein level might perhaps change after longer period of treatment. Western blot analysis was performed using POT1 rabbit monoclonal antibody (Abcam) and TPP1 rabbit polyclonal antibody (Abcam) and the values was normalised using β-Actin rabbit antibody (Sigma). These confirmed previous results obtained with qRT-PCR and revealed that 5-aza-CdR and the combined treatment of 5-aza-CdR and TSA induced the protein levels of POT1 after 72 hours treatment in comparison with DMSO-treated control. However, no significant increase was detected in long-term treatment (Figures 4.16-C and 4.16-D). Furthermore, no substantial difference in TPP1 protein level was observed in long-term treated sample compared to the short-term treatment (Figure 4.15). As shown in section 4.3.6, *POT1* and *TIN2* were significantly up-regulated in 21NT cells after being treated with 5-aza-CdR and TSA at different time points in comparison with DMSO and untreated controls. Therefore, our data indicated that promoter regions of *POT1* and *TIN2* genes may possibly be under the control of DNA methylation. Consequently, Methylation specific PCR (MSP) was used to examine the promoter regions of *POT1* and *TIN2* genes. Methylation specific PCR indicated that the promoter region of *POT1* was partially methylated in untreated (i.e., not exposed to 5-aza-CdR and TSA) breast cancer cell lines (21NT, 21MT-2 and GI101). However, no significant differences in the promoter region of *TIN2* were observed in methylated and unmethylated lanes compared with the control (HMEC1) (Figure 4.4).
The upstream promoter region of \(POT1\) was analysed for methylation in 21N, 21MT-2 and GI101 breast cancer cell lines, using bisulphite sequencing. Contrary to expectation, bisulphite sequencing data showed that no CpG region was methylated in 21MT-2 and GI101 breast cancer cell lines (Figure 4.5). However, 21NT cells was about 30% methylated in the upstream promoter region of \(POT1\) in comparison with HMEC1 (Figure 4.6). The product obtained from bisulphite PCR was sequenced and 100bp of error-free sequence was obtained. This was not enough to get an accurate estimation of the methylation pattern within the promoter region of \(POT1\). Therefore, further sequence analysis should be carried out encompassing a larger region of the \(POT1\) promoter region.

In the current study, we determined the effects of these drugs on cytosine methylation levels in the promoter regions of \(POT1\). The 21NT cells were treated with these drugs for 72 hours to investigate the reduction of cytosine methylation at \(POT1\) promoter region. Bisulphite sequencing data showed that 5-aza-CdR treatment removed all methylation sites of CpG dinucleotide in comparison with DMSO-treated as all cytosine residues were converted to thymidine (Figures 4.18 and 4.19). This finding is consistent with the earlier results showing up-regulation of Shelterin genes. In light of the new data discussed above, both DNA methylation and histone deacetylation appear to be implicated in the silencing of Shelterin and Shelterin-associated genes in breast cancer cell lines. The results presented here suggest that the synergistic effect of 5-aza-CdR may perhaps reduce DNA methylation in association with gene reactivation. It has been hypothesized that 5-aza-CdR and TSA treatments enhance gene transcription by opening promoter region to increased accessibility of assembling transcription factor complexes (Yang, Phillips \textit{et al.} 2001; Margueron, Duong \textit{et al.} 2004). Since these two agents have individually been able to
induce gene expression, they might be expected synergistically have a more potent effect on the gene expression than either alone.
Chapter V

ANALYSIS OF TELOMERE LENGTHS IN THE BREAST CANCER CELL LINE 21NT FOLLOWING EPIGENETIC CHANGES TO THE SHELTERIN GENES
5.1-Introduction

As has been mentioned in early chapters, telomeres consist of repetitive TTAGGG sequences found at the end of mammalian chromosomes, and play an important role in maintaining genomic integrity (Jacobs 2013). Telomeres are maintained by two main processes: (i) a telomere-specific DNA polymerase called telomerase (the primary mechanism), and (ii) the secondary alternative lengthening of telomeres mechanism known as ALT (a rare mechanism found in some tumours) (Conomos, Pickett et al. 2013). The telosomal proteins encoded by Shelterin genes play a part in protecting the ends of telomeres (de Lange 2002). The reverse transcriptase enzyme telomerase is responsible for the addition of hexanucleotide repeats TTAGGG, onto the 3’-end of a telomere, and consequently counter the process of replication-associated telomere shortening (Bryan 1995). The six protein complex Shelterin packages (caps) the ends of chromosomes preventing them from being recognised as a site of DNA damage during DNA replication (Liu, O'Connor et al. 2004; de Lange 2005). In view of the fact that a capacity for limitless replication is a sign of cancer, telomerase or ALT must be activated to overcome the process of telomere erosion (Hanahan and Weinberg 2000). Contrary to normal somatic cells, 85-90% of tumour cells express high levels of telomerase which is responsible for maintaining the 2-3kb telomere length of most cancer epithelial cells (carcinomas) (Kim, Piatyszek et al. 1994).

The role of telomerase activation in human cancer development has been widely studied (Donate and Blasco 2011) and it is important to understand how telomerase activation occurs in breast cancer. It has been reported that the up-regulation of telomerase
is associated with cell immortalization and malignancy (Salhab, Jiang et al. 2008). There is a strong body of evidence suggesting that short telomeres in breast cancer cells precipitate telomere dysfunction and this may be in part related to Shelterin proteins and their level of expression in breast cancer cells (Butler, Hines et al. 2012).

In the previous chapter (IV), we have shown that treatment of the 21NT cells with the DNA demethylating agent 5-aza-CdR and the histone deacetylase inhibitor TSA at different time point results in up-regulation of Shelterin genes mRNA expression. The transcription levels of POT1, TIN2, and TPP1 were significantly increased in treated 21NT cells compared with untreated and DMSO controls ($P<0.05$). The resulting effect of the two agents on telomere length was a question of a considerable interest. The primary aim of this section of the work was to investigate if the up-regulation of Shelterin gene expression had an effect on telomere length. Therefore, changes in telomere length in short-term (72 hours) and long-term (3 weeks, 6 weeks, and 2 months plus retreat for 72 hours) treatment of 21NT cells was examined (Table 5.1).

The assessment of telomere dynamics is critically dependent on the telomere length measurement techniques used. Several techniques are available to measure telomere length; these include Southern blot analysis (Terminal restriction fragment (TRF)), quantitative fluorescence in situ hybridisation (Q-FISH), flow-FISH, the hybridisation protection assay (HPA), quantitative PCR, and single telomere length analysis (STELA). Each method has its benefits and disadvantages. For instance, Southern blot and flow-FISH can determine the average telomere length, while Q-FISH provides information about telomere lengths of individual chromosomes. Quantitative polymerase chain reaction (q-PCR) is another technique to measure telomere length. However, the lack of an appropriate primer
binding site can hamper this method (Forstemann, Hoss et al. 2000; Cawthon 2002). Therefore, an important subsidiary aim of this section of the project was to compare the aforementioned techniques in terms of reliability and accuracy.

**5.2-Materials and methods**

**5.2.1-Interphase Quantitative Fluorescent in situ hybridization (i-QFISH)**

For interphase analysis, samples were produced according to standard cytogenetic methods, with exception of colcemid treatment.

**5.2.1.1-Prehybridization washes**

Cells that were treated with 5-aza-CdR and TSA at different time points (see Section 4.2.3 and Table 5.1) were trypsinized with Trypsin/EDTA. The cell suspension was centrifuged at 12000rcf for 5 minutes. Cells were then treated with 10ml of hypotonic buffer (75mM of KCl) for 30 minutes in a 37°C water bath (this causes cells to swell with water and to burst to release DNA content). The samples were then centrifuged at 1000rcf for 5 minutes. The process of fixation was carried out by removing KCl and adding methanol and glacial acidic acid (3:1) solution. Cell suspensions were dropped onto clean glass slides and aged at 55°C overnight. After 24 hours, microscope slides containing samples were washed with phosphate-buffered saline (PBS) for 5 minutes on the shaker. Subsequently, the samples were treated with 4% formaldehyde for 2 minutes and washed in PBS for 3 times for 5 minutes each. In order to remove unwanted proteins, the cells were treated with 1mg/ml pepsin solution (50ml of water acidified with 0.5ml of 1M HCl, pH 2.0, containing 10% pepsin (Sigma)) for 10 minutes at 37°C in water bath. The slides were then washed 2 times with PBS for 2 minutes each on a shaker platform and then fixed with 4%
formaldehyde for 2 minutes. Afterwards, the samples were washed three times with PBS for 5 minutes each then dehydrated in the 70%, 90%, 100% ethanol for 5 minutes each. Slides were left to dry at room temperature.

Table 5.1 - Represents different time point of 21NT treated cells with 5-aza-CdR, TSA or DMSO

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Period of time under treatment with</th>
<th>Period of time after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treated 21NT</td>
<td>48hrs, 16hrs, 48hrs, 72hrs, 7 days, 3 weeks, 6 weeks, 2 months</td>
<td></td>
</tr>
</tbody>
</table>

5.2.1.2-Hybridization

20µl of the synthetic oligonucleotide PNA (Peptide Nucleic Acid) specific for the telomeric DNA sequence (CCCTAA)_3, labelled with FITC, was added to the slides. Slides were then placed on the heating block for 2 minutes at 70-75°C and left in a dark humidified chamber for 2 hours at room temperature. Stock hybridization mixture (1ml) for the telomeric probe was made up of 700µl deionised formamide, 5µl blocking reagent (10% in maleic acid), 50µl MgCl₂ buffer (2.5M MgCl₂, 9 mM Na₂HPO₄, pH 7.0), 10µl Tris (1M, pH 7.2), 152µl ddH₂O and 83µl of PNA solution (6 µl/ml FITC or Cy3-conjugated PNA) (peptide nucleic acid, Applied Biosystems, MA, USA).

5.2.1.3-Post hybridization washes

After hybridization, the samples were washed twice in 70% formamide solution for 15 minutes each. Then the slides were washed 3 times with PBS for 5 minutes in the dark on a shaker. Slides were then dehydrated in 70%, 90%, and 100% ethanol for 5 minutes each. 15µl of Vectra-shield with fluorescence DAPI mounting medium (Vector, Vectashield) was
added to each slide and covered with a 22x50mm coverslip before sealing with a clear nail varnish.

5.2.1.4-Image capture and telomere length analysis

Images of interphase cells was acquired on a digital fluorescence microscope (Zeiss Axioskop 2) equipped with CCD camera (Photometrics) and Smart Capture software (Digital Scientific, Cambridge, UK) using fixed time exposure of 0.5 sec and magnification of 63x. IP lab software (Digital Scientific), (Figure 5.1) was used to analyse telomere fluorescence intensity per cell. The average signal was calculated as the total intensity of the telomeric signal utilizing the area under curve minus the background signal. The experiment was repeated at least twice and each time 100 cells were quantified for each sample.

Figure 5.1-Representative images of A) LY-R (radio-resistant) and B) LY-S (radio-sensitive) interphase cells after hybridization with telomeric PNA oligonucleotides.
5.2.2-Telomere length determination by flow-FISH

The in vitro measurement of telomere length can be done via different high-throughput techniques. One such technique is called flow-FISH, and is based on flow-cytometry. It works by utilizing a fluorescence tag hybridized to the telomeric repetitive sequence of (C3TAA)$_3$. A modified version of this method first described by Cabuy et al. (2004) was utilized in this work (Cabuy, Newton et al. 2004).

Cells were grown as described previously. Pellets containing 5x10$^5$ cells were resuspended and fixed in 1ml of 70% ethanol. To avoid cellular aggregation, fixative was added drop by drop under continuous shaking. Thereafter, the cells were incubated at 4°C overnight. All the centrifugations between the washing steps were performed at 0.8rcf for 5 minutes. The cell pellets were washed by adding 1ml of PBS, centrifuged, and then the supernatant was discarded carefully. 500μl of hybridization mixture containing 70% formamide, 10mM Tris-HCl pH 7.0, 1% BSA made in PBS, and 0.3μg/ml of fluorescein isothiocyanate (FITC) conjugated peptide nucleic acid (PNA) probe (C3TAA)$_3$ was added to the cell pellet and the mixture was heated at 80°C for 10 minutes in the dark to denature the DNA. The cells were left to hybridise for two hours in the dark at room temperature. Samples without the PNA telomeric probe were used as negative controls. After the hybridization step, pellets were spun down and supernatant was discarded carefully. Post-hybridization washes were carried out to ensure that excess and unbound probe was washed away, therefore reducing the background fluorescence. This was done by adding 500μl of wash solution containing 70% formamide, 10mM Tris buffer (pH 7), 0.1 % BSA and PBS to the cells. The pellet was suspended in the wash solution and the samples were centrifuged at 0.8rcf to collect cells and supernatants were discarded. A second wash was
done again twice using a 500μl of a solution containing PBS and 0.1% BSA; the cells were then centrifuged at 0.8rcf. A second incubation was done with propidium iodide (PI) (Sigma) to quantitatively assess the DNA content of cells. PI is a widely used fluorescence dye that binds directly to DNA by intercalating between the bases. In addition, PI binds to RNA therefore it is important to digest all RNA in the sample by treating it with RNaseA (Invitrogen). The cell pellet obtained above was re-suspended in a solution contained PBS, 0.1% BSA, 10μg/ml of RNase A, and 0.1μg/ml of PI. The tubes were then stored in the dark for one hour at 4°C. After incubation, the samples were centrifuged and supernatant was discarded. The tubes were kept on ice all prior to the measurement with the flow cytometer.

FACSCoulter EPICS XL (Becton Dickinson) was calibrated using flow-check fluorospheres (Beckman Coulter) to check laser alignment on all four channels; this step was performed before each measurement. The instrument was calibrated to measure the FITC telomeric signal on the FL1 channel, and the PI signal on FL3 channel. After calibration, cells were electronically gated for the G0/G1 phase of the cell cycle form the FL3 histogram window. The telomeric fluorescence intensity (TFI) of cells in the G0/G1 stage was recorded. In order to remove the background reading, TFI from the negative control cells was also measured and subtracted from the main sample reading. The experiment was carried out at least three times, and each time TFI readings from a minimum of 5,000 cells and a maximum of 20,000 cells were recorded (Figure 5.2).
Figure 5.2-Examples of a typical profile of flow cytometry of HMEC1 cells. A) Side scatter versus forward scatter dot plot distinguishes each population of cells based on size and complexity of cells. B) Plot of PI/FITC with 3 regions showing the intensity of fluorescence. Positive samples are expected to be in grids C1 and C2 and negative samples in grids C3 and C4. C) Dot plot with a gate encompassing single cells. D) Histogram of a cell cycle was then plotted and only cells in the G0/G1 phase were gated for telomere measurement. E) TFI (Telomere Fluorescence Intensity) units were measured within the middle part of the histogram on the FL1 channel. Twenty thousand cells were detected in five minutes and an average of 1,000 cells was used to measure TFI units.
5.2.3-Terminal restriction fragment (TRF) telomere length analysis

5.2.3.1-Overview

Mammalian telomeric hexanucleotide repeats (T2AG3) and some sub-telomeric DNA sequences do not include restriction sites. Therefore, genomic DNA can be digested with restriction enzymes such as Hinf I and Rsa I to cut genomic DNA into small fragments, while the terminal chromosome fragment remains intact. Digested DNA fragments, including an undisclosed length of sub-telomeric DNA and the terminal section of TTAGGG repeats is called the terminal restriction fragment (TRF). Agarose gel electrophoresis separates the average size of the TRF which can be measured by Southern blotting. It includes hybridisation to a digoxigenin (DIG)-labelled probe precise for hexameric repeats and incubation with a specific DIG-antibody covalently linked to alkaline phosphate (Roche). Alkaline phosphate (AP) is used to visualize the immobilized telomere probe metabolising CDP-Star, a highly sensitive chemiluminescence substrate. During DNA replication, telomeric DNA is eroded which appears as a smear when analysed, showing the characteristically heterogeneous telomere population. This method is commonly used to determine difference in telomere length.
5.2.3.2-TRF Telomere Length Assay

5x10^5 cells were harvested and washed with sterile PBS, pelleted and re-suspended in fresh PBS. Genomic DNA from treated 21NT cell was extracted as described in Section 2.7. TRF length measurement was performed utilizing the Telomere Length Assay (Roche Diagnosis). Approximately 3µg of DNA was digested for 2 hours at 37°C using a mixture of restriction enzymes *Hinf I* and *Rsa I* in a concentration of 20U/µl for each enzyme. The reaction was stopped by adding 5µl of gel electrophoresis loading buffer. The digested genomic DNA was loaded on to 0.8% 1x TAE buffer agarose gel. The same amount of DNA from each sample was carefully loaded in every lane. A DIG labelled molecular weight marker was loaded on either side of the respective samples. The gel was run at 5V/cm in 1x TAE buffer until the bromophenol blue tracking marker reached about 10 centimetres from the starting wells (total run 2-4 hours depending on tank size) (Figure 5.3). The gel was then left for 10 minutes in 0.25M HCl until the bromophenol blue stain changed colour to yellow. Then the gel was rinsed twice with sterile water and was denatured for 30 minutes in 0.5M NaOH, 1.5M NaCl followed by two more rinses. The gel was then neutralized for 30 minutes in 0.5M Tris-HCl, 3M NaCl (pH 7.5). All incubation steps were performed at room temperature with gentle agitation. Southern blotting of the digested DNA was done by capillary transfer using 20x Saline-Sodium Citrate (SSC) onto Hybond N+ membranes (Amersham) overnight.
Figure 5.3-Image of a typical agarose gel after electrophoresis showing smears of gDNA following digestion with restriction enzymes. The gel was then southern blotted and hybridised to the telo-\textit{TTAGGG} probe. Representative result of TRF analysis for telomere length measurement in 21NT treated with 5-aza-CdR and TSA at different time points and control (HMEC1) cells using telomere length Assay. The lanes on the left and right sides show the molecular size marker. Lane1) Untreated, Lane2) DMSO 72hrs, Lane3) 5-aza-CdR 72hrs, Lane4) 5-aza-CdR/TSA 72hrs, Lane5) 5-aza-CdR 3weeks, Lane6) 5-aza-CdR/TSA 3weeks, Lane7) 5-aza-CdR 6weeks, Lane8) 5-aza-CdR/TSA 6weeks, Lane9) DMSO, Lane10) 5-aza-CdR 2 months, Lane11) 5-aza-CdR/TSA 2 months, Lane 12) HMEC1 p5, Lane13) HMEC1 P20, Lane14) The positive control DNA from immortal cell line (Telomere length Assay) respectively. The genomic DNA was digested with \textit{Hinf I} and \textit{Rsa I} enzymes and hybridized with a telomere-specific, digoxigenin (DIG)-labelled hybridization probe. The size markers are indicated on the right.
5.2.3.3-Southern hybridization

After Southern transfer, the membrane was washed in 2x SSC. Pre-hybridization was carried out by adding 18ml of pre-warmed DIG Easy Hyb Granules solution to the membrane for 30 minutes at 42°C with gentle agitation. Before hybridization, the telomere probe was added to the pre-warmed DIG-Easy Hyb Granules solution. The membrane was then hybridized for 3 hours at 42°C in a mixture of DIG-Easy Hyb and Telomere probe (Roche) with gentle agitation. Low stringency washes were carried out by discarding the hybridization solution and washing the membrane two times for 5 minutes in 2x SSC, 0.1% SDS at room temperature. High stringency washes were done twice for 20 minutes at 50°C in pre-warmed 0.2x SSC, 0.1% SDS with gentle agitation. Then, the membrane was washed with 100ml of washing buffer (provided by the kit). In order to pre-block the membrane, 100ml 1x blocking solution was added to the membrane and incubated for 30 minutes at room temperature with gentle agitation. The membrane was then incubated in anti-DIG-AP solution (Roche) for 30 minutes at room temperature with gentle agitation and subsequently washed twice in 100ml of washing buffer. The membrane was then incubated for 5 minutes at room temperature with a detection buffer (Roche), followed by the addition of the substrate solution (CDP-Star) for 5 minutes before exposure to an X-ray film (Amersham Hyperfilm™ ECL) for 5 minutes at room temperature.

5.2.3.4-Densitometry

The average TRF value was calculated by evaluating the telomeric signal (smear) relative to molecular weight standard supplied with the Roche Kit. The exposed X-ray film was scanned with densitometer (a desk scanner G2710, HP) and using ImageQuant software 5.2 (Amersham Biosciences, USA). It showed telomere lengths as smears ranging from 2 to
more than 21 kb. Each sample lane of the scanned image was overlaid with a grid. Then, the telomeric signal in each lane was quantified as a grid object, described as a single column with 30 rows (Figure 5.5). The resolution of the TRF length calculation was determined by the highest of the individual squares of the grid. This grid was placed over the lanes corresponding to the molecular size markers and telomere lane. Afterwards, the data was transferred to a spreadsheet (Microsoft) to quantitate integrate volume. Interpolating molecular sizes for each row were determined by plotting the row number 1-30 against the molecular size ladder and fitting a best (least squares) line. The average labelled TRF length in each lane was calculated in Excel as the mean of the optical density above background (Figure 5.4).

![Figure 5.4-Southern blot analysis of telomere length in 21NT treated cells and controls (HMECs). A) Standard curve used to calculate absolute telomere length measured by Southern blots of TRFs. Y-axis represents log2 of DNA ladder and X-axis represents distance travelled in gel. Correlation coefficient calculated is 0.991. B) Numbers showing converted log2 values of DNA ladder size in kb used in the analysis.](image)
The mean TRF length was defined according to the following formula: \( TRF = \Sigma (OD_i)/\Sigma (OD_i/L_i) \). For each square lane that contains DNA, \( OD_i \) is the chemiluminescent signal and \( L_i \) is the lengths of the TRF at position \( i \) on the gel image. Then the mean TRF length was calculated using the above formula.

Figure 5.5-Chemiluminescent detection of TRFs. The lane on the both sides shows the molecular size marker. Lane 1) Untreated, Lane 2) DMSO-treated 72hrs, Lane 3) 5-aza-CdR 72hrs, Lane 4) 5-aza-CdR/TSA 72hrs, Lane 5) 5-aza-CdR 3weeks, Lane 6) 5-aza-CdR/TSA 3weeks, Lane 7) 5-aza-CdR 6weeks, Lane 8) 5-aza-CdR/TSA 6weeks, Lane 9) DMSO-treated, Lane 10) 5-aza-CdR 2 months, Lane 11) 5-aza-CdR/TSA 2 month, Lane 12) HMEC1 p5, Lane 13) HMEC1 P20, Lane 14) The positive control DNA from immortal cell line (Telomere length Assay) respectively. Gel profiles were read in a densitometer and gained in a grid and data were analysed in Excel spread sheet. Telomeric sizes are then calculated as explained.
5.2.4-Telomere length measurement by quantitative real time PCR

In order to determine telomere length, a real-time PCR technique was used. The relative telomere length was compared with that of a single copy gene. A single copy gene (SCG) control was used for amplification of each sample, and to determine genome copies per sample. We used 36B4 as a single copy gene which encodes the acidic ribosomal phosphoprotein PO (O’Callaghan and Fenech 2011). Genomic DNA was extracted from 21NT treated cells (see Section 4.2.3) and the normal human mammary epithelial cell strain (HMEC1) using the Wizard™ Genomic DNA Kit as described in Section 2.7 (Chapter II). Telomere and single copy gene master mixes were prepared separately. Briefly, two q-PCR master mixes were prepared, one with the telomere primer pair and the other with the single copy gene primer pair (36B4). 10µl of 2x Power SYBER® Green PCR Master Mix (Applied Biosystems); 2µM forward primer (Telomere-F or 36B4-F) (Sigma, Table 5.2); 2µM reverse primer (Telomere-R or 36B4-R) (Sigma, Table 5.2), 4µl of 5ng/µl DNA sample; and nuclease free water up to 20µl were added to the templates.

Telomere and single copy gene q-PCRs were performed in separate 96-well plates. 20µl of telomere master mix was added to each sample well containing 5ng/µl DNA samples, standard well (2µl of the telomere standard) and no template control (NTC) of the first plate. The second plate was 20µl of single copy gene master mix containing 5ng/µl DNA samples, standard well (2µl of the single copy gene standard) and no template control (NTC). A telomere standard curve was established by serial dilutions of the telomere standard (1018400 kb through to 10184 kb dilution) and was used to measure the content of telomeric sequence per sample in kb (Figure 5.6).
A single copy gene (36B4) was used as a control for amplification of every sample performed and to determine genome copies per sample. A single copy gene standard curve was generated by performing serial dilutions of the 36B4 standard (6125000 kb through to 6.125 kb dilution). Plasmid DNA (pBR322) was also added to each standard to maintain a constant 20ng of total DNA per reaction tube. After setting up the reactions, the plate was sealed with a real time plate sealer (MicroAmp, Applied Biosystems) and then centrifuged for one minute at 1000rcf to bring all the contents to the bottom of the well. Real-time PCR runs were performed in triplicate for each of the DNA pools. Each experiment was performed at least three times ensuring the reproducibility and accuracy of the results. The real time PCR reactions were run using the following reaction conditions for both telomere and 36B4 amplifications followed by the construction of a dissociation (or melt) curve:

- 95.0°C for 10 minutes (DNA denaturing step)
- 95.0°C for 15 seconds (DNA denaturing step)
- 60.0°C for 1 minute (DNA Annealing step)
- 95.0°C for 15 seconds
- 60.0°C for 15 seconds

The values (kb/reaction for telomere and genome copies for single copy gene) were exported to an Excel file and used to calculate total telomere length in kb per human diploid genome. The telomere kb length per reaction value was then divided by diploid genome copy number to give a total telomeric length in kb per human diploid genome.
Table 5.2-Oligomers used for telomere length assay in 21NT treated and normal cells (O'Callaghan and Fenech 2011)

<table>
<thead>
<tr>
<th>Oligomer Name</th>
<th>Oligomere Sequence (5´ → 3´)</th>
<th>Amplicon size (bP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Telomere Standard</td>
<td>(TTAGGG)_{14}</td>
<td>84</td>
</tr>
</tbody>
</table>
| 36B4 Standard       | CAGCAAGTGGGAAAAGGTGTAATCCGTCTCCACAGACAAGGCCA  
                      | GGCCTGTTGTTACCGTTGGTTGATGATGATAATGGG          | 75                 |
| Telo-F (Human/Rodent)| CGGTTTGTGTTGGGGTTGGTGGTTGGTTGGTTGGTTGGTTGGTT | >76                |
| Telo-R (Human/Rodent)| GGCTTGCCTACCCCTTACCCCTTACCCCTTACCCCTTACCCCT | >76                |
| 36B4-F (Human)      | CAGCAAGTGGGAAAAGGTGTAATCC       | 75                 |
| 36B4-R (Human)      | CCCATTCTATCATCAACGGGTACCA        | 75                 |

Figure 5.6-Standard curve used to calculate absolute telomere length. A) Standard curve for calculating length of telomere sequence per sample: X-axis demonstrates amount of telomere sequence in kb per reaction with correlation coefficient of 0.997. B) Standard curve for calculating genome copies using 36B4 copy number: correlation coefficient was 0.999.
5.3-Results

5.3.1-Telomere length analysis by Interphase Quantitative Fluorescent in situ Hybridization in 21NT breast cancer cells

The breast cancer epithelial cell line, 21NT, treated with the demethylating agent, 5-aza-CdR and the histone deacetylation inhibitor, TSA, showed up-regulation of expression of Shelterin genes (described in Chapter IV). The aim of the work described in this chapter was to analyse telomere lengths in 21NT cells before and after treatment with demethylating agents. The reasoning behind this was to analyse if up-regulation of some of the Shelterin genes such as POT1 and TPP1 has an effect on average telomere length. As the first method of telomere length measurement we used iQ-FISH. The average length of the telomeres in interphase stage was measured by Smart Capture software.

The average telomere fluorescence intensities (TFI) of 21NT cells treated with 5-aza-CdR and a combination treatment with 5-aza-CdR and TSA at short-term (72 hours) and long-term (3 weeks, 6 weeks and 2 months plus retreated for 72 hours) was measured and compared with those of normal human epithelial cell strain (HMEC1), untreated 21NT and DMSO-treated (the term DMSO treated control refers to 21NT cells that received the solvent DMSO-treated at final concentration of 0.02%) controls. Two mouse lymphoma LY-R (radio-resistant) and LY-S (radio-sensitive) cells were also used as calibration standards with known telomere lengths of 49kb and 7kb respectively (McIlrath, Bouffler et al. 2001). A total 100 interphase cells per cell line were analysed by iQ-FISH to examine the telomere fluorescence intensity for each cell line. Figure 5.7 shows different telomere fluorescence intensity signals in DMSO-treated control, treated 21NT cells and HMEC1 cell strains.
Figure 5.7-Digital image of iQ-FISH. A) DMSO-treated, B and C) 21NT treated with 5-aza-CdR and TSA for 72 hours, D) Normal mammary epithelial cell strain (HMEC1). Telomeres were labelled by PNA-FITC (green) and nuclei were labelled by DAPI (blue). As shown, different samples give different signals and clear signals have been observed in 5-aza-CdR and TSA treatment compared with DMSO-treated control. Magnifications of the images are x65.
Following treatment of the 21NT cells with demethylating agents significantly higher total telomere fluorescence intensity was observed in treated 21NT cells compared with DMSO-treated control ($P<0.001$) (Figure 5.8). The analysis revealed that 21NT treated cells with 5-aza-CdR and a combination treatment with 5-aza-CdR and TSA at different time points, showed approximately a 2-fold increase in telomere fluorescence intensity compared with DMSO-treated and untreated 21NT cells ($P<0.001$) (Figure 5.8). However, 3 weeks treatment showed shorter TFI than the 72 hours, 6 weeks and two month treatment.

**Figure 5.8-Telomere length measurement by iQ-FISH.** Comparison of the mean TFI in 21NT treated and control cell lines. The telomere fluorescence intensity is shown in normal human epithelial cell strain (HMEC), LY-R, LY-S, un-treated 21NT, DMSO-treated and 21NT treated with 5-aza-CdR and a combination treatment with 5-aza-CdR and TSA at 72 hrs, 3 weeks, 6 weeks and 2 months + retreatment for 72 hours. The analysis was performed using untreated 21NT with the lowest TFI compared to 21NT treated at different time points and controls; HMEC1 p6, LY-R, and LY-S. The experiment was repeated at least twice and each time 100 cells were quantified for each sample. Error bars represent SEM. Asterisk indicates difference between DMSO-treated cells and the 21NT treated cell lines (***(P<0.001).
5.3.2-Telomere length analysis by flow-FISH in 21NT treated and control cell lines

Results obtained from iQ-FISH showed a significant increase in telomere fluorescence intensity when the human breast cancer cell line 21NT was treated with 5-aza-CdR and TSA at different time points. To confirm these results, we set out to measure the observed telomere elongation using a high-throughput flow cytometry method. We have therefore measured the telomere length intensity by flow-FISH in all of 21NT treated, untreated, DMSO-treated and HMEC1 cells. Telomere fluorescence intensity was measured in the G0/G1 phase of the cell cycle to make sure data were consistent with those obtained using iQ-FISH. Flow cytometry provides the flexibility to gate cells in different cell cycle (Figure 5.9). Since DNA content is doubled in S-phase, the telomere length measurement is gated in G0/G1. In comparing both methods we sought to confirm that the increase in telomere length observed when cells were treated with 5-aza-CdR and TSA was a reliable result.
Figure 5.9-Example of cell cycle and actual TFI in different cell lines. A and B) represent the histogram window of the cell cycle of DMSO-treated control and TFI, C and D) represent the cell cycle of 21NT treated with 5-aza-CdR for 72 hours and TFI.
Data generated from flow-FISH experiments are shown in Figure 5.10. Interestingly, telomere fluorescence intensity of 21NT treated cells significantly increased after 72 hours, and 3 weeks when compared with DMSO-treated control (**P<0.01, ***P<0.001
respectively). However, these trends did not reach statistical significance after 6 weeks treatment. No higher impact was observed after 2 months post treatment of 21NT cells compared with DMSO-treated control (Figure 5.10).

**Figure 5.10-Telomere length measurement by flow-FISH.** Comparison of the mean Telomere Fluorescence Intensity (TFI) in 21NT treated and control cell lines. The telomere fluorescence intensity is shown in HMEC1, LY-R, LY-S, untreated 21NT (controls), DMSO-treated and 21NT treated with 5-aza-CdR and a combination treatment with 5-aza-CdR and TSA at 72 hrs, 7 days, 3 weeks and 2 months plus retreatment for 72 hours. Error bars represent SEM. Asterisk indicates difference between DMSO-treated cells and the 21NT treated cell lines (**P<0.01, ***P<0.001).

Based on the results obtained from iQ-FISH and flow-FISH methods used for telomere length estimation, flow-FISH proved to be least accurate in comparison with iQ-FISH. Our data revealed that there was low correlation between these two methods (Table 5.3). Although, the increases telomere lengths in treated samples were observed using both
methods, this increase was not expected from untreated samples. Therefore, there are some advantages and disadvantages with each of the two methods that may influence the results. For instance, in the flow-FISH technique, cell clumping, crude experimental processor and fixation method might affect the results (Derradji, Bekaert et al. 2005). Furthermore, with this method, it seems that the probe cannot bind properly to the samples with pellet clumping which can influence the accuracy of the telomere fluorescence intensity. Flow-FISH and iQ-FISH provide the values of fluorescence intensity per cells. However, with both techniques it is not possible to measure the telomere fluorescence intensity signals corresponding to each individual chromosome end.

**Table 5.3**—Comparison of flow-FISH and iQ-FISH results. TFI values represent the telomere fluorescence intensity; *P values indicate the difference between all the treated and untreated samples. HMEC1, LY-R and LY-S cells were used as a positive control.

<table>
<thead>
<tr>
<th>Samples</th>
<th>TFI (flow-FISH)</th>
<th>T-test (flow-FISH)</th>
<th>TFI (iQ-FISH)</th>
<th>T-test (iQ-FISH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>3.25</td>
<td>0.81</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO-treated</td>
<td>12.75</td>
<td>1.44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMEC1 p6</td>
<td>9.15</td>
<td>4.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LY-R</td>
<td>31.5</td>
<td>3.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LY-S</td>
<td>5.85</td>
<td>2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-aza-CdR 72hrs</td>
<td><strong>38.12</strong></td>
<td>0.017</td>
<td>3.24</td>
<td>***1.8E-15</td>
</tr>
<tr>
<td>5-aza-CdR/TSA 72hrs</td>
<td>35.62</td>
<td>0.269744</td>
<td>3.57</td>
<td>***1.2E-15</td>
</tr>
<tr>
<td>5-aza-CdR 3weeks</td>
<td>***41.45</td>
<td>0.001801</td>
<td>2.0</td>
<td>***0.001</td>
</tr>
<tr>
<td>5-aza-CdR/TSA 3weeks</td>
<td>43.85</td>
<td>0.216611</td>
<td>2.59</td>
<td>***7.6E-11</td>
</tr>
<tr>
<td>5-aza-CdR 6weeks</td>
<td>31.75</td>
<td>0.174331</td>
<td>3.34</td>
<td>***9.5E-18</td>
</tr>
<tr>
<td>5-aza-CdR/TSA 6weeks</td>
<td>20.7</td>
<td>0.363277</td>
<td>3.11</td>
<td>***8.6E-16</td>
</tr>
<tr>
<td>5-aza-CdR 2months</td>
<td>12.85</td>
<td>0.61155</td>
<td>3.22</td>
<td>***3.1E-16</td>
</tr>
<tr>
<td>5-aza-CdR/TSA 2months</td>
<td>9.8</td>
<td>0.025613</td>
<td>3.27</td>
<td>***1.4E-15</td>
</tr>
</tbody>
</table>
5.3.3-Telomere length analysis by terminal restriction fragment (TRF) in 21NT treated and control cell lines

The initial observation of increased telomere length in treated 21NT cells was unexpected but in the line with our hypothesis. Could up-regulation of some of the Shelterin genes stabilise telomere lengths and even elongate them in the presence of telomerase enzyme. In order to be more confident of the initial data, we set out to confirm the above findings using other methods of telomere length measurement (Terminal Restriction Fragment, TRF).

The result from the analysis of the 13 samples in Figure 5.11 is shown in Table 5.4. LY-R and LY-S samples were not considered as the enzymes used in the assay do not cut the mouse DNA samples. The data showed that there was the most increase in telomere length for 21NT cells treated with 5-aza-CdR at 3 weeks and the combination with 5-aza-CdR and TSA treatment for 72 hours (as compared with their respective DMSO-treated and untreated 21NT controls). Samples from short-term (72 hours) treatment with 5-aza-CdR yielded telomere length approximately the same size as those with long-term treatment with combination of 5-aza-CdR and TSA (3 weeks) treatment. However, only minimal differences were observed in 6 weeks and 2 months treatment groups. Therefore, it seems that the drugs lose their effectiveness over long term treatment period (2 months) (Figure 5.11). The telomere length in the positive DNA control from an immortal cell line was 10.18 kb which was consistent with the size recommended in the kit (Roche). A 1.13 kb difference in telomere size was observed in HMEC1 controls at different passages. It could be hypothesized that telomere shortening is correlated with increasing the passage number.
Table 5.4-Changes in telomere length (kb) determined by TRF analysis in control and treated samples

<table>
<thead>
<tr>
<th>Samples</th>
<th>Telomere length (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMEC1 p5</td>
<td>8.95</td>
</tr>
<tr>
<td>HMEC1 p20</td>
<td>7.82</td>
</tr>
<tr>
<td>Positive control DNA</td>
<td>10.18</td>
</tr>
<tr>
<td>untreated 21NT</td>
<td>1.45</td>
</tr>
<tr>
<td>DMSO-treated</td>
<td>1.35</td>
</tr>
<tr>
<td>5-aza-CdR 72hrs</td>
<td>2.33</td>
</tr>
<tr>
<td>5-aza-CdR/TSA 72hrs</td>
<td>3.30</td>
</tr>
<tr>
<td>5-aza-CdR 3 weeks</td>
<td>4.56</td>
</tr>
<tr>
<td>5-aza-CdR/TSA 3 weeks</td>
<td>2.45</td>
</tr>
<tr>
<td>5-aza-CdR 6 weeks</td>
<td>2.36</td>
</tr>
<tr>
<td>5-aza-CdR/TSA 6 weeks</td>
<td>2.32</td>
</tr>
<tr>
<td>5-aza-CdR 2 months / retreat</td>
<td>1.94</td>
</tr>
<tr>
<td>5-aza-CdR +TSA 2 months /retreat</td>
<td>1.55</td>
</tr>
</tbody>
</table>

Figure 5.11-Telomere length measurement by telomere restriction fragment length (TRF). Comparison of the telomere length in 21NT treated and control cell lines. The telomere length is shown in normal human epithelial cell strain (HMEC1 p5 and P20), the positive control DNA from immortal cell line (Roche), untreated 21NT cells, DMSO-treated, and 21NT treated with 5-aza-CdR and a combination treatment with 5-aza-CdR and TSA at 72hrs, 3 weeks, 6 weeks and 2 months plus retreatment for 72 hours. The analysis was performed with untreated 21NT cells with the lowest telomere length (kb) compared with 21NT treated cells at different time points and to the adjacent controls; HMEC1 p5, HMEC1 p20, and positive control DNA.
5.3.4-Telomere length analysis by quantitative real time PCR in 21NT treated and control cells

In order to confirm results obtained from TRF, iQ-FISH, and flow-FISH, the q-PCR technique was also performed. The average telomere length was measured by q-PCR in treated 21NT cells at different time points, untreated 21NT cells, DMSO-treated and HMEC1 controls. As shown in Figure 5.12, HMEC1 showed a mean telomere lengths of around 7.3 kb. In comparison with untreated and DMSO-treated controls, the short-term (72 hrs) and 3 weeks treatment of 21NT cells with 5-aza-CdR showed increase telomere lengths ranging from 4.5 kb to 4.7 kb. The average telomere length in untreated 21NT cells was approximately 2.5 kb which was consistent with published results (Cuthbert, Bond et al. 1999). The mean telomere length of the combination treatment of 21NT cells with 5-aza-CdR and TSA for 72 hours and 3 weeks had a significant telomere length increase of about 3.4 kb and 3.7 kb, respectively (P<0.05). However, no higher impact was observed with 6 weeks and 2 month treatment of 21NT cells with 5-aza-CdR and 5-aza-CdR/TSA in comparison with 72 hours and 3 weeks treatment (Figure 5.12).

Comparing the results obtained using TRF showed that 21NT treated cells had the highest increase in telomere length at 3 weeks of treatment with 5-aza-CdR (~4.56 kb) which was similar to the q-PCR result (~4.61kb) described earlier in this chapter (Figures 5.11 and 5.12).
Figure 5.12-Telomere length measurement by q-PCR. Comparison of the telomere length in 21NT treated and control cell lines. The telomere length is shown in the normal human epithelial cell line (HMEC1 p5), untreated 21NT, DMSO-treated, and 21NT treated with 5-aza-CdR and a combination treatment with 5-aza-CdR and TSA at 72 hrs, 3 weeks, 6 weeks and 2 months plus retreatment for 72 hours. The analysis was performed with untreated 21NT and DMSO-treated with the lowest telomere length (kb) compared with 21NT treated at different time points and to the adjacent controls; HMEC p5, untreated 21NT and DMSO-treated controls. Error bars represent SEM. Asterisk indicates difference between DMSO-treated cells and 21NT treated cell lines (*P<0.05).

Results from using the q-PCR and TRF techniques for telomere length measurement were approximately similar and showing an increase in telomere length (Table 5.5). However, there are some differences between two methods. Like q-PCR, the TRF method was performed using genomic DNA to measure telomere length. However, unlike with q-PCR, the TRF assay requires larger amounts of DNA. A significant drawback of the TRF method versus the q-PCR analysis is the fact that TRF estimates the length of telomeric
sequences that also contain subtelomeric repeats. These can vary in length based on the last restriction site at a given chromosome arm. Therefore increasing the TRFs heterogeneity influences the length of subtelomeric repeats and this prevents detection of the true length of telomere repeats (Vera and Blasco 2012). Previous findings by Steinert et al. (2004) showed that the mean length of subtelomeric portions is about 2 to 4 kb of sequence that is resistant to enzymatic digestion (Steinert, Shay et al. 2004). Unlike q-PCR and TRF methods, there was a considerable discrepancy between flow-FISH and iQFISH. Consequently, these two methods seem to be not as reliable and accurate as q-PCR and TRF. However, it appears that TRF and q-PCR techniques have both their benefits and disadvantages; it seems that molecular analyses in comparison with cytogenetic techniques are more quantitative and can measure changes in telomere length with better accuracy. The q-PCR method, in comparison with all the other methods used here, appears to be most accurate and reliable technique.

Table 5.5-Comparison of TRF and q-PCR results. This table represents the differences in telomere length measurements by TRF and q-PCR methods.

<table>
<thead>
<tr>
<th>Samples</th>
<th>TRF (kb)</th>
<th>q-PCR (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>1.45</td>
<td>2.44</td>
</tr>
<tr>
<td>DMSO-treated</td>
<td>1.35</td>
<td>2.64</td>
</tr>
<tr>
<td>HMEC1 p5</td>
<td>8.95</td>
<td>7.32</td>
</tr>
<tr>
<td>5-aza-CdR 72hrs</td>
<td>2.33</td>
<td>4.64</td>
</tr>
<tr>
<td>5-aza-CdR/TSA 72hrs</td>
<td>3.30</td>
<td>3.67</td>
</tr>
<tr>
<td>5-aza-CdR 3 weeks</td>
<td>4.56</td>
<td>4.61</td>
</tr>
<tr>
<td>5-aza-CdR/TSA 3 weeks</td>
<td>2.43</td>
<td>3.98</td>
</tr>
<tr>
<td>5-aza-CdR 6 weeks</td>
<td>2.36</td>
<td>2.81</td>
</tr>
<tr>
<td>5-aza-CdR/TSA 6 weeks</td>
<td>2.32</td>
<td>2.45</td>
</tr>
<tr>
<td>5-aza-CdR 2 months</td>
<td>1.94</td>
<td>3.04</td>
</tr>
<tr>
<td>5-aza-CdR/TSA 2 months</td>
<td>1.55</td>
<td>3.55</td>
</tr>
</tbody>
</table>

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5.4-Discussion

Telomere length dysregulation plays a major role in cancer development (Butler, Hines et al. 2012). Telomeric attrition is involved in genomic instability and in early events in tumorigenesis. Maintaining telomere structure and function relies on the interaction between telomere length and the Shelterin complex (Hu, Zhang et al. 2010). It has been shown in several independent studies that regulating the expression of telomere proteins can be implicated in telomere length regulation in cancer (Gao, Zhang et al. 2011; Butler, Hines et al. 2012). For instance, one previous study in gastric cancer (and precancerous gastric lesions) reported that over-expression of TRF1, TRF2, TIN2, and TERT was associated with the reduction of telomere length (Hu, Zhang et al. 2010). Additionally, recent findings by Butler et al. (2012) observed that the over-expression of POT1, TIN2, TRF1, and TRF2 in breast cancers was associated with a decrease in mean telomere length.

Since the 21NT cells has shorter telomeres (~3kb) (Cuthbert, Bond et al. 1999) than normal HMECs (~8kb) (Sputova, Garbe et al. 2013), we asked the question whether the increase in telomere length in 21NT cells treated with 5-aza-CdR and TSA could be due to demethylation and/or chromatin remodelling leading to an increase in Shelterin gene expression. This might suggest a mechanism linking telomere shortening and down-regulation of Shelterin genes in breast cancer cell lines.

The assessment of telomere dynamics is critically dependent on the telomere length measurement techniques chosen. In this study four different methods: TRF, iQ-FISH, flow-FISH and q-PCR were used to measure telomere length.
Two main lines of investigations were covered in the work described this chapter. First, the telomere length measurements were obtained from 21NT cells treated over a time course ranging from 72 hours to 2 months using the four different methodologies; all four methods were compared in terms of reliability and accuracy.

The telomere fluorescence intensity (TFI) of 21NT cells treated with 5-aza-CdR and TSA at different time points were measured by iQ-FISH and flow-FISH. The iQ-FISH results showed the TFI of 21NT treated cells was significantly higher in comparison with control (DMSO-treated) cells ($P<0.001$). No substantial difference in telomere lengths observed between 72 hours, 6 weeks and 2 month of treatment (Figure 5.8). We have also measured the telomere fluorescence intensity of 21NT treated, untreated, control (DMSO-treated) and HMEC1 cells in G0/G1 phase of cell cycle by flow-FISH. During the G0/G1 phase the cells stopped dividing; hence, before telomeres replication in late S phase, the average telomere fluorescence intensity in G0/G1 was examined. The average TFI of 21NT treated cells increased after 72 hours, 3 weeks and 6 weeks in comparison with the DMSO-treated control ($P<0.01$ and $P<0.001$ respectively). Nonetheless, it was observed that at 2 months following initial treatment of the 21NT cells, no difference in TFI was observed compared with the DMSO-treated control (Figure 5.10). The average telomere length determined by TRF showed that the telomere length in 21NT cells treated with 5-aza-CdR for 3 weeks had a higher increase in comparison with 72 hours, 6 weeks and 2 months treatment. However, the telomere length after short-term treatment (72hrs) increased compared with the DMSO-treated and untreated controls (Figure 5.11). Results of q-PCR showed approximately a two-fold increase in telomere length of 21NT cells for 72 hours and 3 weeks which were similar in comparison with TRF technique (Figure 5.12). No substantial differences in
telomere length have been observed in 6 weeks and 2 months retreated samples in comparison with 72 hours and 3 weeks treatment. The same trend held true when we looked at the expression levels of POT1 and TIN2 at different time points (previously discussed in Chapter IV). These results indicated that 5-aza-CdR and TSA lose their effectiveness in the longest time point of treatment (6 weeks and 2 months retreatment) (Figure 5.12). In other words, 5-aza-CdR treatment is reversible and not permanent.

All these findings support an emerging view that Shelterin genes POT1, TIN2 and TPP1 positively and negatively regulate telomere length elongation by telomerase. The TPP1-POT1 complex is presumably activating telomerase processivity under the certain circumstances. This complex covers the 3' overhang of single stranded telomeric DNA and inhibits binding of the telomere to telomerase (Wang, Podell et al. 2007). Moreover, disruption of this complex (TPP1-POT1) results in inhibition of POT1 to localize to telomeric DNA (Liu, Safari et al. 2004). It is moreover likely that the association of POT1-TPP1-TIN2 is important for recruitment of telomerase to the telomere. The data in this chapter have demonstrated that up-regulation of Shelterin genes through demethylation of their promoters co-operate with an effect on telomere length.

mRNA expression of Shelterin genes was silenced in breast cancer cell lines by DNA methylation and histone deacetylation. As discussed earlier, (Chapter IV), POT1, TIN2 and TPP1 were significantly induced in 21NT cells treated with DNA methylation inhibitor 5-aza-CdR and the histone deacetylation inhibitor, TSA (P<0.05). This further supports the hypothesis that these genes were silenced by DNA methylation and histone deacetylation. The results suggest that after treatment with 5-aza-CdR and TSA, Shelterin gene expression was increased. Correlation between 5-aza-CdR and TSA induced Shelterin gene expression
and increased telomere length. However, it is recognised that 5-aza-CdR and TSA will affect expression of many genes. Nonetheless, the possible association shown here is of interest.
Chapter VI

OVER-EXPRESSION OF HUMAN POT1 IN 21NT BREAST CANCER CELL LINE REGULATES TELOMERE LENGTH ELONGATION
6.1-Introduction

Earlier in this project (Chapter IV), when the 21NT breast cancer cell line was treated with 5-aza-CdR and TSA, evidence for up-regulation of Shelterin genes was observed. Significant re-expression of POT1 and TIN2 was seen after short-term and long-term treatment of 21NT cells. Moreover, previous results showed that up-regulation of Shelterin genes was positively correlated with increased telomere length.

The telomere binding proteins (TBPs) have been proposed to regulate telomerase enzyme activity at the chromosome level. POT1 is the only protein that is able to bind directly to the 3’ single strand of telomeric DNA via its oligonucleotide/oligosaccharide-binding (OB) fold domain (Baumann and Cech 2001; Lei, Podell et al. 2004; Loayza, Parsons et al. 2004; Gao, Zhang et al. 2011). It has been found that TPP1 recruits POT1 to telomeres. Moreover, the amino terminus of TPP1 has a telomerase-interacting domain, signifying that TPP1 plays a role in the recruitment of telomerase to chromosome ends (Tejera, Stagno d’Alcontres et al. 2010). Additionally, in the present of POT1, the Shelterin components RAP1, TRF1, and TRF2 localise to the telomere. Therefore, the Shelterin complex may require POT1 in order to maintain telomere length (Ramsay, Quesada et al. 2013) and it seems that POT1 is implicated in telomere length maintenance. Therefore, this gene was chosen for further analysis to investigate the effect of POT1 in telomere length elongation. In this connection, our working hypothesis was that ectopic over-expression of POT1 in 21NT breast cancer cells may regulate telomere length.
6.2-Materials and methods

6.2.1-Transformation of POT1 cDNA into bacterial cells

The full length of human POT1 gene (2kb, variation 1) was cloned into the plasmid vector pcDNA/FRT/V5-His-TOPO (a kind gift from Roger Reddel (Colgin, Baran et al. 2003)). This vector contains hygromycin for selection in mammalian cells. For amplification, 100ng of plasmid pcDNA5-POT1 was added into a vial of One Shot® TOP10 Chemically Component E. coli and incubated on ice for 30 minutes. The sample was heat-shocked at 42°C for 30 seconds and then incubated on ice for 2 minutes. After that, 250µl of SOC medium was added to the tube. The tube was capped tightly and shakes horizontally in a Gallenkamp orbital shaker at 200rpm at 37°C for one hour. Afterwards, 200µl of SOC medium was spread on pre-warmed LB-agar (Sigma) plate containing 50µg/ml ampicillin. Plates were incubated at 37°C overnight. Then, 6 single ampicillin resistant bacteria clones were picked and used to inoculate 5ml of LB-Broth (Sigma) media containing 50µg/ml ampicillin. The cultures were grown overnight in an orbital shaker at 200rpm at 37°C.

6.2.1.1-Isolation of plasmid DNA with Alkaline Protease Solution

Samples were purified from cultured transformed bacteria using Wizard® Plus SV Minipreps DNA purification Kit (Promega). The bacterial cells were collected by centrifugation at 16000rcf for 5 minutes. The supernatant was removed and the pellet was re-suspended by adding 250µl of Cell Re-suspension Solution. Then 250µl of Cell Lysis Solution was added to each sample and mixed by inverting it 4 times. Afterwards, 10µl of Alkaline Protease Solution was added to the tubes, mixed by inversion, incubated at room temperature for 5 minutes. 350µl of Neutralization Solution was then added and mixed.
Subsequently, the tubes were spun at 16000 rcf at room temperature for 10 minutes. The entire mixture was transferred into Spin Column and centrifuged at top speed for 1 minute at room temperature. The flow-through was discarded and spin column was placed back in the same collection tube. The columns were washed twice with 750µl of Wash Solution prepared with ethanol and centrifuged at 16000 rcf for 1 minute. The elution of plasmid DNA was performed by placing the columns in clean 1.5ml micro-centrifuge tubes and adding 100µl of Nuclease-Free Water to the centre of the membrane. The sample was eluted by centrifugation at full speed for 1 minute. Then, the tubes containing plasmid DNA were stored at -20°C until further processing. The concentration of plasmid DNA was adjusted. Then, for plasmid confirmation by restriction enzyme analysis, 1µg of extracted plasmid DNA were cut in a 20µl of reaction volume containing 2µl of BamH1, 2µl of Buffer, 0.1 µl of Bovine serum albumin (BSA) and made up to 20µl with sterile distilled water. The reaction mixture was incubated at 37°C for 2 hours. After digestion, DNA samples were run on 0.8% agarose gel for 2 hours (Figure 6.1).

![Image of a typical 0.8% of agarose gel electrophoresis with digested plasmid DNA to check the size of plasmid.](image)

The lane on the left side shows the molecular size marker. The 7kb band seen on the gel represents of POT1 gene attached to the 5kb plasmid vector.
6.2.1.2-QIAGEN® plasmid purification Maxi-prep Kit

The Maxiprep purification was carried out to obtain the most pure form of DNA and was used according to the manufacturer’s instructions. Briefly, a single transformed bacterial colony, which was confirmed as having the POT1 insert, was grown in 5ml of LB-Broth media containing 50µg/ml ampicillin on an orbital shaker at 200rpm overnight at 37°C. Then, 2ml of cultured media was added to 100ml of LB-Broth media containing 50µg/ml ampicillin and was placed in an orbital shaker at 200rpm overnight at 37°C. The bacterial cell pellets were collected by centrifugation at 16000rcf for 15 minutes at 4°C. The cell pellet was re-suspended in 10ml of Buffer PI (50mM Tris-HCl pH 8, 10mM EDTA, and 100µg/ml RNase A). 10ml of Buffer P2 (200 mM NaOH, 1% SDS) was added to the samples and mixed thoroughly by inversion 4 to 6 times, and incubated at room temperature for 5 minutes. After that, 10ml of chilled Buffer P3 (3M potassium acetate pH 5.0) was added to the tubes and mixed by vigorously inverting 6 times. The lysate was poured into the barrel of a QIAfilter Cartridge and then were incubated at room temperature for 10 minutes. The cap was removed from the QIAfilter Cartridge outlet nozzle and the plunger was gently inserted into the QIAfilter Cartridge. Afterwards, the cell lysate was filtered into the equilibrated QIAGEN-tip. The supernatant was loaded into a QIAGEN-tip column previously equilibrated with 10ml of Buffer QBT (750mM NaCl, 50mM MOPS pH 7.0, 15% iso-propanol, 0.15% TritonX-100) and allowed to empty by gravity flow. The columns were washed twice with 10ml of Buffer QC (1.0M NaCl, 50mM MOPS pH 7.0, 15% iso-propanol) and the plasmid DNA was eluted with 5ml of Buffer QF (1.25M NaCl, 50mM Tris-HCl pH 8.5, 15% iso-propanol). Plasmid DNA was precipitated by adding 3.5ml of iso-propanol and centrifuged at 16.1rcf for 30 minutes at 4°C. The supernatant was carefully decanted DNA pellet was
washed with 2ml of room-temperature 70% ethanol and spun down at 16,000 rcf for 10 minutes. The DNA was dried and re-suspended in 200µl of DNase-free water. The samples were kept on ice for 30 minutes and the concentration of plasmid DNA was measured.

6.2.1.3-Transfection procedure using GeneJuice®

One day prior to transfection, 15x10^5 21NT cells still in the exponential phase of growth were seeded into p100 tissue culture dish. The cells grew overnight to reach about 80% confluency. For each 100-mm dish to be transfected, 800µl of serum free modified Eagle medium was placed into a sterile 1.5ml tube. 18µl of GeneJuice® Transfection Reagent (Novagen) was dropped directly to the serum-free medium. The tube was mixed thoroughly by vortexing and incubated at room temperature for 5 minutes. Three tubes were prepared; one was a transfection reagent control (GeneJuice reagent/serum-free medium), another was POT1 plasmid and the final tube was an empty vector control (pcDNA3.1/hygro 5.6kb plasmid kindly provided by Dr Evgeny Makarov). After that, 10µg of POT1 plasmid and 10µg of pcDNA (control) were added to each tube of GeneJuice reagent/serum-free medium mixture by gentle pipetting and incubated at room temperature for 15 minutes. The cells were washed with PBS and 10ml of fresh culture medium was added. Then, prepared precipitates were added to each plate and incubated for 24 hours. After incubation for overnight, the culture medium was aspirated and divided in 10 plates. For the purposes of this study, we wanted to isolate stable clones that over-expressed POT1. To do this, 21NT transfected cells were routinely cultured in modified Eagle’s medium with 400U/ml Hygromycin B as a selection marker. This selection marked that only clones which have picked up the vector carrying the selectable marker will grow. Cells that do not contain the
vector will die after several weeks in culture. Subsequently the incubation was continued for 3 to 4 weeks, allowing for growth and selection of 21NT-transfected colonies.

**6.2.1.4-Picking of cell colonies**

In preparation for picking cell colonies, cloning cylinders of varying sizes were cleaned and sterilised by submersing them in 100% pure ethanol (Hayman) for one day prior to use. The position of colonies was identified under a microscope and colonies well separated from one other were chosen for isolation. Each clone was marked by a permanent marker on the bottom of the plate. The media was removed and washed with pre-warmed PBS. After gentle swirling and aspiration of PBS, an appropriately sized cloning cylinder was removed from 100% pure ethanol solution with sterile forceps and firmly pressed into pre-autoclaved Vaseline to create a thick layer on the bottom of the cylinder. The cloning cylinder was placed carefully over the colony based on the circle drawn on the bottom of the dish and presses down firmly to form a tight seal. Then 100-250µl of TrypLE Express (depending on the cylinder size) was pipetted into the cylinder. This process was repeated if several colonies were to be isolated from the same plate, and the cells were incubated at 37°C for 3 minutes. After incubation, the cells were checked under the microscope to make sure that they were detached. Subsequently, the cells were retro-pipetted several times and transferred to a 12 well-plate containing 1ml of pre-warmed complete medium. This step was repeated several times until all colonies had been picked and transferred to the 12 well-plates. Each clone was cultured until the cells reached 90% confluence; they were then harvested and transferred to a P60 plate. Once the cells had reached 90% confluence on a P100 dish they were used for further analysis.
6.2.2-Quantification of telomerase activity using RQ-TRAP assay

6.2.2.1-Protein isolation

Cell lines to be analysed were grown to 80-90% confluence on a P100 dish. At this point cells were harvested and total cell number was determined. The cell pellet was obtained by centrifuging at 15,000rcf for 5 minutes. The supernatant was aspirated and the pellet was washed once with pre-warmed PBS. Sample pellets were left on wet ice and 200µl CHAPS lysis buffer (TRAPEZE® 1x CHAPS MILLIPORE Company) per $10^5-10^6$ cells was used to re-suspend the pellets. All samples were retro-pipetted several times and the cell suspension was transferred to a fresh tube. The suspension was incubated on wet ice for 30 minutes before centrifugation at 12,000rcf for 20 minutes at 4°C. 150µl of the supernatant was transferred into a fresh tube and determined the protein concentration for all the samples including the one was going to be used as the standard curve. The remaining extract was aliquoted and immediately stored at -80°C until required.

6.2.2.2-Determination of protein concentration

The protein concentration of samples was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific). Pierce BCA Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. The assay was performed according to manufacturer’s guidelines. A standard calibration curve was set up using bovine serum albumin (BSA) diluted in CHAPS lysis buffer, ranging from concentrations 0-2µg/ml (Figure 6.2). All unknown sample protein concentrations were measured against the standard curve (Table 6.1).
Table 6.1 - Preparation of diluted BSA standards for BCA analysis

<table>
<thead>
<tr>
<th>Tube</th>
<th>Volume of dH₂O</th>
<th>Volume of BSA</th>
<th>Final BSA concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>90</td>
<td>10</td>
<td>0.2</td>
</tr>
<tr>
<td>C</td>
<td>80</td>
<td>20</td>
<td>0.4</td>
</tr>
<tr>
<td>D</td>
<td>70</td>
<td>30</td>
<td>0.6</td>
</tr>
<tr>
<td>E</td>
<td>60</td>
<td>40</td>
<td>0.8</td>
</tr>
<tr>
<td>F</td>
<td>50</td>
<td>50</td>
<td>1</td>
</tr>
<tr>
<td>G</td>
<td>25</td>
<td>75</td>
<td>1.5</td>
</tr>
<tr>
<td>H</td>
<td>0</td>
<td>100</td>
<td>2</td>
</tr>
</tbody>
</table>

200µl of Working Reagent (BCA protein assay reagent A/B diluted 50:1) was prepared for each aliquot of protein extract and BSA protein standard concentration. To each 5µl of protein lysate, 200µl of the Working Reagent was added and the samples were vortexed thoroughly on a shaker for 30 second. The tube was incubated at 37°C for 30 minutes in water bath and then allowed to cool at room temperature. Subsequently, 100µl of each sample was added to 96-well plate. The A562 of the standards and protein lysates was then measured using a plate reader (BP800, BioHit). A standard curve was prepared by plotting the blank-corrected measurement for each BSA standard against its concentration. The standard curve was then used to determine the protein concentration of each study sample.
6.2.2.3-Quantitative telomere-repeat amplification (TRAP) Assay

Telomerase activity was measured using a quantitative TRAP assay (Paraskeva, Atzberger et al. 1998). Bench-top surfaces and pipettes were cleaned with RNase Zap (Ambion) in preparation for the assay. Samples to be analysed were thawed on wet ice and diluted to a final concentration of 250ng/µl in CHAPS lysis buffer. Stock solutions were immediately quick-frozen and stored at -80°C to prevent protein degradation. Reaction mixtures for each of the samples to be analysed were made up using the following components and volumes:

-12.5µl of standard iTaq™ Universal Syber®-green (BIO-RAD)

-1µl telomerase primer (ACX primer) (0.05µg/µl) (5’-GCGCGG (CTTACC) 3CTAACC-3’)

-1µl telomerase primer (TS Primer) (0.1µg/µl) (5’-AATCCGTCGAGCAGAGTT-3’)

![Standard Curve](image)

**Figure 6.2** - Standard curve used in protein quantification.

\[
y = 0.0977x + 0.0775 \\
R^2 = 0.9945
\]
- 1µl protein samples

- 15.5µl nuclease free water

- Total volume = 25µl

Reaction mixtures were thawed on ice to prevent protein degradation, vortexed and centrifuged briefly. Samples were assayed in triplicate and each assay run included a telomerase positive, negative and non-template control. As an additional negative control, 10µl of each target sample was heat-treated to inactivate the enzyme by incubating at 85°C for 10 minutes. The microtitre plate was then loaded into the thermocycler block and PCR amplification parameters were carried out with the following reaction conditions. The reaction mixture was first incubated at 25°C for 20 minutes to allow the telomerase in the protein extracts to elongate to TS primer by adding TTAGGG repeat sequence. PCR was then started at 95°C for 10 minutes to activate Taq polymerase followed by a two-step PCR amplification of 35 cycles at 95°C for 30s and 60°C for 90s. Telomerase activity is reported as standard cell equivalents. To quantify telomerase activity, a standard curve was generated from serially diluted telomerase positive prostate cell line; PC-3/hTERT extracts (10^6-10^2). The threshold cycle values (C_t) of the unknown samples were read off against the standard curve, which gives the level of telomerase activity compared with PC-3/hTERT cells. All RQ-TRAP reactions were analyzed in triplicate and error bars created using standard error.
6.3-Results

6.3.1-POT1 over-expression facilitates telomere length elongation on 21NT cells

POT1 is one of a six proteins which makes up the Shelterin complex that specifically binds to the G-rich single strand tail of mammalian chromosomes. POT1 function in cancer cells has been extensively studied in order to understand how this protein maintains telomere function and regulates the telomerase enzyme activity in disease states. Previous work by Colgin et al. (2003) showed that the over-expression of full length POT1 in telomerase positive HT1080 cells increased telomere length in over-expressed cloned cells. Therefore, based on these findings we hypothesized that over-expression of POT1 in 21NT cells might positively regulate telomere length.

6.3.2-Determination of relative POT1 mRNA levels in 21NT transfected clones

In order to evaluate the effects of POT1 (variation 1) over-expression in 21NT cancer cells, these cells were transfected with human POT1. cDNA from two stable clones plus non-transfected 21NT cells, and ten vector control clones were synthesized from total RNA (Figure 6.3).
Figure 6.3-Images of 21NT transfected clones at 10x magnification. Representative images of 21NT transfected clones growing after 3-4 weeks in selection before being picked and cultured as a separate cell line. A) Image of POT1-1/pcDNA5, B) POT1-3/pcDNA5, C) POT1-4/pcDNA5, D) empty vector control clone (pcDNA3.1-1). POT1 transfected cells appeared to grow more slowly in comparison with empty vector control clone.

The expression levels of POT1, in 21NT transfected cells were quantified using qRT-PCR and normalized against endogenous GAPDH. As shown in Figure 6.4, POT1 mRNA levels were elevated 3-4 fold in the POT1-1/pcDNA5 and POT1-3/pcDNA5 compared with non-transfected 21NT cells. This difference was statistically significance when compared with the level in non-transfected 21NT cells ($P<0.5$). There were no substantial differences in expression of POT1 in vector control clones (pcDNA3.1-1) in comparison with 21NT control cells (Figure 6.4).
6.3.3-Western blot analysis

In order to confirm over-expression of POT1 in transfected 21NT cells at the protein level, western blot analysis was performed. The western blot analysis was carried out using POT1 rabbit monoclonal antibody (Abcam) and the values were normalised using β-Actin rabbit antibody. A 71-KD band which corresponds to the size of the POT1 protein was evident in non-transfected 21NT cells, two vector control clones, PC-3 included for comparison, and normal mammary epithelial cell strain (Figure 6.5-A). After imageQuant 5.0 densitometry analysis, the POT1 protein levels were found to be elevated more than 30% in the 21NT cells over-expressing POT1 compared with vector controls, 21NT cells, PC-3 cells,
and normal mammary epithelial cell strain (HMEC1) controls which was broadly in line with the qRT-PCR results (Figures 6.4 and 6.5-B).

Figure 6.5-Western blot analysis of POT1 expression in 21NT transfected and control cell lines. A) A 12% SDS-PAGE gel indicating POT1 protein expression and β-Actin ratio levels in western blots of transfected 21NT cells, two vector controls, PC-3, non-transfected 21NT and normal mammary epithelial cell strain (HMEC-p10). B) Densitometric analysis of POT1 protein normalised to total β-Actin protein and reported as optical densitometry (OD) units.
In order to investigate the interaction between *TPP1* and *POT1*, the mRNA levels of *TPP1* in *POT1* over-expressing 21NT cloned was quantified. The mRNA expression of *TPP1* was increased in the POT1-1/pcDNA5 and POT1-3/pcDNA5 in comparison with vector controls and non-transfected 21NT. However, these increased did not reach a statistical significance (Figure 6.6).

**Figure 6.6** Represents comprehensive expression levels of *TPP1* after over-expression of *POT1* in transfection of 21NT cells. Data shows the average expression of *TPP1* in 21NT un-transfected control, two vector control clones and two clones of *POT1* transfection relative to GAPDH. The un-transfected 21NT cells were used as the calibrator. RQ indicates relative quantification and error bars represent SEM.
6.3.4-Telomere length analysis of 21NT transfected cells

Previous studies by Colgin et al. (2003) showed that over-expression of POT1 in telomerase-positive HT1080 cells results in increased telomere length. In addition, subsequent studies have confirmed that reduction of POT1 by RNA interference (RNAi) causes loss of telomeric single-stranded overhangs and induces chromosomal instability and apoptosis (Yang, Zheng et al. 2005).

In order to obtain a better and clear understanding of the exact role and function of POT1 in protection and maintenance of telomeres in human breast cancer, we needed to determine whether over-expression POT1 may play a role in telomere length maintenance. For this analysis the q-PCR technique was used as it was the most accurate and quickest (Chapter V) to measure telomere length. After about 4 weeks, genomic DNA was extracted from two stable clones, non-transfected 21NT cells, two vector control clones, PC-3 was included for comparison and a normal mammary epithelial cell strain (HMEC1) using Wizard™ Genomic DNA Kit as described in Section 2 (Chapter II). As shown in Figure 6.7, PC-3 and HMEC1 controls showed telomere length ranging from 4.5 to 7.2 kb respectively. The average telomere length of 21NT non-transfected and empty vector control clones ranged from 2.5 to 3 kb, while the POT1 over-expressing clones were between 4 to 5 kb. This represents an increase in telomere length of approximately 2kb around 4 weeks after transfection. Overall, the results showed that telomeres were elongated in two clones whereas no elongation was observed in the two vector control clones (Figure 6.7). Therefore, it seems that POT1 is a positive regulator of telomere length.
Figure 6.7-Telomere length measurement by q-PCR. Comparison of the telomere length in 21NT transfected and control cell lines. The telomere length is shown in normal human epithelial cell line (HMEC p5), non-transfected 21NT, and two vectors control clones, PC-3 and two stable colons. The analysis was performed with non-transfected 21NT and two vector control clones with the lowest telomere length (kb) compared with two stable clones to the adjacent controls; HMEC1 p5 and PC-3 controls. Error bars represent SEM.

6.3.5-Analysis of telomerase enzyme activity

Telomerase activity within stable clones was measured in order to search for a link between telomerase activity and increased telomere elongation. The quantitative TRAP assay was carried out using PC-3/hTERT as a positive control (provided by Dr Terry Roberts). This cell line expressed high levels of exogenous hTERT and as a result has high telomerase enzyme activity, telomerase positive non-transfected 21NT cells, and the telomerase negative HMEC1 cell strain; all were used as control samples. To quantify telomerase activity, PC-3/hTERT control was serially diluted and the relevant standard curve plotted.
Based on the equation obtained, the mean $C_t$ values for unknown samples calculated and telomerase activity was calculated relative to the telomerase positive control (Figure 6.8). Negative controls for each individual sample to determine heat-sensitivity, were also included by heat-treating (HT) an extract of each sample. The results, presented in Figure 6.8, show that approximately two-fold reduction in telomerase enzyme activity was observed in two stable clones compared with 21NT cells, non-transfected, and PC-3/hTERT controls. Our data suggested that telomere elongation by POT1 is not mediated by increased telomerase activity.

![Figure 6.8-Quantitative telomerase activity](image)

**Figure 6.8-Quantitative telomerase activity.** All values were acquired based on $C_t$ values. Relative telomerase enzyme activity was obtained utilizing a standard curve generated by the serial dilution of PC-3/hTERT. The telomerase enzyme activity is shown in normal human epithelial cell line (HMEC1 p5) as a negative control, non-transfected 21NT, PC-3/hTERT as a positive control, two vector control clones and 21NT POT1 expressing clones. The analysis was performed with the highest telomerase enzyme activity assigned to the PC-3/hTERT sample and the activity in the unknown worked out as a percentage PC-3/hTERT sample. Error bars represent SEM. Asterisk indicates significant difference between 21NT cells and transfected cells (* $P<0.05$).
6.4-Discussion

Based on the results obtained from work described in Chapter V, which showed a significant increase in telomere length after 72 hours and 3 weeks treatment of 21NT cells with 5-aza-CdR and TSA, it was of interest to explain whether any one of the Shelterin proteins may play a role in this phenomenon. In other words could forced over-expression of any of the Shelterin genes stabilise telomere length in breast cancer cells? Or conversely could reduced expression of Shelterin genes lead to telomere lengthening (Yang, Zheng et al. 2005). It was previously reported that after each cell division, the 3’ single stranded telomeric DNA becomes approximately 50 to 100bp shorter in the absence of telomerase activity (Stewart, Ben-Porath et al. 2003). Therefore, it has been suggested shortening of telomeric DNA occurs at the 3’ overhang. It has been shown that POT1 is the only Shelterin protein that binds directly to the G-rich tail of telomeric DNA (Martinez and Blasco 2010). Therefore, it can be considered that POT1 is possibly one of the most important candidates within the Shelterin complex to participate in telomere elongation in breast cancer cells. The role of POT1 in telomere length regulation has been the subject of intense investigation. For instance, Kendellen et al. (2009) showed that deletion of the OB fold of the POT1 domain induces telomere elongation (Kendellen, Barrientos et al. 2009). However, findings by Colgin et al. (2003) appeared to be contradictory to this. They observed that over-expression of POT1 in telomerase-positive cells resulted in telomere length elongation.

Based on the relevant publications highlighted above, and the work described in the Chapter V, showing that significant re-expression of POT1 in 21NT cells was observed after treatment with 5-aza-CdR and TSA (see Chapter IV), we set out to find what effect over-expression of POT1 has on telomere length. To test whether over-expression of POT1
induces telomere lengthening, 21NT cells were stably transfected with human *POT1* (variation 1) cDNA. The mRNA levels of *POT1* increased more than three-fold compared with the empty vectors and non-transfected 21NT controls. The over-expression of POT1 at the protein levels was confirmed by western blotting. POT1 protein levels were elevated approximately 40% in the 21NT stable transfectants in comparison with controls which were broadly consistent with the qRT-PCR.

In order to determine the effect of over-expression of *POT1* on the function of *TPP1*, the mRNA levels of *TPP1* was quantified. The expression levels of *TPP1* were increased in the POT1-1/pcDNA5 and POT1-3/pcDNA5 compared with empty vector controls and non-transfected 21NT cells. However, these increases did not reach a statistical significance. TPP1 has been previously identified as a partner of POT1 and binds directly to TIN2 and form a part of Shelterin protein complex (Takai, Kibe *et al.* 2011). Consistent with these observations, over-expression of *POT1* may perhaps play an important role to elevate the expression of *TPP1* in 21NT transfected cells. Therefore, it seems that the interaction of POT1 and TPP1 regulates telomere lengthening and enhance POT1 affinity for 3’ single stranded telomeric DNA.

To investigate the effect over-expression of *POT1* has on telomere length elongation; the average telomere length was examined by q-PCR. The results showed that the average telomere length of the *POT1* over-expressing clones was about 2 to 3 kb longer in comparison with 21NT non-transfected and empty vector control clones. The same trend held true when we looked at telomere length at different time point treatments (previously discussed in Chapter V).
Based on these results we asked the question whether the increased telomere length was the result of telomerase activity? Therefore, telomerase enzyme activity was assessed by a quantitative TRAP assay. Surprisingly, there was about a 2-fold reduction in telomerase activity in the \textit{POT1} transfected clones which had increased telomere length when compared with non-transfected 21NT cells and empty vector controls. As expected, telomerase activity in normal diploid HMEC1 cells was extremely low.

The result showed that telomere elongation by over-expressing \textit{POT1} (variation 1) is not due to a direct effect of the telomerase enzyme. These results are interesting in light of and consistent with the previous finding by Colgin \textit{et al.} (2003) showing that telomere elongation by over-expression of \textit{POT1} was not accompanied by an increase in telomerase activity. In addition, Yang \textit{et al.} (2007) observed a significant increase in telomere length following over-expression of \textit{POT1} (variation 1) in the HT1080 human fibrosarcoma cell line. They reported that this telomere lengthening was likely to be telomerase dependent as they did not observe an increase telomere length in telomerase negative human fibroblasts (Yang, Zhang \textit{et al.} 2007).

It has been found that \textit{POT1} inhibits telomerase activity presumably by obstructing access to the 3' overhang (Colgin, Baran \textit{et al.} 2003). However, this does not mean that \textit{POT1} completely inhibits activation of telomerase at the 3' telomeric overhang. Hence, it seems likely that \textit{POT1} negatively regulates telomerase access to the telomere (Colgin, Baran \textit{et al.} 2003; Yang, Zhang \textit{et al.} 2007). It is conceivable that telomere length may be regulated by the interaction of \textit{POT1} with TPP1 complex. Moreover, it is possible that the interaction of \textit{POT1}-TPP1 complex with telomeric single-stranded DNA regulates telomere elongation, suggesting this association appears to play a fundamental role in the capping...
function of POT1. In addition, the interaction of POT1 with TPP1 is necessary for telomerase dependent telomere lengthening as TPP1 can directly correlate with one of the catalytic subunit of telomerase enzyme, i.e. hTERT (Yang, Zhang et al. 2007).

Two theories about the function of telomere regulation have been described; “open” and “closed” states. It is argued that the “closed” conformation protects telomeric DNA from end-to-end fusion, implying that this condition is possibly implicated in the T-loop model. However, in the “open” state the telomere allows telomerase to access to the chromosome end (Colgin, Baran et al. 2003). This hypothesis leads to speculation that one possible prominent role for POT1 is to bind directly the 3’ overhang of telomeric DNA and stabilize telomeres in the “open” state. Therefore, it is conceivable that over-expression of POT1 displaces the T-loop formation and helps telomeres remaining in the “open” conformation (Colgin, Baran et al. 2003). Thus, according to this theory, we can assume that POT1 may allow telomerase access to telomere which results in increased telomere length. Collectively, according to our finding, it could be hypothesized that POT1 may perhaps be a negative regulator of telomerase activity to regulate telomere length (Colgin, Baran et al. 2003). It seems that POT1 may possibly regulate a localized activation of telomerase at the telomere end in telomerase-positive cancer cell lines. Taken together, it is interesting to speculate that POT1 may be a useful target for developing anti-cancer therapeutic agents against cancer.
Chapter VII

GENERAL DISCUSSION AND FUTURE DIRECTIONS
Maintenance and regulation of chromosome ends, at the telomere is fundamental for genome stability. Telomeres are made up of hexameric repeats to which Shelterin proteins bind in order to protect chromosome ends from end-to-end fusion, thus preventing them from being recognised as sites of DNA double strand breaks (Butler, Hines et al. 2012). Telomere dysfunctions have been implicated in most epithelial carcinomas. For instance, in 150 cases of breast tumours, more than 50% had significantly altered (mainly shorter) telomere lengths in comparison with normal breast tissues (Meeker, Hicks et al. 2004). The relationship between telosomal DNA-binding proteins and telomere length maintenance has become a popular area of interest recently. Butler et al. (2012) demonstrated that the mRNA levels of TRF1, TRF2, TIN2 and POT1 were correlated with telomere length in breast tumours. Furthermore, earlier investigations had shown that down-regulation of POT1 correlates with telomere length dysfunction in gastric carcinoma (Kondo, Oue et al. 2004). Based on the advances highlighted above, this project aimed to examine whether the expression of Shelterin and Shelterin-associated genes is altered in breast cancer cell lines and if so whether telomere length maintenance is affected. Answers to distinct questions relating to the evaluation of changes in telomere length in breast cancer cell lines were sought. First, we asked whether there is up-regulation or down-regulation of Shelterin and Shelterin-associated genes in breast cancer cell lines. To answer this, the mRNA and protein levels of these genes in a panel of ten breast cancer cell lines were quantified (Chapter III). Second, in an attempt to understand the causes and consequences of any observed alteration in the regulation of Shelterin and Shelterin-associated genes in breast cancer cell lines, the epigenetic regulation of these genes were assessed (Chapter IV). Third, changes in telomere length in breast cancer cell lines following epigenetic changes were measured with
different techniques (Chapter V). Finally, and most importantly, the aim was to identify a single Shelterin gene (POT1) to study in greater detail. Ectopic over-expression of POT1 in the 21NT breast cancer cell lines was performed in order to understand more about the molecular mechanisms that may possibly contribute to alterations in telomere length regulation (Chapter VI).

Previous work done by Salhab et al. (2008) demonstrated an increase in the mRNA levels of TNKS1, hTERT, EST1, and TEP1 in breast cancer tissues whereas TNKS2 and POT1 were down-regulated in comparison with normal breast tissue. More recently, Gao et al. (2011) reported over-expressed levels of POT1 in gastric cancer tissues. Furthermore, TRF1, TRF2 and TIN2 were significantly over-expressed in precancerous lesions, gastric cancer tissues, and lymph node metastases in comparison with normal gastric mucosa tissues (Hu, Zhang et al. 2010; Gao, Zhang et al. 2011). Different patterns of mRNA expression of Shelterin and Shelterin-associated genes may result in telomere length dysfunction in breast cancer cell lines. Therefore, the focus of the first set of experiment (Chapter III) in this study was to quantify mRNA and protein levels of these genes in different breast and prostate cancer cell lines. Some of the Shelterin genes have different splice variants. Therefore, in order to investigate a difference between each splice variant, their mRNA levels were quantified by qRT-PCR (Chapter III).

Results showed that POT1 splice variant (SV) 1 and 2, TRF1 (SV1 and SV 2) and TRF2, SMG6 (SV1), TIN2 (SV1 and SV2), TEP1, TNKS2, and RAP1 were significantly down-regulated at the mRNA level in breast cancer cell lines in comparison with RNA extracted from normal mammary breast tissue. However, interestingly, TPP1 mRNA levels were higher in most breast cancer cell lines compared with normal breast tissue. Tissues are mixed populations
of tightly connected cells. Gene expression can vary from one cell to another dependent on their distinct function. Therefore, differences in the expression of Shelterin and Shelterin-associated genes between individual tissues and cells may be observed. Hence, in order to validate telosomal gene expression results further, a normal breast mammary epithelial cell strain (HMEC1) was analysed for mRNA levels of POT1 and TPP1. The mRNA levels of POT1 were considerably lower in all cancer cell lines in comparison with HMEC1. The protein levels of Shelterin genes were next examined and it was found that 21NT, 21MT2 and HS578-T expressed considerably higher levels of POT1 protein compared with HMEC1 which was not in agreement with the mRNA expression data. As expected, TPP1 protein was over-expressed in most breast cancer cell lines compared with HMEC1 (Chapter III). However, the level of TPP1 protein was lower in 21NT and 21MT-2 than HMEC1 control, which again did not correlate with the mRNA expression data.

It is commonly believed that mRNA expression levels correlate with protein levels within cells but clearly this is not always the case. For instance, Schwanhausser et al. (2011) quantified protein and mRNA levels of 5279 unique proteins in NIH3T3 mouse fibroblast to analyse the correlation between expression levels of protein and mRNA. These investigations found proteins to be on average five times more stable than their mRNAs. Moreover, Tian et al. (2004) used multipotent mouse EML cells and their differentiated progeny MPOR cells to map the abundance ratios of 425 proteins and compared them to the amount of their corresponding mRNAs. They showed that 150 genes have alterations at their protein and/or mRNA levels between the two cell types. In addition, the EML cells showed a 5-fold higher level of c-kit ligand protein (aka stem cell factor) but no change in mRNA levels. Thus, based on these two papers and other published data from independent
researchers, it could be concluded that the concentration of proteins and their mRNA is not always proportional to each other and concentration of one cannot be used universally to estimate the concentration of the other. In contrast to the findings of the above cited studies is that mRNA and protein levels of housekeeping genes are generally stable.

The observed relationship between the mRNA and protein levels of TPP1 may be explained by the above reasoning. TPP1 was expressed at high mRNA and low protein levels. However, there is also an unexpected relationship observed in these results; POT1 had a high level of protein and low level of mRNA in most breast cancer cell lines compared with HMEC1. Furthermore, the POT1-encoded protein had lower expression levels in the normal control HMECs compared with 21NT cancer cell line. Previous work by Marks et al. (1991) showed that high p53 protein levels were associated with inactivating mutations in ovarian cancer cells (Marks, Davidoff et al. 1991). In addition, the results from a recent study indicated recurrent somatic mutations in the POT1 gene in cancer cells (Ramsay, Quesada et al. 2013). Therefore, the observed discrepancy in our results may be explained by stable mutant forms of the POT1 protein within 21NT cells. Furthermore, despite the higher protein content of POT1 in 21NT cells, this mutated POT1 protein may not be capable of binding to the other Shelterin components properly which leads to telomere dysfunction. This may explain the higher protein levels observed within 21NT breast cancer cells in comparison with HMECs.

Several cellular factors (i.e., mutation, DNA methylation, histone acetylation, chromatin remodelling, etc.) in theory could affect the expression of Shelterin and Shelterin-associated genes in breast cancer cell lines. Based on previous published data and our
results presented in Chapter III, POT1 mRNA was found to be significantly down-regulated in malignant breast tissues and cancer cell lines (Salhab, Jiang et al. 2008). Moreover, it had been previously reported that POT1 was the most mutated Shelterin gene in a wide range of cancers such as; gastric, papillary thyroid, breast and leukaemia cancer cell lines (Poncet, Belleville et al. 2008; Shen, Gammon et al. 2010; Wan, Tie et al. 2011; Cantara, Capuano et al. 2012). Prior studies have shown that POT1 is the only Shelterin protein which is able to bind directly to the G-strand telomeric single strand DNA sequence overhang via its OB fold and plays a critical role in regulating telomere length (Lei, Podell et al. 2004; Yang, Zhang et al. 2007). Additionally, in the presence of POT1, the Shelterin components TIN2, TRF1, and TRF2 localises to the telomeres. As a result of that, Shelterin proteins may require the POT1 component to maintain telomere length (Ye, Hockemeyer et al. 2004; Wang, Podell et al. 2007).

Currently, the catalogue of somatic mutations in cancer (COSMIC) database shows over 127 somatic mutations in POT1, 41 in TPP1, and 249 in RAP1. However, at the time part of this study was carried out in 2009, the COSMIC database did not report any mutations within POT1 and any other Shelterin genes. Hou et al. (2006) reported a single mutation within exon12 of POT1 in HeLa and HO8910-PM cells. Therefore, the first focus of the work described in Chapter IV was to screen for exon12 mutations within breast cancer cell lines. The results revealed that no mutations within exon12 of the POT1 gene were present in any of 10 breast cancer cell lines. This finding does not of course exclude mutations which may be present in other exons of POT1. A more comprehensive study to look for mutations within all Shelterin genes within the breast cancer cell lines would have been useful.
However, the scale of such a study would have been too large to accommodate in this project.

Epigenetic alterations to DNA and histones can result in silencing of genes (Kondo, Shen et al. 2003). The down-regulation of Shelterin genes in breast cancer cell lines (Chapter III) could be due to epigenetic modification (methylation) of the promoter of these genes or histone acetylation/deacetylation of chromatin at this locus. To study this, the promoter regions of POT1 and TIN2 genes in breast cancer cell lines were analysed. Methylation Specific PCR (MSP) showed that POT1 was partially methylated in untreated breast cancer (i.e., not exposed to 5-aza-CdR and TSA) cell lines. However, in TIN2 no significant differences were observed in methylated and unmethylated lanes in all breast cancer cell lines. These results provided insight into a plausible mechanism to explain the observed expression data based on DNA hypermethylation in the breast cancer cell lines.

A previous study by Zemliakova et al. (2003) looked at promoter methylation of five genes which were methylated in breast cancer tissues. They found that the promoter region of p16 (56%), RB1 (17%), CDH1 (79%), P15 (2%) and MGMT (8%) were methylated in breast cancer tissues (Zemliakova, Zhevlova et al. 2003). In addition, it was reported that BRCA1 was hypermethylated in breast and ovarian cancers (Esteller, Silva et al. 2000). Furthermore, a previous study showed that the RECK gene was hypermethylated in hepatocellular carcinoma (HCC) in comparison with non-tumour tissues. It has been also reported that hypermethylation of the RECK promoter may be associated with silencing of RECK mRNA expression. Indeed, it was implied that the expression of RECK was likely regulated by DNA

Based on published studies highlighted above, the 21NT cell line was then used as a model system for treatment with 5-aza-CdR and TSA, two epigenetic modifying agents. If Shelterin genes expression were silenced through promoter methylation/histone modification, the treatments should reactivate them.

The National Cancer Institute database reported that these epigenetic modification agents have been used as anti-cancer drugs in about 100 clinical trials (Ghoshal, Datta et al. 2005). The drugs have been used to treat different types of leukemia, sickle cell anemia and β-thalassemia (Saunthararajah, Hillery et al. 2003). Work by Mirza et al. (2010) showed that p53 and p21 were up-regulated in MCF-7 breast cancer cells treated with 5-aza-CdR. Demethylation of p53 and p21 promoters in MCF-7 cells after treatment with 5-aza-CdR resulted in an up-regulation of these genes. It was found that the mRNA levels of POT1, TIN2, and TPP1 were significantly increased in 21NT cells treated with both agents in comparison with DMSO-treated control.

The novel findings described in this project showed that 5-aza-CdR and TSA were most effective on modulating TIN2 and POT1 mRNA levels after relatively short term (48 and 72 hour) treatment and again after 3 weeks treatment of 21NT cells. In addition, the biphasic response of POT1 and TIN2 gene expression was seen with an optimal peak at 72 hours, which then declined and the expression was increased significantly again at 3 weeks treatment (correlated with telomere length see Chapter V). However, these agents together were found to lose their effectiveness during long term treatment (6 weeks and 2 month
retreatment) as no substantial difference was observed at these time points. Thus, the treatment was not permanent and is reversible. It is well established that the process of DNA methylation is carried out by DNA methyltransferases (DNMTs) enzymes. These enzymes catalyse the transfer of a methyl group from S-adenosyl-L-methionine (SAM) to the 5 position of cytosine (Robertson 2001). DNMT1, the main methylation maintenance enzyme, preferentially methylates hemi-methylated DNA during the process of DNA replication. In this scenario, 5-aza-CdR is incorporated exclusively into DNA as a cytidine analogue, and thereby inhibits DNA methylation via irreversible covalent binding to DNMT1 (Maslov, Lee et al. 2012). By this mechanism, it could be argued that the lowest expression of POT1 and TIN2 between 72 hours and 3 weeks may have resulted from an increase in the expression of DNA methyltransferases 1 enzyme, leading to hypermethylation of POT1 and TIN2 after several replications. Further investigations will be required to examine which factors are involved to decrease the transcription levels of POT1 and TIN2 in the interval between 72 hours and 3 weeks treatment. For instance, if this assay were to be repeated in the future, the expression of DNMT1 could be analysed along with POT1 and TIN2.

It has been reported in several independent studies that 5-aza-CdR has therapeutic value for cancer treatment (Venturelli, Armeanu et al. 2007; Cai, Kohler et al. 2011; Liu, Zhang et al. 2012). Recent work by Kang et al. (2013) reported that Runt-related transcription factor 3 (RUNX3), a tumour suppressor gene, was hypermethylated in MCF-7 (breast cancer cell line). The work reported that 5-aza-CdR induces apoptosis and inhibits cell proliferation by demethylating the promoter region of RUNX3 and reactivating its expression (Kang, Dai et al. 2013). Therefore, based on previous investigations, it should be noted that, used as a chemotherapeutic drug, 5-aza-CdR causes cell death via induction of
apoptosis pathways. Consistent with these observations, approximately 72 hours after treatment with 5-aza-CdR, a significant reduction in 21NT cell number was observed. Therefore, theoretically by 7 days of treatment, cells which have become resistant to apoptosis but retained the unmethylated status will start to grow out of the population and continue to grow up to 3 weeks accompanied by an increase in gene expression. Further studies in vitro will be required to determine which factors are involved in the modulation of gene expression. For instance, the Terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) assay may be a useful method to detect apoptotic programmed cell death.

In order to support the results described in this thesis, the promoter region of POT1 was analysed for CpG demethylation in DNA samples from 5-aza-CdR treated 21NT cells. The bisulphite sequencing data obtained was entirely consistent with the results showing up-regulation of POT1, as all potential methylation sites within the CpG Island were demethylated after the treatment of 21NT cells with 5-aza-CdR. These data confirmed that 5-aza-CdR inhibits epigenetic mechanisms including DNA methylation. This appears to have the effect of reversing the silencing of Shelterin and Shelterin-associated genes in breast cancer cell lines. It is suggested that 5-aza-CdR and TSA enhance gene transcription by opening promoter regions to increase accessibility of assembling transcription factor complexes (Yang, Phillips et al. 2001; Margueron, Duong et al. 2004). It should be stressed that 5-aza-CdR in combination with TSA reactivated expression of Shelterin and Shelterin-associated genes, while TSA alone mostly had little effect on the expression of aforementioned genes. However, prior studies by others showed that TSA reactivates the expression of estrogen receptor (ER) in breast cancer cell lines (Yang, Ferguson et al. 2000). It is therefore, likely that the combined effect of 5-aza-CdR with TSA on gene expression has
dual activity on DNA demethylation and histone acetylation respectively. Since these two agents were individually shown to enhance gene expression, they seem to act synergistically. We can speculate that silencing of these genes is controlled by epigenetic modifications. The findings described in Chapter IV open up interesting avenues for future work. In the first instance, the promoter regions of other Shelterin and Shelterin-associated genes should be examined in all breast cancer cell lines. A second experimental approach should focus on delineating histone modifications such as acetylation of lysine residues on histone H3 and H4, together with the methylation of lysine 9 on histone H3, H9, H27 and H3K36 as well as the role of histone methylation (Wozniak, Klimecki et al. 2007). The application of chromatin immunoprecipitation (ChIP) technology followed by real-time PCR to quantify the degree of histone methylation in 21NT cells and other breast cancer cell lines would be central to such work.

Results obtained thus far demonstrated an up-regulation of some Shelterin genes when breast cancer cells were treated with epigenetic modulators. How does this affect telomere length maintenance? Previous studies indicated that dysregulation of Shelterin genes expression can result in telomere length dysfunction in a variety of cancers, including breast cancer (Butler, Hines et al. 2012). For instance, Hu et al. (2010) showed that overexpression of TRF1, TRF2 and TIN2 in gastric cancer tissues were correlated with a reduction in telomere length. Moreover, deficiencies in Shelterin regulation have recently been implicated in telomere length dysfunction during liver carcinogenesis (El Idrissi, Hervieu et al. 2013). However, the majority of studies have shown only that altered expression of Shelterin proteins affects telomere length, but they have not pinpointed a mechanism. To date, no previous investigations have reported effects of 5-aza-CdR and TSA treatment on
telomere length maintenance in cancer cells. Hence, following telomere length measurement by several reliable methods, it was clear that short-term (72 hrs) and 3 weeks treatment of 21NT cells with 5-aza-CdR resulted in an increase telomere lengths around of 4.7 kb. Therefore, it would seem that, in cancer, Shelterin expression is down-regulated through epigenetic modification of DNA and histone proteins. Up-regulation of Shelterin genes, through the use of epigenetic modifying agents, are directed towards the telomeres where they influence telomere length elongation. Indeed, our data may suggest that the regulation of the Shelterin protein complex is needed to maintain telomere length regulation in breast cancer cell lines.

The POT1-TPP1 complex covers the single strand 3' overhang and prevents binding of the telomere to telomerase (Wang, Podell et al. 2007). It remains likely that the association of POT1-TPP1-TIN2 plays a key role in recruiting telomerase to the telomere. Therefore, our results clearly showed that Shelterin genes particularly POT1, TIN2 and TPP1, were significantly induced in 21NT cells following treatment with the DNA methylation inhibitor, 5-aza-CdR and the histone deacetylation inhibitor, TSA. This further supports the hypothesis in this thesis that demethylation of the Shelterin genes TIN2, POT1 and TPP1, stabilises the Shelterin complex that functions to regulate and maintain telomere length elongation.

Based on results discussed above, three of the Shelterin proteins were significantly up-regulated after treatment with 5-aza-CdR and TSA; this ultimately led to telomere elongation. In the final Chapter of the thesis, it was attempted to identify the most important gene of the three. In this respect it was previously reported that the single-stranded 3' in the absence of telomerase DNA becomes approximately 50 to 100 bases
shorter after each cell division (Stewart, Ben-Porath et al. 2003). This may well be linked with POT1 as it is the only Shelterin protein that directly binds to the 3' ends of telomere (Baumann and Price 2010).

Previous noteworthy studies showed that reduction of POT1 by RNAi results the loss of the 3' overhangs (Yang, Zheng et al. 2005). Furthermore, it had also been observed that over-expression of POT1 in telomerase-positive cells resulted in telomere length elongation (Colgin, Baran et al. 2003; Yang, Zhang et al. 2007). Therefore, based on the results from previous Chapters, it was concluded that over-expression of POT1 in 21NT cells may provide an improved understanding of telomere regulation in cancer cell lines. Thus, the full length of human POT1 (variation 1) gene was over-expressed in 21NT cells to determine the effect on telomere length elongation. The results revealed that the average telomere length of the POT1 over-expressing clones was 2 to 3 kb longer in comparison with 21NT non-transfected and empty vector control clones. However, increased telomere length by ectopic over-expression of POT1 is not likely to be due to a direct effect on telomerase enzyme activity since the latter was not increased.

Based on a previous finding by Colgin et al. (2003) telomere length elongation by over-expressing POT1 did not show an increase in telomerase activity. Such a result may validate our hypothesis and could support the finding that telomere elongation by POT1 is not accompanied by an increase in telomerase activity. However, this does not exclude the possibility that POT1 completely inhibits activation of telomerase at the chromosome ends. Furthermore, our results showed approximately a 2-fold decrease in telomerase activity in transfected 21NT cells. Therefore, it is likely that POT1 initially allows telomerase to synthesise telomeres and leads to telomere length elongation up to 2 kb, after a certain
length is reached, subsequently inhibits telomerase access and prevents further elongation. Therefore, it could be argued that POT1 negatively regulates the access of telomerase to the telomeres (Colgin, Baran et al. 2003). In order to further examine the role of telomerase activity and POT1-mediated telomere lengthening, POT1 over-expression should be carried out within a telomerase-negative HMEC cell strain.

Two models of telomere length regulation have been described. The “open” state permits the access of telomere to telomerase and the “closed” conformation shields telomere DNA from end-to-end fusion (Colgin, Baran et al. 2003). With an “open” conformation, POT1 may possibly stabilise the telomere, via binding to the 3’ overhang of the telomere end. Therefore, POT1 could be a negative regulator of telomerase activity in order to maintain telomere length. However, it is also possible that POT1 controls a localised activation of the telomerase enzyme at the telomere end. Our data highlights the importance of a potential fundamental role for POT1 in regulating Shelterin genes stability. It is likely that the interaction of POT1 with TPP1 form a part of the Shelterin complex via by binding TIN2. This develops the concept that, by virtue of these interactions, such a complex appears to form a stable sub-complex to interact with other Shelterin genes to protect and maintain telomere length.

In the past, several studies used genetically modified mice and cultured human cell lines to investigate the role of Shelterin genes in telomere length maintenance in aging and cancer (Martinez and Blasco 2010; Lu, Wei et al. 2013). For instance, inhibition of the expression of Shelterin genes, or over-expression of dominant negative forms of these proteins, (in cultured human cell lines) or knockout of Shelterin component (in mouse embryonic fibroblasts (MEFs)) results in telomere loss, T-loop recombination and telomere
fusion (Lu, Wei et al. 2013). In addition, conditional knockout of Rap1, Tpp1, and Trf1 in mice showed high incidence of oncogenesis and deletion of Pot1 induces mouse dyskeratosis congenita (DKC) (Hockemeyer, Palm et al. 2008; Martinez and Blasco 2010). Depletion of Shelterin components in mice pinpointed telomere dysfunction as the major driving force which leads to a more rapid pathological aging phenotype in comparison with those induced by telomerase deficiency. Moreover, genetic variation in the Shelterin complex has been observed in human pathological aging and cancer (Lu, Wei et al. 2013). Taken together, these observations indicated that, Shelterin proteins in telomere biology and disease play fundamental role in the context of the mammalian organism.

Results obtained in this thesis have shown that the use of chemotherapeutic epigenetic modifying drugs, such as 5-aza-CdR and TSA, induce and increase the expression of several Shelterin and Shelterin-associated genes in breast cancer cell lines. Up-regulation of these genes ultimately leads to an increase in telomere length. The effect of 5-aza-CdR and TSA on mammalian telomeres has not been widely reported in the literature to-date making the results presented here novel. We can now speculate on how the use of therapeutic agents such as 5-aza-CdR and TSA to increase telomere lengths of cancer cells may benefit clinical outcome. Research into the role of Shelterin and telomerase in cancer has found that telomerase re-activation functions to maintain telomeres at a critically short length (Low and Tergaonkar 2013). Telomeres in this unstable state are still prone to genetic damage via end-to-end fusions and translocations. This will have the effect of damaging the genome of the cancer cell further giving rise to further clonal evolution and a more advanced disease. If drugs such as 5-aza-CdR and TSA are used to treat cancer, they could induce telomere lengthening. This may have the effect of stabilizing the telomere and
reducing the amount of genetic damage the cell will undergo thereby stopping the clonal evolution of the cancer cell population. These tumours may be more susceptible to further treatment as a result. Drugs such as 5-aza-CdR and TSA are non-specific and cause global cellular demethylation/deacetylation which in the context of this work can be considered off-target effects. In order to specifically target telomere maintenance, it may be better to concentrate one or more Shelterin components rather than telomerase itself.

It is conceivable that targeting telomere length and telomerase will be an effective pharmaceutical strategy for cancer treatment. Manipulating telomere length (e.g. by controlling expression of telomerase components such as hTERT) might be expected to be beneficial for treating aging related diseases and cancer (Holysz, Lipinska et al. 2013; Lu, Wei et al. 2013). In addition, targeting Shelterin protein components may possibly more effective that targeting telomerase, especially POT1 which is implicated to regulate telomere length and capping (Martinez and Blasco 2010; Lu, Wei et al. 2013).

We observed that the over-expression of POT1 negatively affected telomerase activity and resulted telomere lengthening. Increases in telomere length may stabilize cancer cells and render them less prone to telomeric fusion. As a result the clonal evolution of cancer cell populations may be reduced and making them more susceptible to further drug treatment. Therefore, targeting POT1 in breast cancer cells allows for simultaneous investigation of telomere length and regulates the access of telomerase to telomere.

Gene therapy is the use of DNA as a drug to treat disease by delivering therapeutic DNA into a patient’s cells. The most common form of gene therapy involves using DNA that encodes a functional, therapeutic gene to replace a mutated gene potentially in cancers. This relies on the efficient transfer of a nucleic acids which encodes a therapeutic protein,
into cells by a number of methods using viral vectors. Viruses such as retrovirus, lentivirus and adenovirus can be used in vivo to introduce genetic material into their host cell as a part of their replication process (Roth and Cristiano 1997; Cross and Burmester 2006). For instance, in clinical trials, the in vivo strategy involves the direct delivery of DNA (usually via a viral vector) to resident cells of the target tissue. There are two requirements for such a strategy: firstly, that target cells be easily accessible for infusion or injection of virus, and secondly, that the transfer vector readily and specifically infects, integrates, and then expresses the therapeutic gene in target cells and not surrounding cells at effective levels for extended time periods (Selkirk 2004). Thus, gene therapy can be used to deliver POT1 into cells may be considered as a potential mechanism to treat breast cancer.
References


List of websites


Anatomy and physiology of the male and female: http://cnx.org/content/m46392/latest/

National statistic 2013: http://www.cancer.gov/cancertopics/types/breast

Publication:

Appendix 1:

All sequences of POT1 exon12:

GTTGGGAAGCTTCTTAGAATCTATAGCGCTCCATACCAACTTCAATCAATGAATTCAGAGAATCAGACAATGTT
AAGTTTAGAGTTTAGCTATGTGAAGGTACCAGTTACGGTGCGGGGAAATCAGGGTCTTGCCAGAAAGTAACTCT
GATGTGGATCAACTGAAAA

HMEC1
GTTGGGAAGCTTCTTAGAATCTATAGCGCTCCATACCAACTTCAATCAATGAATTCAGAGAATCAGACAATGTT
AAGTTTAGAGTTTAGCTATGTGAAGGTACCAGTTACGGTGCGGGGAAATCAGGGTCTTGCCAGAAAGTAACTCT
GATGTGGATCAACTGAAAA

21NT
GTTGGGAAGCTTCTTAGAATCTATAGCGCTCCATACCAACTTCAATCAATGAATTCAGAGAATCAGACAATGTT
AAGTTTAGAGTTTAGCTATGTGAAGGTACCAGTTACGGTGCGGGGAAATCAGGGTCTTGCCAGAAAGTAACTCT
GATGTGGATCAACTGAAAA

21MT-2
GTTGGGAAGCTTCTTAGAATCTATAGCGCTCCATACCAACTTCAATCAATGAATTCAGAGAATCAGACAATGTT
AAGTTTAGAGTTTAGCTATGTGAAGGTACCAGTTACGGTGCGGGGAAATCAGGGTCTTGCCAGAAAGTAACTCT
GATGTGGATCAACTGAAAA

BT20
GTTGGGAAGCTTCTTAGAATCTATAGCGCTCCATACCAACTTCAATCAATGAATTCAGAGAATCAGACAATGTT
AAGTTTAGAGTTTAGCTATGTGAAGGTACCAGTTACGGTGCGGGGAAATCAGGGTCTTGCCAGAAAGTAACTCT
GATGTGGATCAACTGAAAA

BT474
GTTGGGAAGCTTCTTAGAATCTATAGCGCTCCATACCAACTTCAATCAATGAATTCAGAGAATCAGACAATGTT
AAGTTTAGAGTTTAGCTATGTGAAGGTACCAGTTACGGTGCGGGGAAATCAGGGTCTTGCCAGAAAGTAACTCT
GATGTGGATCAACTGAAAA

HCC1143
GTTGGGAAGCTTCTTAGAATCTATAGCGCTCCATACCAACTTCAATCAATGAATTCAGAGAATCAGACAATGTT
AAGTTTAGAGTTTAGCTATGTGAAGGTACCAGTTACGGTGCGGGGAAATCAGGGTCTTGCCAGAAAGTAACTCT
GATGTGGATCAACTGAAAA

GI101
GTTGGGAAGCTTCTTAGAATCTATAGCGCTCCATACCAACTTCAATCAATGAATTCAGAGAATCAGACAATGTT
AAGTTTAGAGTTTAGCTATGTGAAGGTACCAGTTACGGTGCGGGGAAATCAGGGTCTTGCCAGAAAGTAACTCT
GATGTGGATCAACTGAAAA

Figure S1-POT1 exon12 in cancer and control cell lines.
MCF-7
GTGGGAAGCTTTCTTAAATCTATAGCCTTCATAAAGTCTATCAATGAAATGCAGAGAATCAGACAAATGTT
AAGTTTAGGTTTCTACATGAGGTAGTTACGTCTGGCATGGAATCCAGGATTGGTCTTGGCCAGAAAGTAACTCT
GATGTGGATCAACTGAAAA

HS578-T
GTGGGAAGCTTTCTTAAATCTATAGCCTTCATAAAGTCTATCAATGAAATGCAGAGAATCAGACAAATGTT
AAGTTTAGGTTTCTACATGAGGTAGTTACGTCTGGCATGGAATCCAGGATTGGTCTTGGCCAGAAAGTAACTCT
GATGTGGATCAACTGAAAA

PB1
GTGGGAAGCTTTCTTAAATCTATAGCCTTCATAAAGTCTATCAATGAAATGCAGAGAATCAGACAAATGTT
AAGTTTAGGTTTCTACATGAGGTAGTTACGTCTGGCATGGAATCCAGGATTGGTCTTGGCCAGAAAGTAACTCT
GATGTGGATCAACTGAAAA

PC3
GTGGGAAGCTTTCTTAAATCTATAGCCTTCATAAAGTCTATCAATGAAATGCAGAGAATCAGACAAATGTT
AAGTTTAGGTTTCTACATGAGGTAGTTACGTCTGGCATGGAATCCAGGATTGGTCTTGGCCAGAAAGTAACTCT
GATGTGGATCAACTGAAAA

Figure S1-POT1 exon12 in cancer cell lines.

Appendix 2:

HMEC1: TAGTTAATTGGTTAGTGG
21MT-2: TAGTTAATTGGTTAGTGG
GI101: TAGTTAATTGGTTAGTGG
21NT: CGTTTAATTGGTTAGTGG

Figure S2-Sequence of POT1 promoter region in untreated cancer and control cell lines.

Appendix 3:

DMSO: CGTTTAATTGGTTAGTGG
5-aza-CdR: TAGTTAATTGGTTAGTGG

Figure S3-Sequence of POT1 promoter region in 21NT treated with 5-aza-CdR and DMSO for 72 hours.