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Epigenetic Regulation of the Telomerase Gene Promoters

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A thesis submitted to the University in partial fulfilment of the requirements for the Degree of Doctor of Philosophy

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Abstract

The presence of active telomere maintenance mechanisms in immortal cells allows the bypass of senescence by maintaining telomere length. In most immortal cell lines and tumours, telomere maintenance is attributable to telomerase reactivation. However, a number of immortal cell lines and tumours can achieve telomere maintenance in the absence of detectable telomerase activity by the alternative lengthening of telomere (ALT) mechanism. Some stem cell populations also have telomerase activity and in telomerase biology and a key issue is how the complex pattern of telomerase gene expression is maintained in the adult stem cell. Epigenetic mechanisms have been implicated in the regulation of telomerase gene expression and here we show that specific modifications within the chromatin environment of the hTR and hTERT promoters correlate with expression of hTR and hTERT in ALT, normal and telomerase-positive tumour cell lines.

Lack of expression of hTR and hTERT is associated with repressive histone modifications, while, hTR and hTERT expression is associated permissive histone modifications. Methylation of lysine 20 H4 was not linked to gene expression but instead was specific to the hTR and hTERT promoters of ALT cells providing an insight into the differences between ALT and telomerase-positive cells as well as a novel marker for the ALT phenotype. Basal transcription machinery dynamics were also shown to be different between normal and cancer cells at the telomerase gene promoters. Modulation of the chromatin environment was also shown to cause re-expression or increased expression of hTR and hTERT further supporting the role of the chromatin environment in controlling telomerase gene expression.

Epigenetic mechanisms are also shown to be involved in the repression of hTERT transcription in human mesenchymal stem cell (hMSCs) and modulation of the chromatin environment is shown to allow re-expression of hTERT expression, while the disruption of telomerase gene expression in human haematopoietic stem cells (hHSCs) in chronic myeloid leukemia (CML) and the role of the chromatin environment was also studied. These data establishes how epigenetic mechanisms can contribute to transcriptional regulation of telomerase and also highlights the potential importance of epigenetics in senescence and tumourigenicity.

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List of Accompanying Material

Atkinson, S. P., Hoare, S. F., Glasspool, R. M. and Keith, W. N. (2005) Lack of telomerase gene expression in alternative lengthening of telomere cells is associated with chromatin remodeling of the hTR and hTERT gene promoters. Cancer Res, 65, 7585-90.

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Authors Declaration

I am the sole author of this thesis. All of the references have been consulted by myself in the preparation of this manuscript. Unless otherwise acknowledged all of the work presented in this thesis was performed personally.

Abbreviations

5-azadC	5-azadeoxycitidine
ADP	Adenosine Di-phosphate
ALT	Alternative Lengthening of Telomeres
AP CML	Accelerated Phase CML
APB	ALT-associated PML Bodies
ATM	Ataxia-telangiectasia, mutated
ATP	Adenosine Triphosphate
ATR	ATM and Rad3-related
BP CML	Blast Phase CML
BRCA1	Breast cancer 1 protein
CBP	CREB binding protein
CDK	Cyclin Dependent Kinase
CDKi	Cyclin Dependent Kinase Inhibitor
cDNA	Complementary DNA
CHD1	Chromo-ATPase/helicase-DNA-binding
ChIP	Chromatin Immunoprecipitation
Chk1, 2	Checkpoint Kinase 1, 2
CHX	Cyclohexamide
CML	Chronic Myeloid Leukaemia
CP CML	Chronic Phase CML
CtBP	C-terminal Binding Protein
DNA	Deoxyribonucleic acid
DSB	Double Strand Break
EGF	Epidermal growth factor
ER	Oestrogen Receptor
ERE	Oestrogen Receptor Element
ERK	Extracellular-Signal Regulated Kinase
EZH2	Enhancer of Zeste Homolog
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCN5	General Control of amino-acid synthesis 5
GKM	Gate Keeper Mutation
GLP	G9a related protein
GMAT	Genome-wide mapping technique
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
hHSC	Human haematopoietic stem cells
HIF	Hypoxia Inducible Factor
hMSC	Human mesenchymal stem cells
HMT	Histone Methyltransferase
HP1	Heterochromatin Protein 1
HPC	Haematopoietic Progenitor Cell
HRE	Hypoxia Response Element
hTERT	Human Telomerase Reverse Transcriptase
HTLV-1	Human T-Cell Lymphotropic Virus
hTR	Human Telomerase RNA component
HYPB	Huntingtin interacting protein B
IM	Imatinib Mesylate
INHAT	Inhibitor of histone acetyltransferase
IP	Immunoprecipitation
JHDM1	JmjC domain-containing histone demethylase 1
KRAB-ZFP	Kruppel-associated box-zinc finger protein

LSD1	Lysine-specific demethylase 1
MAPK	Mitogen-Activated Protein Kinase
MDM2	Mouse Double Minute 2
MLL	Mixed-lineage leukemia
mRNA	Messenger Ribonucleic Acid
MZF2	Myeloid Specific Zinc Finger Protein 2
NAD+	Nicotinamide Adenine Dinucleotide molecule
NBF	Neutral buffered formalin
NF-Y	Nucleic Factor Y
NSD1	Nuclear receptor binding SET domain protein
PCAF	p300/CBP associated factor
PcG	Polycomb
PCR	Polymerase Chain Reaction
PML	Promyelocytic leukemia protein
PRC1, 2	Polycomb repressive complex 1, 2
Q-PCR	Quantitative-PCR
rDNA	ribosomal DNA
RNAi	RNA interference
RT	Reverse Transcriptase
RT-PCR	Reverse Transcription-PCR
RXR	Retinoid X Receptor
SAGE	Serial Analysis of Gene Expression
SAHF	Senescence-associated Heterochromatin Foci
SET	Su(var), Enhancer of zeste, Trithorax
SIR2	Silent Information Regulator 2
SMYD3	SET and MYND domain containing 3
SUMO	Small ubiquitin-related modifier
TAF(II)250	TBP-associated Factor (II) 250
TFIIB	Transcription Factor IIB
TFIID	Transcription Factor IID
TRAP	Telomeric repeat amplification protocol
TRF1, 2	Telomeric repeat binding factor 1, 2
TSA	Trichostatin A
USF	Upstream Stimulatory Factor
VDR	Vitamin D3 receptor
VHL	Von Hippel-Lindau protein
WDR5	WD40-repeat protein
WT1	Wilms Tumour 1 protein

Units

oC	Degrees Celsius
bp	Base Pairs
g	Gram
hr	Hour
k	Kilo
l	Litre
m	Milli
µ	Micro
mins	Minutes
n	Nano
nt	Nucleotide

rpm	Revolutions per minute
sec	Second
cm^2	Centimetre Squared
M	Molar

1 Introduction

The term epigenetics is defined as mechanisms through which changes in gene function can occur without a change in the DNA sequence. Such mechanisms include covalent modifications of histones, DNA methylation, ATP-dependent chromatin remodelling, incorporation of variant histones, looping and changes in local conformation of DNA and alterations of higher-order chromatin structure. Telomerase gene (hTR and hTERT) regulation has been intensely studied but the regulation of the telomerase gene promoters by covalent changes to histone proteins has not been fully resolved. Given the importance of telomerase in tumourigenesis and senescence, fully resolving different modes of gene regulation may be of importance.

1.1 Regulation of Senescence

1.1.1 Senescence

Mammalian cells have a finite replicative potential *in vitro* and *in vivo* as upon reaching this limit, cells enter a state referred to as cellular senescence and cease to divide. This was first observed in normal human fibroblasts (Hayflick, 1965). Senescent cells do however remain viable and metabolically active although they cannot re-initiate DNA replication in response to mitogenic stimuli and become resistance to apoptosis. As these cells do not proliferate, it has been proposed that cellular senescence is a major barrier to cancerous transformation. Senescence is also associated with altered cellular morphology, size and, importantly, alterations in the expression of numerous genes involved in many aspects of cell physiology (Shelton *et al.*, 1999; Hardy *et al.*, 2005; Grillari *et al.*, 2000; Untergasser *et al.*, 2002; Yoon *et al.*, 2004; Guo *et al.*, 2004; Mason *et al.*, 2004).

Therefore the regulation of multiple genes is involved in the senescence phenotype and the complexity of the phenotype suggests that epigenetic mechanisms may be involved through regulating gene expression. Indeed, it is now apparent that in addition to multiple genetic pathways, epigenetics plays an important role in senescence (Bandyopadhyay and Medrano, 2003; Young and Smith, 2000) and also in the bypass of senescence leading to tumourigenicity (Esteller, 2006; Santos-Rosa and Caldas, 2005; Baylin and Ohm, 2006). Therefore, the study of epigenetics, alongside genetics may allow further understanding of senescence and tumourigenesis.

1.1.2 The Role of Telomeres and Telomerase in Senescence

1.1.2.1 Telomeres and Telomerase

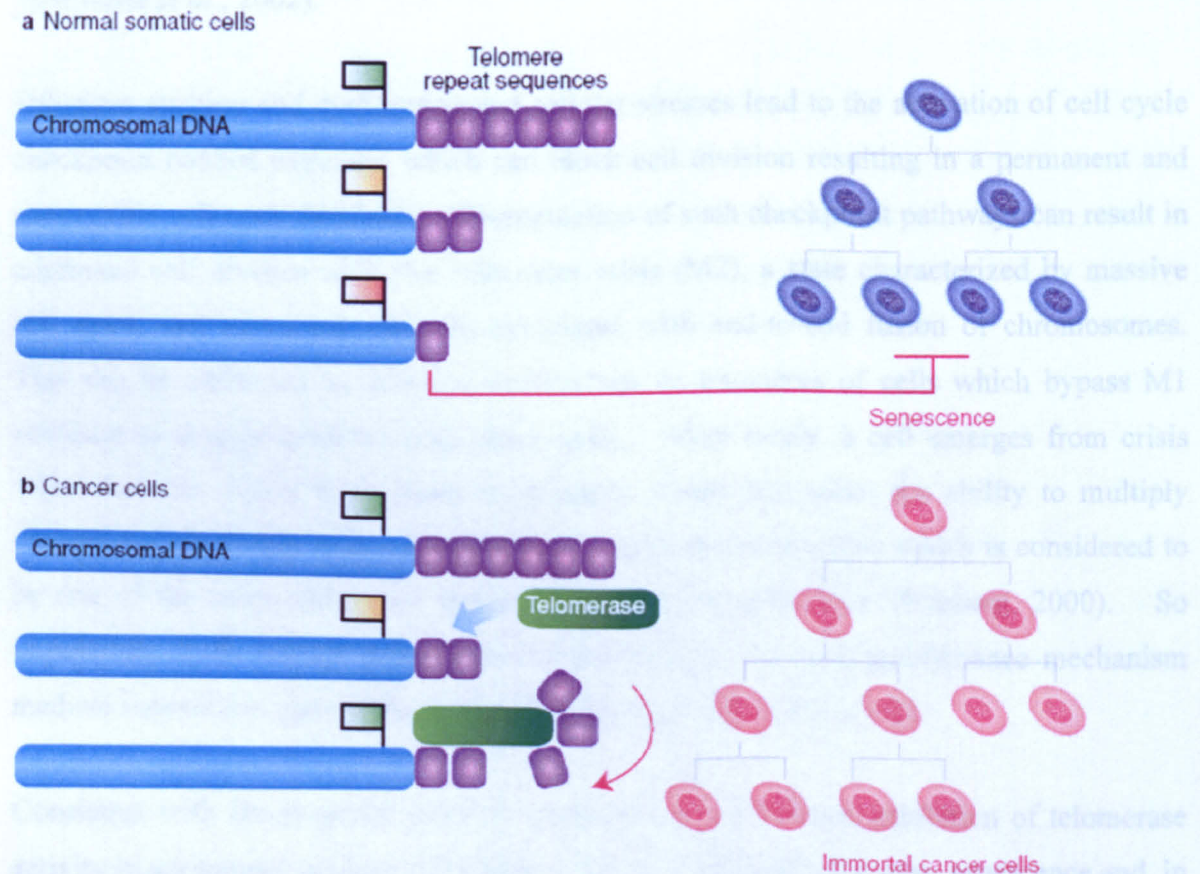
Telomeres are DNA-protein complexes forming capping structures that stabilise chromosomal ends and prevent them from recognition as DNA double strand breaks and from loss of coding sequence at the chromosome end during DNA replication. In humans, telomeric DNA is composed in the 5' to 3' direction of a hexameric oligonucleotide sequence (TTAGGG) repeated approximately 1000 to 2000 times (Moyzis *et al.*, 1988; Blackburn, 1991). Telomeres are usually maintained by the enzyme telomerase which catalyses the addition of the telomeric repeats on to the ends of chromosomes, thus maintaining their length despite continued cell division (Morin, 1989). Telomerase is minimally composed of two components and functions by utilising an RNA template subunit and a reverse transcriptase subunit to catalyse the addition of the telomeric repeats onto the 3' ends of eukaryotic chromosomes (Greider and Blackburn, 1987). In humans the subunits are known as hTR (Telomerase RNA template component) and hTERT (Telomerase Reverse Transcriptase component) and have been described as the minimal functional requirements for telomerase activity (Autexier *et al.*, 1996; Weinrich *et al.*, 1997).

1.1.2.2 Telomeres, Senescence and Immortality

In adult humans, most normal cells have low or no detectable telomerase activity whereas telomerase activity is observed in an estimated 85% of malignant tumours (Shay and Bacchetti, 1997). Telomerase activity in cancer cells allows for the unchecked growth of cancer cells (See Figure 1.1). However, certain normal cells do exhibit telomerase activity, including germ line cells (Wright *et al.*, 1996; Kim *et al.*, 1994), activated lymphocytes (Bodnar *et al.*, 1996), some stem cell populations (Hiyama *et al.*, 1995; Broccoli *et al.*, 1995; Chiu *et al.*, 1996), basal keratinocytes (Harle-Bachor and Boukamp, 1996) and endothelial cells (Hsiao *et al.*, 1997). The lack of telomerase activity and therefore telomere elongation or maintenance in normal cells, coupled with the fact that the machinery which replicates the bulk of chromosomal DNA is intrinsically incapable of fully replicating the chromosome termini (the end replication problem) (Olovnikov, 1973), leads to the loss of approximately 50-100bp of telomeric sequence at each cell division (Harley *et al.*, 1990; Hastie *et al.*, 1990). The loss of telomeric tract during cell division also presents a limit to the maximum number of divisions a cell can undertake and

Figure 1.1 Telomeres and Telomerase in Normal Somatic Cells and Cancer

a, In normal human somatic cells, which do not express telomerase, telomeres will shorten with age until they reach a critical short length where they will signal for the cell to enter senescence. **b**, In cancer cell, telomerase can become reactivated leading to the maintenance of telomere length and therefore telomeres will not reach the critical short length where the cell enters to enter senescence. [Picture from Keith et al (Keith *et al.*, 2002)].



therefore provides a limit to its lifespan (Allsopp *et al.*, 1992). This is the Hayflick limit (or Mortality Stage 1 [M1]) (Hayflick, 1965) and when cells reach this limit they enter senescence, or initiate programmed cell death. This response to telomere shortening is thought to have evolved as a tumour suppressive barrier against excessive clonal expansion. Senescence can also be triggered by several other mechanisms, including cellular stresses, such as activated oncogenes, DNA damage, oxidative stress and sub-optimal cell culture condition and has also been observed by a change in the protected status of shortened telomeres, rather than by the complete loss of telomeric DNA (Karlseder *et al.*, 2002).

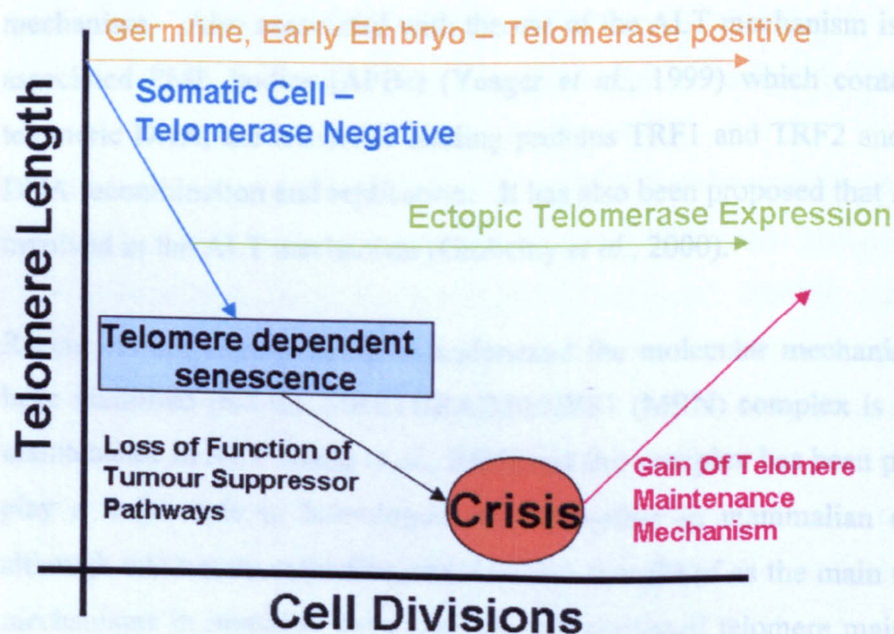
Telomere attrition and dysfunction and cellular stresses lead to the activation of cell cycle checkpoint control pathways which can block cell division resulting in a permanent and irreversible cell-cycle blockade. Dysregulation of such checkpoint pathways can result in continued cell division until the cells enter crisis (M2), a state characterized by massive cell death and karyotypic changes associated with end-to-end fusion of chromosomes. This can be attributed to telomere dysfunction, as telomeres of cells which bypass M1 continue to shorten until the cells enter crisis. Very rarely, a cell emerges from crisis which has the ability to maintain its telomere length and gains the ability to multiply without limits (Wright *et al.*, 1989), a trait termed immortalisation which is considered to be one of the prerequisites for tumour formation (Hanahan and Weinberg, 2000). So importantly, both tumour suppressor mechanisms and telomere maintenance mechanism mediate senescence, immortalisation and tumourigenicity (Figure 1.2).

Consistent with the proposed roles of telomerase in senescence, inhibition of telomerase activity in telomerase positive cells leads to the shortening of telomeres, senescence and, in some cases, cell death (Hahn *et al.*, 1999b; Zhang *et al.*, 1999; Herbert *et al.*, 1999) while reintroduction of *hTERT* into telomerase negative normal cells can reactivate telomerase activity and increase replicative lifespan (Bodnar *et al.*, 1998; Rufer *et al.*, 2001; Yang *et al.*, 1999; Vaziri and Benchimol, 1998). Detectable telomerase activity in an estimated 85% of malignant tumours (Shay and Bacchetti, 1997) and strong links between telomerase activity and malignant cells (Kim *et al.*, 1994) demonstrates the importance of telomerase activity in tumourigenesis and also suggests that telomerase activation is the most common mechanism for telomere maintenance in cancer cells.

However, a number of immortal cell lines and tumours lack telomerase activity yet have the ability to elongate their telomeres, which suggests the existence of nontelomerase-dependent routes to immortalisation (Reddel, 2003).

Figure 1.2 Senescence Mediated by Telomere Length and Cell Cycle Checkpoint Genes

Telomere length after development continually decreases in length with each population doublings in normal human somatic cells until telomeres become short and dysfunctional and signal for senescence and the cell will stop dividing. If cell cycle checkpoints are lost, cells can continue to divide beyond senescence, with continued attrition of telomere length, until they reach crisis in which most cells die. A rare cell will arise from crisis with the ability to maintain and/or elongate their telomeres and become immortal.



1.1.2.3 Alternative Lengthening of Telomeres

It is known that the alternative lengthening of telomeres (ALT) mechanism can be used to maintain telomere length in the absence of telomerase activity (Bryan *et al.*, 1997). In cells where ALT has been activated, telomere length is maintained by a recombination-based DNA replication mechanism rather than the catalytic addition of short repeats utilised by telomerase (Dunham *et al.*, 2000). ALT cells exhibit elevated levels of post-replicative inter-telomeric exchange (Londono-Vallejo *et al.*, 2004) and this may be responsible for the heterogeneity in telomere lengths observed in ALT cell lines (Bryan *et al.*, 1997) which is a characteristic now taken as a marker for the use of the ALT mechanism. Also associated with the use of the ALT mechanism is the presence of ALT associated PML bodies (APBs) (Yeager *et al.*, 1999) which contain the protein PML, telomeric DNA, the telomeric binding proteins TRF1 and TRF2 and proteins involved in DNA recombination and replication. It has also been proposed that APBs may be actively involved in the ALT mechanism (Grobelny *et al.*, 2000).

Recent studies endeavouring to understand the molecular mechanisms behind ALT and have identified that the MRE11/RAD50/NBS1 (MRN) complex is required for telomere maintenance in ALT (Jiang *et al.*, 2005) and this complex has been previously proposed to play a major role in homologous recombination in mammalian cells. Interestingly, although telomerase activation and ALT are thought of as the main telomere maintenance mechanisms in immortal cells and tumours, proposed telomere maintenance mechanisms other then telomerase activation or ALT have also been reported (Cerone *et al.*, 2005; Fasching *et al.*, 2005; Marciniak *et al.*, 2005).

It has been noted that activation of ALT in tumours cells is a much rarer event that activation of telomerase (Bryan *et al.*, 1997) and it has been hypothesised that ALT may normally occur under selective pressure when expression of telomerase is prevented. ALT is commonly activated in tumours of mesenchymal origin, including osteosarcomas, soft tissue sarcomas and glioblastoma multiforme (Henson *et al.*, 2005; Ulaner *et al.*, 2003; Hakin-Smith *et al.*, 2003; Johnson *et al.*, 2005), and it is understood that in normal mesenchymal tissues telomerase activity is more tightly repressed that in epithelial cells. This would lead to the selective pressure for ALT activation over telomerase activity in these cell types.

1.1.2.4 Telomerase and Transformation

It is widely accepted that cancer is the result of multiple alterations in gene expression caused by genetic and epigenetic mechanisms. This suggests that the bypass of senescence, gain of immortality and transformation would require alterations in many genes for such processes to occur. Consistent with this is the finding that although expression of telomerase is sufficient to immortalise most normal cells (Bodnar *et al.*, 1998; Rufer *et al.*, 2001; Yang *et al.*, 1999; Vaziri and Benchimol, 1998) it has been shown that alone, telomerase expression does not lead to neoplastic transformation (Jiang *et al.*, 1999; Morales *et al.*, 1999; Wootton *et al.*, 2003; Klinger *et al.*, 2006). However, multiple studies have shown that telomerase activity in combination with inactivation of tumour suppressor checkpoint pathway proteins can contribute to transformation.

In normal human fibroblasts it was observed that only a combination of hTERT, SV40 Large T antigen, which inactivates p53 and pRB, and H-RAS expression allowed establishment of a malignant phenotype (Hahn *et al.*, 1999a). Also, combinations of hTERT and H-RAS or c-MYC can transform Leiden human diploid fibroblasts, which have an INK4a/ARF mutation resulting in $p16^{INK4a}$ deficiency (Drayton *et al.*, 2003), while abrogation of $p16^{INK4a}$ and p53 combined with the expression of SV40 small t antigen, hTERT and H-RAS also leads to the transformation of normal human fibroblasts. (Voorhoeve and Agami, 2003). These studies suggest that multiple events are required to transform normal human cells.

However, some groups have shown that expression of telomerase activity is sufficient for neoplastic transformation. *hTERT* transduced fibroblasts have been shown acquire malignant changes during extended lifespan (Milyavsky *et al.*, 2003; Zongaro *et al.*, 2005) as have *hTERT* transduced human mesenchymal stem cells (hMSCs) (Serakinci *et al.*, 2004). Amongst other neoplastic changes, the hTERT transduced fibroblasts and hMSCs both showed inactivation of the tumour suppressor genes $p16^{INK4a}$ and $p14^{ARF}$ after extended population doublings.

It has also been shown that transformation by the expression of combinations of oncogenes can occur without the need for expression of hTERT in normal human fibroblasts (Lazarov *et al.*, 2002; Seger *et al.*, 2002) but tumours derived from these cells had gained telomerase activity suggesting that immortalisation did not require telomerase activity but long term growth of cancer cells requires it. In an interesting study, the ALT mechanism has also been shown to be the equivalent to telomerase activity in neoplastic transformation of

human cells by oncogenic Ras (Sun *et al.*, 2005a) thus indicating that telomerase activity is not always needed for neoplastic transformation.

However the fact that telomerase activity is detected in an estimated 85% of malignant tumours (Shay and Bacchetti, 1997) and the strong links between telomerase activity and malignant cells (Kim *et al.*, 1994) suggests the importance of telomerase activity in tumourigenesis. So, immortalisation may not be sufficient for tumourigenesis but may provide the necessary prerequisite for accumulating the total number of genetic events required for malignancy by allowing an extended lifespan in which neoplastic changes can accrue.

1.1.3 The Role of Tumour Suppressor Genes in Senescence

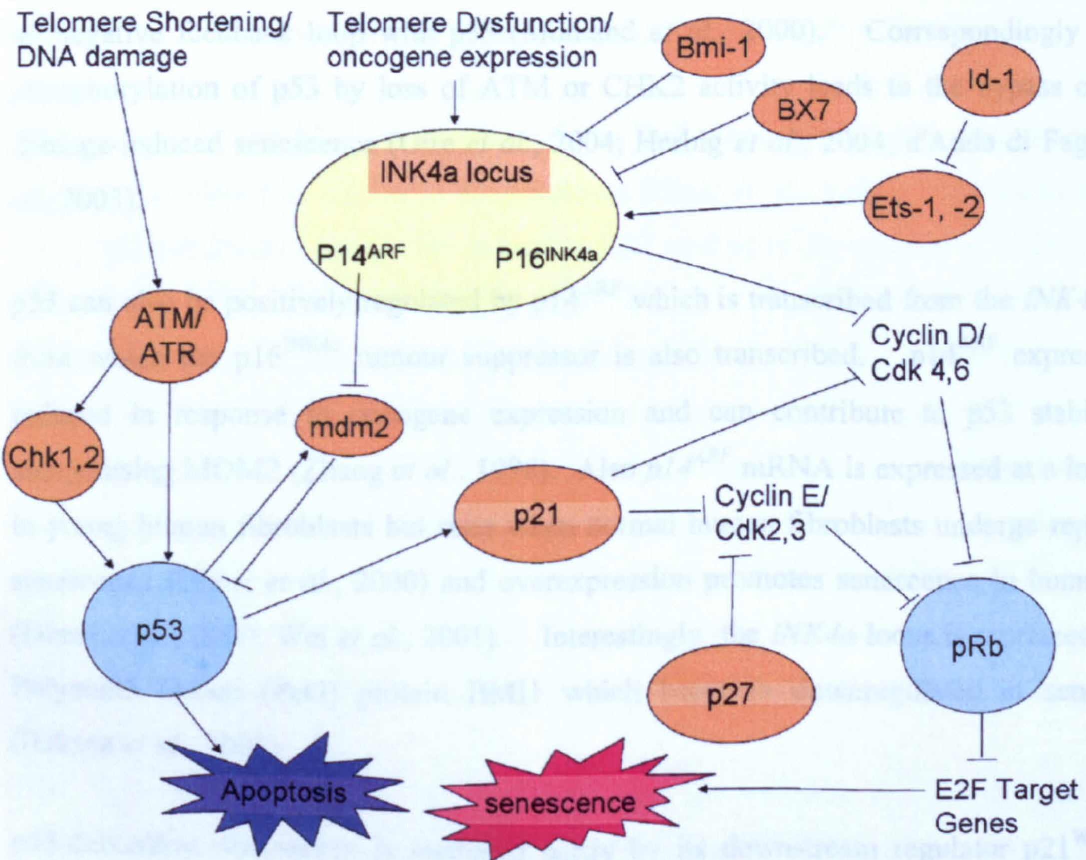
Multiple genetic pathways are known to mediate senescence and dysregulation of parts of these pathways can lead to bypass of senescence and contribute towards tumourigenesis. Two main tumour suppressor pathways in human cells are the p53-p21^{WAF1}-p14^{ARF} pathway and the p16^{INK4a}-pRB pathway (See Figure 1.3).

1.1.3.1 p53-p21^{WAF1}-p14^{ARF} Pathway

p53 is a sequence specific DNA binding tumour suppressor protein that is frequently inactivated in human cancers (Hollstein *et al.*, 1991). Loss of p53 function is sufficient for cells to escape senescence and to acquire up to 20–30 extra population doublings (Shay *et al.*, 1991; Hara *et al.*, 1991; Bond *et al.*, 1994). Studies have also shown that expression of wild type p53 in human cancer cell lines that lack wild type p53 can cause senescence or apoptosis (Shaw *et al.*, 1992; Sugrue *et al.*, 1997) while p53 activity was shown to be increased upon senescence and its transcriptional activity and DNA binding-activities were elevated (Atadja *et al.*, 1995; Bond *et al.*, 1996). Short or dysfunctional telomeres can be recognised as DNA double strand breaks (DSBs) (Karlseder *et al.*, 2002) and the occurrence of telomere dysfunction in serially cultivated normal human somatic cells correlated to an increase in p53 and senescence (d'Adda di Fagagna *et al.*, 2003; Vaziri and Benchimol, 1996). Cells react to DSBs by triggering the DNA damage checkpoint response, which arrest cell-cycle progression, and this response relies on the upstream protein kinases ATM and ATR and downstream transducer kinases CHK1 and CHK2 which can sense short or dysfunctional telomeres and transduce this as a DNA damage response (d'Adda di Fagagna *et al.*, 2003; Karlseder *et al.*, 1999). This leads to the phosphorylation of p53 which increases p53 stability and accumulation of p53 leading to

Figure 1.3 The Role of Tumour Suppressor Genes pRB and p53 in Senescence

See text for details



senescence (Siliciano *et al.*, 1997; Tibbetts *et al.*, 1999; Canman *et al.*, 1998; Banin *et al.*, 1998). p53 phosphorylation also inhibits MDM2s ability to promote p53 degradation (Shieh *et al.*, 1997) and MDM2 itself a direct transcriptional target of p53 thereby forming an negative feedback loop with p53 (Momand *et al.*, 2000). Correspondingly loss of phosphorylation of p53 by loss of ATM or CHK2 activity leads to the bypass of DNA damage-induced senescence (Gire *et al.*, 2004; Herbig *et al.*, 2004; d'Adda di Fagagna *et al.*, 2003).

p53 can also be positively regulated by p14^{ARF} which is transcribed from the *INK4a* locus from which the p16^{INK4a} tumour suppressor is also transcribed. p14^{ARF} expression is induced in response to oncogene expression and can contribute to p53 stability by antagonising MDM2 (Zhang *et al.*, 1998). Also *p14^{ARF}* mRNA is expressed at a low level in young human fibroblasts but rises when normal human fibroblasts undergo replicative senescence (Dimri *et al.*, 2000) and overexpression promotes senescence in human cells (Dimri *et al.*, 2000; Wei *et al.*, 2001). Interestingly, the *INK4a* locus is repressed by the Polycomb Group (PcG) protein BMI1 which becomes downregulated at senescence (Itahana *et al.*, 2003).

p53-dependent senescence is mediated partly by its downstream regulator p21^{WAF1} (el-Deiry *et al.*, 1994). p53 stabilisation at senescence allows p53 mediated transcription of multiple genes, including *p21^{WAF1}*, which becomes elevated at senescence in several cellular systems (Alcorta *et al.*, 1996; Tahara *et al.*, 1995). p21^{WAF1} is a cyclin-dependent kinase inhibitor (CDKi) which binds to and inactivates cyclin E-CDK2/3 complexes and cyclin D1-CDK4 and cyclin D1–CDK6 complexes to promote senescence by preventing phosphorylation of pRB tumour suppressor (Xiong *et al.*, 1993; Harper *et al.*, 1993). Exogenous expression of p21^{WAF1} induces senescence in early passage human diploid fibroblasts (McConnell *et al.*, 1998), while p21^{WAF1} inactivation leads to the abrogation of senescence and the induction of crisis (Brown *et al.*, 1997).

1.1.3.2 p16^{INK4a}-pRB Pathway

The Retinoblastoma (pRB) gene product is a tumour suppressor that regulates multiple cellular processes such as growth, differentiation and apoptosis, and is mutated or inactivated in many type of human cancers (Weinberg, 1995). The tumour suppressive activity of pRB derives from its ability to inhibit cell cycle transit by repressing the transcription of genes required for the G1-S-phase transition (Narita *et al.*, 2003; Weintraub *et al.*, 1995; Bremner *et al.*, 1995). Loss or inactivation of pRB in normal

human fibroblasts leads to the bypass of senescence and entrance into crisis, similar to that which happens with the abrogation of the p53-p21^{WAF1} pathway (Wei *et al.*, 2003; Sage *et al.*, 2003; Shay *et al.*, 1991). Correspondingly, it was found that re-introducing pRB into pRB$^{-/-}$ cells leads to the induction of senescence (Xu *et al.*, 1997).

pRB activity is regulated by phosphorylation and dephosphorylation events and these events are mediated by Cyclin/CDK complexes (Hinds *et al.*, 1992). At senescence pRB is hypophosphorylated and is able to bind to E2F leading to the repression of gene targets (Stein *et al.*, 1990; Futreal and Barrett, 1991; Narita *et al.*, 2003). Conversely, pRB is phosphorylated by Cyclin D/CDK4 or 6 in G1 and Cyclin E/CDK2 at the G1-S boundary and allows the transcription of genes essential to cell cycle progression by E2F (Bremner *et al.*, 1995; Hinds *et al.*, 1992; Buchkovich *et al.*, 1989; Chen *et al.*, 1989; Sherr and Roberts, 1995). Another CDK inhibitor, p27^{KIP1} also plays in important role in senescence by the inhibition of the phosphorylation of pRB by Cyclin E/CDK2 and increased formation of the p27^{Kip1}-Cyclin E-CDK2 ternary complex and decreased associated kinase activity are associated with pRB-mediated senescence (Alexander and Hinds, 2001).

The CDKi p16^{INK4a} acts as an upstream regulator of pRB and keeps pRB in an unphosphorylated form in senescent human cells by the inhibition of Cyclin D/CDK4 or 6 complexes and is a potent mediator of cell cycle arrest (Lukas *et al.*, 1995; Serrano *et al.*, 1993). p16^{INK4a} is induced by the accumulation of cell population doublings and the action of oncogenes (Serrano *et al.*, 1997; Zhu *et al.*, 1998; Alcorta *et al.*, 1996), oncogenic signalling (Lin *et al.*, 1998) or DNA damage or stress signals (Robles and Adami, 1998; Shapiro *et al.*, 1998). p16^{INK4a} is inactivated in a broad range of tumours (Kamb *et al.*, 1994; Nobori *et al.*, 1994) and this provides compelling evidence for the role of p16^{INK4a} in senescence. Interestingly, epigenetic silencing of p16^{INK4a} is a common mechanism for loss of expression in tumours suggesting the importance of epigenetic mechanisms in senescence (Merlo *et al.*, 1995; Huschtscha *et al.*, 1998). It is also noted that the pRB expression is also epigenetically silenced in some retinoblastoma tumours (Greger *et al.*, 1989).

Cellular lifespan is extended upon the inactivation of the p16^{INK4a}/pRB pathway (Huschtscha *et al.*, 1998; Narita *et al.*, 2003; Bond *et al.*, 2004) or by the overexpression of CDK4, the principle target of p16^{INK4a} (Morris *et al.*, 2002). Upstream regulators of p16^{INK4a} such as the Polycomb Group (PcG) proteins BMI1 and CBX7 are also capable of mediating senescence by repressing the *INK4a* locus leading to decreased levels of p16^{INK4a} (Itahana *et al.*, 2003; Gil *et al.*, 2004). The ETS1 and ETS2 transcription factors

can bind and activate the *INK4a* locus increasing levels of $p16^{INK4a}$, while ID1 represses $p16^{INK4a}$ expression (Ohtani *et al.*, 2001).

1.2 The Role of the Histone Modifications in the Regulation of Transcription

Studies have shown that expression of genes differs greatly in presenescent, senescent and transformed cell lines (Shelton *et al.*, 1999; Hardy *et al.*, 2005; Grillari *et al.*, 2000; Untergasser *et al.*, 2002; Yoon *et al.*, 2004; Guo *et al.*, 2004; Mason *et al.*, 2004), but how this is controlled is still relatively unknown. It is known that epigenetic processes such as DNA methylation and post-translational modification of histones are sufficient to alter gene expression and can therefore play a role in many cellular processes. Therefore it is plausible to suggest that changes in the chromatin environment can play a role in the huge changes in gene expression involved in senescence and also in the bypass of senescence towards transformation and malignancy.

The term epigenetics is defined as mechanisms through which changes in gene function can occur without a change in the DNA sequence. Such mechanisms include covalent modifications of histones, DNA methylation, ATP-dependent chromatin remodelling, incorporation of variant histones, looping and changes in local conformation of DNA and alterations of higher-order chromatin structure. These processes are required for processes involving DNA, due to the nature of the packaging of DNA in the nucleus. DNA is packaged as chromatin in the form of approximately 147bp of DNA wound twice around a histone octamer consisting of two copies of the H2A, H2B, H3 and H4 histones to form the structural subunit of chromatin; the nucleosome. This packaging strategy allows approximately 1 metre of DNA to be packaged properly into the 10μm size of an average cell nucleus, but also represents a major obstacle to many processes involving DNA including transcription. Epigenetic modifications are necessary to allow such processes to proceed by altering chromatin structure to change of accessibility of a region of DNA.

Sections of the genome can be labelled euchromatin or heterochromatin based on the transcriptional permissivity of the chromatin and therefore the accessibility of the DNA. Euchromatin is gene rich and is not tightly wrapped around histones meaning that genes within the region are frequently transcribed to mRNA in the cell, while heterochromatin is darkly staining and tightly packaged or coiled throughout the cell cycle and that is, for the most part, genetically inactive. Certain histone modifications are associated with

compacting the binding of DNA to histones, therefore repressing the binding of regulatory factors which may mediate expression of the DNA content therein, while certain modifications are linked to the relief of this compaction, permitting access of regulatory factors to the DNA. Various amino-acid residues mainly on the histone amino-terminal tail can be covalently modified by acetylation, methylation, phosphorylation, SUMOylation, ubiquitination and ADP-ribosylation (Figure 1.4). Patterns of such modifications signify the transcriptional status of the chromatin and therefore the transcriptional status of DNA therein. This is known at the "histone code" hypothesis (Jenuwein and Allis, 2001; Strahl and Allis, 2000) and entails that combinations of histone modifications can be read as a code and translated into an output signal, for example; gene transcription on or off.

1.2.1 Covalent Histone Modifications Linked to the Repression of Gene Transcription

Methylation of specific lysine residues are understood to be linked to gene repression and the formation of heterochromatin. Heterochromatin is generally characterised by hypo-acetylated histones and methylation of lysines 9 and 27 of histone H3 (K9 H3 and K27 H3) and methylation of lysine 20 histone H4 (K20 H4).

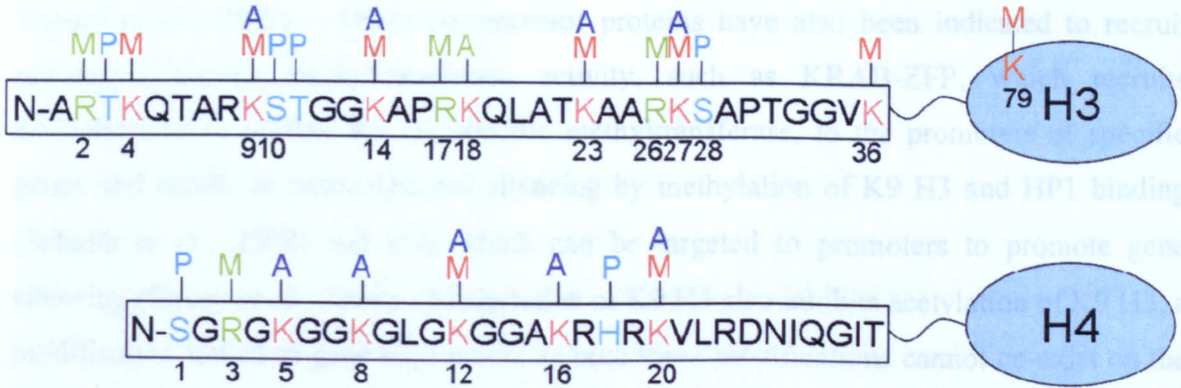
1.2.1.1 Methylation of Lysine 9 Histone H3

Methylation of K9 H3 has roles in heterochromatin formation and transcriptional silencing of euchromatic genes (Boggs *et al.*, 2002; Peters *et al.*, 2002; Nakayama *et al.*, 2001). The histone methyltransferases (HMTs) SUV39H1 and SUV39H2 mediate tri-methylation of K9 H3 (Rea *et al.*, 2000) and loss of the SUV39H enzymes leads to increased genomic instability and increased tumour risk demonstrating that the maintenance of heterochromatic domains is essential for cellular stability (Peters *et al.*, 2001). The HP1 proteins (α, β and γ) are known to bind methylated K9 H3 to mediate transcriptional repression by compacting chromatin through association with other HP1 proteins (Bannister *et al.*, 2001; Lachner *et al.*, 2001). HP1 has also been shown to recruit SUV39H1 (Stewart *et al.*, 2005), DNA-methyltransferases (Fuks *et al.*, 2003) and Histone Deacetylases (HDACs) (Vaute *et al.*, 2002) which are all involved in the generation of a repressive chromatin environment.

However, the function and regulation of K9 H3 methylation is different in heterochromatin than in euchromatin with the mono- and di-methylated modification restricted to

Figure 1.4 Histone Modifications of Histones H3 and H4.

This diagram shows the main residues that can be modified and the main modifications know to occur on the histone tails and globular domain of histone H3 and H4 (m – methylation; a, acetylation; p, phosphorylation) [Redrawn from Santos-Rosa et al (Santos-Rosa and Caldas, 2005)].



euchromatin in mammals (Rice *et al.*, 2003; Peters *et al.*, 2003) with the HMTs G9a and GLP being the major enzymes which catalyse mono- and di-methylation of K9 H3 in euchromatin (Tachibana *et al.*, 2005). However, it is known that pRB can recruit SUV39H1 to the promoters of several S-phase genes in differentiating cells in order to methylate K9 H3 and allow binding of HP1 allowing gene repression (Nielsen *et al.*, 2001; Vandel *et al.*, 2001). Other co-repressor proteins have also been indicated to recruit repressive histone methyltransferase activity, such as KRAB-ZFP, which recruits SETDB1/ESET, another K9 H3 specific methyltransferase, to the promoters of specific genes and results in transcriptional silencing by methylation of K9 H3 and HP1 binding (Schultz *et al.*, 2002) and G9a which can be targeted to promoters to promote gene silencing (Gyory *et al.*, 2004). Methylation of K9 H3 also inhibits acetylation of K9 H3, a modification linked to gene expression, as both these modifications cannot co-exist on the same lysine residue, while SUV39H1 recruitment can be inhibited by methylation of lysine 4 H3 (K4 H3), a modification also linked to gene expression (Nishioka *et al.*, 2002a).

Demethylation of K9 H3 has also recently been observed with the discovery of the demethylase LSD1 (Metzger *et al.*, 2005) which has been shown to be able to demethylate a di-methylated K9 H3.

Recently, a new role for methylation of K9 H3 has been uncovered with the discovery of dynamic K9 H3 di- and tri-methylation occurring in the transcribed region of active genes in mammalian chromatin (Vakoc *et al.*, 2005). This study links the presence of methylated K9 H3 and HP1γ to active transcription as their presence is associated with elongation by RNA polymerase II and are rapidly removed from repressed genes. Methylation of K4 H3 was also observed to be present at the coding sequence of transcribed genes, to a level similar to that of methylation of K9 H3, showing that these markers can actually be found on the same chromatin fragment in *cis*. G9a has also recently been described as functioning as a transcriptional coactivator for nuclear receptors, cooperating synergistically with GRIP1, CARM1 and p300 (Lee *et al.*, 2006) further suggesting a role for methylation of K9 H3 in transcriptional activation rather than repression.

1.2.1.2 Methylation of Lysine 27 Histone H3

Methylation of K27 H3 is also a modification linked to transcriptional repression and maintenance of stable epigenetic silencing (Cao *et al.*, 2002; Dellino *et al.*, 2004; Kirmizis *et al.*, 2004). Methylation of K27 H3 is mediated by G9a (Tachibana *et al.*, 2001), which

can also methylate K9 H3, and the polycomb group proteins EZH2 (Cao *et al.*, 2002) and SUZ12 (Cao and Zhang, 2004b). Methylation of K27 H3 allows the binding of Pc, a member of the Polycomb Group (PcG) proteins, similar to the binding of HP1 to methylated K9 H3. The roles of methylation of K27 H3 include the silencing of homeotic genes during development (Cao *et al.*, 2002), X-inactivation (Plath *et al.*, 2003), genomic imprinting (Czermin *et al.*, 2002; Muller *et al.*, 2002) and heterochromatin formation (Peters *et al.*, 2003).

1.2.1.3 Methylation of Lysine 20 Histone H4

Methylation of K20 H4 is also linked to gene silencing and is considered to be a marker of mammalian heterochromatin (Schotta *et al.*, 2004; Kourmouli *et al.*, 2004). The HMT NSD1 mediates K20 H4 methylation as well as K36 H3 methylation (discussed below) (Rayasam *et al.*, 2003) while the enzyme PR-SET7/SET8 has also been shown to methylate K20 H4 and mediate gene silencing (Nishioka *et al.*, 2002b; Fang *et al.*, 2002). The HMTs SUV4-20H1 and SUV4-20H2 can also catalyse tri-methylation of K20 H4 which precludes histone H4 hyperacetylation, a euchromatic modification, aiding the formation of silent chromatin (Sarg *et al.*, 2004).

Interestingly, tri-methylation of K20 H4 has been observed to accumulate in older tissues and also accumulates in cells in the stationary phase of growth (Sarg *et al.*, 2002) while another study demonstrated that heterochromatin in cancer cells is less trimethylated at K20 H4, further reinforcing the critical role this modification has in the silencing and compaction of chromatin (Fraga *et al.*, 2005). This model suggests that the loss of methylation of K20 H4 could account for genomic instability observed in cancers, although mechanisms of how methylation of K20 H4 is lost are unknown. It is known however that the SUV4-20H enzymes directly interact with the RB proteins (Gonzalo *et al.*, 2005) and a lack of pRB could the lead to the loss of this modification and the silencing properties it entails. Studies have also indicated a link between methylation of K9 H3 and K20 H4 and transcriptional repression as the SUV4-20H enzymes have been shown to bind to HP1 and methylation of K20 H4 is abolished at pericentric regions in the absence of SUV39H1 and SUV39H2 (Schotta *et al.*, 2004; Kourmouli *et al.*, 2005).

Although this modification has been linked to silent chromatin, the role of this modification has been complicated by the finding of the presence of mono-methylated K20 H4 at euchromatic regions of the genome, such as promoter sequences and coding regions of active genes (Talasz *et al.*, 2005). Mono-methylation of K20 H4 was also linked to

hyperacetylation and seems to parallel data from studies of the finding of methylation of K9 H3 in the coding regions of actively transcribed genes.

Methylation of K20 H4 has also been linked to DNA repair in Drosophila. The loss of the SET9 gene, which mediates K20 H4 methylation in Drosophila, leads to hypersensitivity to DNA double strand breaks rather than disruption of heterochromatin formation (Sanders *et al.*, 2004b). This seems to be due to the inability of Crb2 to be recruited to DSBs due to the lack of methylated K20 H4. The actions of Crb2 are shown to need constitutive methylation of K20 H4 for its role in DNA damage response, so the loss of methylation of K20 H4, as previously observed tumour cells, could lead to the abrogation of the DNA damage response increasing the likelihood of cells accumulating neoplastic changes. Interestingly 53BP1, the human Crb2 homologue, binds to methylated K79 H3 (Huyen *et al.*, 2004), a modification linked to gene expression and transcriptional activity.

Demethylation of K20 H4 has yet to be shown but recent findings that LSD1 can function as a K9 H3 and, as will be discussed later, a K4 H3 demethylase dependent on the complex in that it exists, suggests that this enzyme may function as non-specific histone demethylase which could therefore play a role in demethylation of K20 H4 (Metzger *et al.*, 2005; Shi *et al.*, 2004).

1.2.2 Covalent Histone Modifications Linked to Permissiveness of Gene Transcription

Methylation of specific lysine residues are also understood to be linked to gene activation and the formation of euchromatin. Euchromatin is characterised by hyper-acetylated histones and methylation of lysines 4, 36 and 79 of histone H3 (K4 H3, K36 H3 and K79 H3).

1.2.2.1 Methylation of Lysine 4 Histone H3

Elevated levels of methylation of K4 H3 have been observed at active genes, gene promoters and transcriptional start sites (Santos-Rosa *et al.*, 2002; Schneider *et al.*, 2004a; Bernstein *et al.*, 2005; Liang *et al.*, 2004; Schubeler *et al.*, 2004). While di-methylation of K4 H3 can be found at active and inactive chromatin domains, tri-methylation of K4 H4 marks exclusively for active transcription (Santos-Rosa *et al.*, 2002). A number of human enzymes are known to be able to methylate K4 H3 including SMYD3 (Hamamoto *et al.*,

2004), SET7/9 (Wilson *et al.*, 2002), hSET1 (Wysocka *et al.*, 2003) and MLL (Milne *et al.*, 2002).

It is proposed that methylation of K4 H3 can influence gene expression through a number of mechanisms. Methylation of K4 H3 can act as a docking site for protein modules as di-methylated K4 H3 can be bound by WDR5, a component of an MLL complex required for global tri-methylation (Wysocka *et al.*, 2005). MLL also associates with the K16 H4 acetyltransferase MOF (Dou *et al.*, 2005) thereby promoting the formation of a more permissive chromatin environment. Methylation of K4 H3 can also mediate the binding of the ATP-dependent chromatin remodeller CHD1, which is linked to the regulation of transcription of certain genes (Sims *et al.*, 2005). As well as promoting the binding of some proteins, methylation of K4 H3 has been shown to preclude repressive complexes including deacetylase complexes (Zegerman *et al.*, 2002) and can inhibit K9 methylation by SUV39H enzymes (Wang *et al.*, 2001). So, in general methylation of K4 H3 inhibits repressive histone modifications and promotes permissive histone modifications, therefore leading to a chromatin environment more likely to allow gene expression.

The reversibility of this modification has recently been illustrated with the discovery of the enzyme LSD1 which, as well as being able to demethylate K9 H3, has been shown to be able to demethylate K4 H3 (Shi *et al.*, 2004).

Interestingly, methylation of K4 H3 is also proposed to play a role in a type of cellular memory by marking for recently transcribed genes. This occurs due to the interaction of the RNA polymerase II molecule with a K4 H3 methyltransferase (Ng *et al.*, 2003). This was established in yeast, and demonstrated that the yeast K4 H3 HMT Set1, is recruited by RNA polymerase II to the 5' portion of active mRNA coding regions. This led to K4 H3 hypermethylation within the coding region which was observed to persist for considerable time after transcriptional inactivation indicating that this modification could provide a molecular memory of recent transcriptional activity.

1.2.2.2 Methylation of Lysine 36 Histone H3

Methylation of K36 H3 is linked to transcriptional activation and is postulated to have a role in transcriptional elongation (Morris *et al.*, 2005; Bannister *et al.*, 2005). Methylated K36 H3 has been found to be concentrated at active genes, peaking at the 3' end of the gene (Bannister *et al.*, 2005) while other studies in yeast have provided a mechanism by which this could occur by showing the interaction of a K36 H3 specific methyltransferase

with an elongating RNA polymerase II molecule (Kizer *et al.*, 2005). In humans, two proteins have been identified which are able to catalyse the methylation of K36 H3; NSD1 (Rayasam *et al.*, 2003) and the Huntingtin interacting protein B (HYPB) (Sun *et al.*, 2005b).

Interestingly, recent studies in yeast have shown that methylated K36 H3 can be bound by a chromodomain containing factor which recruits deacetylase activity (Keogh *et al.*, 2005; Joshi and Struhl, 2005), further complicating the role of methylation of K36 H3 in transcriptional control. A proposed model indicates that after the RNA polymerase II molecule has passed through an "open" coding region of chromatin, it must be "shut" again and methylation of K36 H3, while associated with transcription, may mediate the condensation of chromatin after transcription has occurred by mediating the binding of a chromatin silencing protein to a modification previously mediating the permissivity of the chromatin environment.

The reversibility of this modification has also recently been described with the identification of a JmjC domain-containing protein, called JHDM1 (JmjC domain-containing histone demethylase 1) which specifically demethylates K36 H3 (Tsukada *et al.*, 2005).

1.2.2.3 Methylation of Lysine 79 histone H3

Methylation of K79 H3 is catalysed by hDOTL (Feng *et al.*, 2002) which unusually does not contain a SET domain unlike all other lysine methyltransferases. It has been shown to be a marker for euchromatin in yeast and mammalian cells (Im *et al.*, 2003; Schubeler *et al.*, 2004) and recent studies have shown that the fusion of hDOTL to a Mixed Lineage Leukaemia (MLL) fusion partner results in the upregulation of a number of genes involved in leukaemia, concomitant with methylation of K79 H3 (Okada *et al.*, 2005). Methylation of K79 H3 has also been linked to the sensing of double stranded breaks through the binding of 53BP1 (Huyen *et al.*, 2004). Higher order structural changes that occur upon the formation of double stranded breaks are thought to reveal methylated K79 H3 which are then sensed and bound by 53BP1 which then activates the DNA DSB checkpoint pathway by signalling through ATM.

1.2.2.4 Histone H3 and H4 Acetylation

In general, histone hyperacetylation occurs in euchromatic regions and correlates to gene expression (Schubeler *et al.*, 2004; Bernstein *et al.*, 2005). Acetylation of promoter proximal histones is associated with gene expression (Liang *et al.*, 2004; Forsberg *et al.*, 2000; Roh *et al.*, 2005) with K9 and K14 H3 acetylation highly localised to active transcriptional start sites (Liang *et al.*, 2004). These modifications also strongly correlate with methylation of K4 H3 (Bernstein *et al.*, 2005). While histone hyperacetylation correlates with gene expression, histone hypoacetylation is linked to heterochromatin and gene repression (Su *et al.*, 2004). Acetylation is very low in condensed chromatic regions and inactive genes show similar levels (Litt *et al.*, 2001; Gilbert and Sharp, 1999). Reversible histone acetylation is mediated by the actions of histone acetyltransferases (HATs) and histone deacetylases (HDACs).

Histone lysine acetylation is thought to play several roles in gene regulation. Initial studies provided evidence that acetylation of histones allowed flexibility and accessibility of chromatin (Krajewski and Becker, 1998) and enhanced binding of transcription factors to DNA (Vettese-Dadey *et al.*, 1996). Further studies have shown that acetylation of H3 and H4 can counteract nucleosome fibre compaction (Bertin *et al.*, 2004) and recent studies have definitively shown that acetylation of histones increases chromatin accessibility as measured by studying the nuclear distribution of microinjected flourescein-labelled dextrans (Gorisch *et al.*, 2005).

Acetylation can also preclude repressive modifications or repressive complexes in order to promote a more permissive chromatin environment. Acetylation of K16 H4, mediated by MOF, is observed to be enriched in transcriptionally active gene sequences (Taipale *et al.*, 2005) and precludes tri-methylation of K20 H4 (Sarg *et al.*, 2004) and histone H3 acetylation can preclude the binding of INHAT, a repressive chromatin modifying complex known to mediate transcriptional repression (Schneider *et al.*, 2004b). Acetylation of K14 H3 and phosphorylation of serine 10 H3 (S10 H3) can cause dissociation of HP1 from methylated K9 H3 (Mateescu *et al.*, 2004) and acetylation of K9 H3 precludes methylation of K9 H3, as these two modifications cannot exist on the same lysine moiety.

Acetylated lysines can also be bound by bromodomain containing proteins or complexes which can affect transcription, such as TFIID, part of the basal transcription machinery, which can bind to promoter sequences by recognizing acetylated K9 and K14 (Agalioti *et*

al., 2002). Studies in yeast have also shown that acetylated K14 H3 can be bound by a chromatin remodelling enzyme in order to mediate gene expression (Kasten *et al.*, 2004).

As described, transcriptional activation frequently correlates with additional acetylation of histones at promoter nucleosomes and targeting HAT activity to promoters can enhance protein expression levels in mammalian cells (Kwaks *et al.*, 2005). Transcription factors can recruit co-activators with HAT activity to regulatory DNA sites (Utley *et al.*, 1998). MYC binding is concomitant with H4 acetylation by the recruitment of the TIP60 histone acetyltransferase complex to chromatin (Frank *et al.*, 2003). Acetylation may also allow the passage of RNA polymerase II through nucleosomes (Protacio *et al.*, 2000) and the HAT CBP/p300 has been shown to co-localise with RNA polymerase II at transcriptionally active domains (von Mikecz *et al.*, 2000). Conversely, transcriptional repressors can recruit corepressors with HDAC activity, such as the Mad/Max heterodimer which can recruit HDAC activity (Sommer *et al.*, 1997). pRB can also recruit HDACs to promoters to repress gene expression (Ferreira *et al.*, 1998; Luo *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998; Brehm *et al.*, 1998) and has also been shown to recruit other enzymes such as SUV39H1 leading to the repression of gene transcription (Gonzalo *et al.*, 2005; Nielsen *et al.*, 2001; Vandel *et al.*, 2001).

1.2.3 Epigenetics and Cancer

It is now understood that epigenetics can impact on gene expression and this area has been one of the most researched fields over recent times and the importance of epigenetic mechanisms in the initiation and progression of cancer has now become apparent. Enzymes which control histone modifications through modifying histone tails, and also other epigenetic mechanisms represent an additional level of transcriptional control which can become dysregulated in tumourigenesis. In many cancers, histone modifying enzymes are mutated, fused to other activators/repressors, deleted, duplicated, amplified, overexpressed or aberrantly expressed (Santos-Rosa and Caldas, 2005). This can lead to the dysregulation of multiple genes mediating many cellular processes due to the important role that the chromatin environment has to play in global gene regulation. Many excellent reviews have illustrated this point well and suggest an epigenetic model of cancer to complement the genetic model (Santos-Rosa and Caldas, 2005; Jones and Baylin, 2002; Hake *et al.*, 2004; Esteller, 2006; Feinberg, 2004).

The epigenetic model of cancer hypothesises that epigenetic modifications can play roles in tumour initiation and progression (Feinberg, 2004). Epigenetics is hypothesised to

impact on tumour initiation by interacting with the genetic alterations, in that epigenetic alterations may determine the effect of subsequent genetic insults by altering the frequency of neoplasia after a genetic insult. Another excellent review, discussed in more detail in Chapter 5, suggests that epigenetics is at the very root of tumourigenicity, by disruption of gene expression at the stem cell level (Feinberg *et al.*, 2006), impacting on both the fields of epigenetics and cancer stem cell biology.

Overall, this further suggests a role for epigenetic disruption in the key early events in tumourigenesis, such as the bypass of senescence leading towards transformation and further suggests that the regulation of genes involved in senescence and tumourigenicity may become altered by epigenetic mechanisms.

1.3 Epigenetic Mechanisms of Senescence

Although a great deal is already known about how chromatin regulates cell proliferation through its effects on transcription, less is known about how chromatin regulates senescence. However, recent studies have begun to shown how modulation of the chromatin environment can play an important role in the senescence phenotype.

1.3.1 Histone Methylation and Senescence

Initial studies have previously established that chromatin becomes reorganised with increased population doublings with large scale affects evident upon senescence (Macieira-Coelho, 1991) and recent papers have now shown that the formation of heterochromatin can play an important role in the senescence phenotype. Studies have shown that treatment with oncogenic RAS can induce the accumulation of $p16^{INK4a}$ and hypophosphorylated pRB with a senescence-like arrest in the IMR90 normal human fibroblast cell line (Narita *et al.*, 2003). Intriguingly, electron microscopy then found that the senescence-like arrest was associated with the formation of distinct heterochromatic structures which were named Senescence Associated Heterochromatin Foci or SAHF (Narita *et al.*, 2003).

Importantly, these heterochromatic foci were formed by newly deposited heterochromatin rather than from redistributed heterochromatin and were concentrated for repressive histone modifications such as methylation of K9 H3 and HP1 while methylated K4 H3 and acetylated K9 H3 were excluded. It was also established that active transcription was not occurring at the sites of these foci and chromatin features indicative of these foci formed

on E2F-responsive promoters of genes known to be downregulated at senescence. This indicates that these chromatin modifications are vitally important for the stable senescence-like arrest attained. The genes that gained heterochromatic features and became downregulated included the Cyclin A gene and the PCNA gene and their promoters were observed to be bound by pRB, thereby linking pRB binding to the formation of the SAHF. pRB has been shown to interact with factors involved in the formation of heterochromatin (Ferreira *et al.*, 1998; Luo *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998; Brehm *et al.*, 1998; Vandel *et al.*, 2001; Nielsen *et al.*, 2001) and so pRB binding to regulatory sequences could be sufficient to form these heterochromatic foci to silence gene expression. Importantly, the authors showed in the same study that promoters of genes known to be upregulated at senescence gained euchromatic patterns at their promoters and lost the heterochromatic features at senescence at the same time as the SAHF were being formed suggesting that the formation of the SAHFs may be directed towards certain genes and not an indiscriminate global effect. Further studies into the formation of SAHF have also indicated roles for the chromatin regulators HIRA and ASF1 and were shown to be rate limiting for the formation of SAHF and required for senescence (Zhang *et al.*, 2005).

These studies suggest a model in which the formation of distinct promoter specific heterochromatin structures accompanies the senescence process and indicates a role for pRB-mediated heterochromatin formation at growth regulatory genes during senescence, leading to the stable repression of these genes. Upregulation of $p16^{INK4a}$ during senescence can inhibit the phosphorylation of pRB leading it to bind to E2F and cause a permanent growth arrest by silencing growth regulatory genes by altering the chromatin state of the promoter sequences. From this it was proposed that failure of certain processes in these cells (INK4a or pRB mutations) may lead to bypass of senescence and progression to cancer due to the inhibition of repression of the chromatin environment of the growth regulatory genes (Narita *et al.*, 2003).

These studies have also shown that chromatin changes that occur during senescence can affect the "gene expression profile" of the cell as increased repressive modifications on proliferation-promoting genes and decreases in repressive modifications at senescence associated genes were observed. In addition, it has also been observed that many genes upregulated in senescence are actually physically clustered (Zhang *et al.*, 2003a), further suggesting that re-organisation of chromatin domains may be responsible for the upregulation of these genes by the formation of a more euchromatic chromatin environment.

Several other papers have recently provided more evidence of the link between chromatin remodelling and senescence. Utilising a mouse model, Braig *et al* have demonstrated that oncogenic RAS could halt proliferation of primary lymphocytes by a SUV39H1-dependent and K9 H3 methylation-related senescent growth arrest (Braig *et al.*, 2005). In the same study, the deletion of SUV39H1 in primary lymphocytes led to the improper methylation of K9 H3 ultimately leading to the promotion of RAS-driven lymphanogenesis, indicating a necessity for proper regulation of the chromatin environment at senescence and also a role for senescence in tumour suppression in RAS driven oncogenesis.

This study also correlates with other work which demonstrated that loss of methylation of K9 H3 and K20 H4 resulted in genomic instability in a variety of normal cells and was associated with increased tumour risk (Peters *et al.*, 2001). Decreased expression of SUV39H1 has also been observed in the early premalignant stage of tumour development in the context of hepatocarcinogenesis induced by methyl deficiency in rats (Pogribny *et al.*, 2006), although beyond this point in cancer development, SUV39H1 expression increased. The loss of trimethylation of K20 H4 was also observed at both preneoplastic and tumour stages of liver cancer in the same study and corresponded to a decrease in the K20 H4 HMT SUV4-20H2. These studies provide evidence that improper heterochromatin maintenance can lead to genomic instability and tumourigenicity.

In another study, Michaloglou et al demonstrated that $BRAF^{E600}$-mediated oncogenic signalling in melanocytes has been shown to induce a senescence like phenotype including the formation of SAHF (Michaloglou *et al.*, 2005). BRAF is a protein kinase and downstream regulator of RAS and the V600E mutation is an oncogenic mutation observed frequently in human naevi (moles). A further study focusing on RAS utilised a mouse model with a conditional oncogenic K-RASV12 allele activated by the Cre recombinase (Collado *et al.*, 2005). Upon activation of the oncogenic RAS allele, the mice developed multiple lung adenomas (pre-malignant) and a few lung adenocarcinomas (malignant) and markers for senescence were evident in the adenomas but not in the carcinomas. Interestingly HP1-γ was strongly positive in the adenomas, whereas the carcinomas were negative. As the HP1 proteins can bind methylated K9 H3 to mediate gene silencing, this study further reinforcing the role of the chromatin environment in senescence growth arrest.

Although the gain of heterochromatin by increased levels of repressive histone modifications in senescence is apparent, the gain of repressive histone modifying activity and gene silencing has also been observed to allow extension of lifespan and is involved in

cancer while the loss of heterochromatin has been linked to the onset of senescence (Howard, 1996). The Polycomb Group (PcG) proteins act by forming multiprotein complexes that can repress target gene expression by catalysing the addition of repressive modifications of histones. Loss of function of the PcG protein BMI1 was shown to impair the progression of normal lymphocytes into S-phase and these cells underwent premature senescence, connecting transcriptional repression by PcG proteins with cell-cycle control and senescence (Jacobs *et al.*, 1999). BMI1 has also been shown to be downregulated in senescing human fibroblasts (Itahana *et al.*, 2003) while BMI1 over-expression has also been observed in tumours and tumour cell lines (Bea *et al.*, 2001; Vonlanthen *et al.*, 2001; Itahana *et al.*, 2003; Mori *et al.*, 2005) and has been shown to be required for the short term survival of cancer cells (Liu *et al.*, 2006). Further studies have also shown that overexpression of BMI1 is able to transform and immortalise some human cells (Leung *et al.*, 2004; Jacobs *et al.*, 1999; Dimri *et al.*, 2002). Importantly the INK4a locus from where $p16^{INK4a}$ and $p14^{ARF}$ are transcribed from is a critical downstream targets for BMI1 with regard to its effects on cell proliferation and senescence (Itahana *et al.*, 2003). It is proposed that by inhibiting the expression of $p16^{INK4a}$ and $p14^{ARF}$ BMI1 can lead to the promotion of cell cycle progression.

EZH2 is another PcG protein, and has been shown to be able to methylate K27 H3 (Cao and Zhang, 2004a). EZH2 is overexpressed in some cancers, again linking the formation of heterochromatin to the extension of lifespan (Varambally *et al.*, 2002; Kleer *et al.*, 2003; Bracken *et al.*, 2003). EZH2 expression is also known to be repressed by pRB suggesting a possible mechanism for EZH2 overexpression in cancers (Bracken *et al.*, 2003). Interestingly, the EZH2 expression is also suppressed by activated p53 is seen to be specifically downregulated in senescent cells (Tang *et al.*, 2004). The corresponding loss of K27 H3 HMT activity may correspond to loss of heterochromatin and activation of genes such as $p16^{INK4a}$ and $p14^{ARF}$ leading to senescence (Villeponteau, 1997) while overexpression of EZH2 may lead to the proper maintenance of heterochromatin allowing the elongation of lifespan in cancer cells by bypassing senescence. These studies establish a link between the loss of PcG-mediated silencing and the senescence phenotype and PcG mediated silencing and tumourigenesis.

Overall, that the gain of heterochromatic features can lead to senescence by the repression of proliferation-promoting genes, although the repression of some genes by chromatin based gene silencing can contribute to tumourigenesis.

1.3.2 DNA Methylation and Senescence

Other epigenetic mechanisms such as DNA methylation have also been shown to be involved in the senescence phenotype. DNA methylation has been shown to decrease with age (Wilson and Jones, 1983) and this loss was hypothesised to function as a "counting mechanism" for senescence so the senescence phenotype would be signalled when a cell lost a certain degree of DNA methylation (Hoal-van Helden and van Helden, 1989; Wilson and Jones, 1983) similar to the model in which errors in the maintenance of heterochromatic domains could lead to senescence (Howard, 1996). Treatment with 5-azadeoxycitidine (5-azadC), a DNA demethylating agent, has been shown to reduce the replicative lifespan of normal human fibroblasts supporting the "counting mechanism" hypothesis (Holliday, 1986; Fairweather *et al.*, 1987).

Also in agreement with this hypothesis are the findings that DNA methyltransferase activity is elevated in cancer cells *in vitro*, in tumours *in vivo* (Belinsky *et al.*, 1996) and in cells transformed by oncogenic RAS or SV40 large T antigen (Slack *et al.*, 1999; Rouleau *et al.*, 1995), while DNA methyltransferase activity decreases in serially passaged normal human fibroblasts (Issa *et al.*, 1994). Therefore the loss of DNA methylation with age may eventually have deleterious consequences such as dysregulation of gene expression. Consistent with this view, it has been hypothesised that DNA methylation may repress a set of growth inhibitory genes (Young and Smith, 2001; Jones and Baylin, 2002). As the number of cell population doublings increases and DNA methylation decreases, the repression of such growth inhibitory genes may become less stringent leading to their eventual expression, contributing to senescence.

However, it has also been shown that a striking feature of some tumours (both benign and malignant) is that global DNA methylation is reduced (Feinberg and Vogelstein, 1983; Goelz *et al.*, 1985). This loss of gene silencing is also linked to chromosomal instability which will contribute to tumourigenesis (Eden *et al.*, 2003; Gaudet *et al.*, 2003). So, paradoxically, it has been shown that the loss of DNA methylation can contribute to senescence *and* tumourigenicity. However, it is acknowledged that gene specific methylation is variable and that many tumour suppressor genes show promoter specific DNA methylation leading to gene silencing, including *RB1* (Sakai *et al.*, 1991) and $p16^{INK4a}$ (Gonzalez-Zulueta *et al.*, 1995). It is likely that global loss of DNA methylation which leads to chromosomal instability will trigger the senescence phenotype as a tumour-suppressor mechanism but the promoter specific alterations in key tumour-suppressor genes may allow the bypass of senescence and tumourigenicity.

Overall, the data suggests that genome wide loss of heterochromatin may lead to genetic instability which is detected and the cell will signal for senescence to occur. However, gene specific gain of heterochromatic features can also lead to senescence by the repression of proliferation-promoting genes.

1.3.3 Histone Acetylation and Senescence

The acetylation status of promoter proximal histones has also been linked to senescence. Promotion of histone acetylation by HDAC inhibitors can induce senescent-like phenotype in human fibroblasts several population doublings after treatment (Ogryzko *et al.*, 1996; Munro *et al.*, 2004; Place *et al.*, 2005). It was also demonstrated that in senescing normal human fibroblasts, HDAC1 became downregulated (Place *et al.*, 2005) indicating that overall the promotion of acetylation can lead to senescence.

However histone deacetylation has also been shown to have a role in the extension of cellular lifespan. The yeast protein Sir2, identified as an NAD^+-dependent HDAC involved in rDNA silencing, has been shown to be able to contribute to the replicate lifespan of yeast (Kaeberlein *et al.*, 1999; Imai *et al.*, 2000). Enhanced activity of the *sir2* gene was also found to be associated with increased longevity in worm (Hekimi and Guarente, 2003), flies (Rogina and Helfand, 2004) and rodents (Cohen *et al.*, 2004). Drosophila Sir2 has further been shown to be involved in epigenetic gene silencing by the PcG proteins and is physically associated with a complex containing the Drosophila homolog of EZH2 further implicating Sir2 in the control of senescence (Furuyama *et al.*, 2004). SIRT1, the human homologue of Sir2, was also described to be associated with longevity and is believed to act primarily by inhibiting cellular senescence (Yeung *et al.*, 2004; Brunet *et al.*, 2004; Vaziri *et al.*, 2001). Although it is likely that SIRT1 and other Sir2 homologues main actions are through the deacetylation of transcription factors and not on histones, SIRT1 has been shown to have K16 H4 deacetylase activity (Michishita *et al.*, 2005). Recently a SIRT1 inhibitor, Sirtinol, was shown to induce a senescence-like growth arrest in human cancer cells (Ota *et al.*, 2005) in agreement with the above data, showing that HDAC inhibitors could lead to senescence.

Histone acetyltransferases have also been demonstrated to have a role in senescence. The HATs p300 and CBP were studied in senescing human melanocytes and interestingly, levels of both proteins were observed to decrease as these cells underwent replicative senescence and that this decrease was senescence specific (Bandyopadhyay *et al.*, 2002). p300 deficient fibroblasts were also shown to rapidly senesce in another similar study (Yao

et al., 1998). This decrease in the levels of the HAT expression led to dramatic decreases in total histone H4 acetylation levels while total histone H4 protein levels remained at a similar level to proliferating cells. Specifically, the Cyclin E gene promoter was shown to lose promoter histone H4 acetylation and become repressed at senescence while in proliferating cells Cyclin E is highly expressed and promoter histone H4 is highly acetylated. Cyclin E is a critical regulator of senescence and so heterochromatin formation at the promoter sequences in senescence may play an important role in stably downregulating this gene and the acquisition of the senescent phenotype. Other studies of the Cyclin E promoter during senescence showed that pRB and HDACs also bound to the promoter to repress transcription, similar to previous studies linking the senescent phenotype to the pRB/p16^{INK4a} pathway and the formation of SAHF (Narita *et al.*, 2003). A recent review of p53's role in global regulation of chromatin acetylation indicates roles for p53 in the control of gene expression by acetylation (Allison and Milner, 2004) and p53's intimate relationship with senescence further suggests a role of the regulation of the chromatin environment in senescence.

These studies suggest that the increases in global histone acetylation can lead to senescence; perhaps mediated by the re-expression of previously heterochromatically silenced genes which when activated, promote senescence, while de-acetylation may serve to repress genes which contribute to the senescence phenotype.

1.4 Aims of this Project

Modulation of the chromatin environment is now understood to play a major role in the transcriptional control of gene expression and this suggests that the chromatin based regulatory mechanisms will be important for multiple cellular processes. Modulation of the chromatin environment has also been observed in senescence, suggesting that the control of the chromatin environment could also have important roles in the bypass of senescence and tumourigenesis. Telomerase has long been indicated to play important roles in senescence and tumourigenesis, but knowledge about their transcriptional control is lacking. Transcriptional control of *hTR* and *hTERT* is deemed to be an important level of control of *hTR* and *hTERT* gene expression and previous studies on the role of epigenetics in telomerase gene regulation have been limited. The aims of this project are to better understand how alterations of the chromatin environment can affect the expression hTR and hTERT by utilising two different model systems; normal, telomerase positive tumour cells and ALT cells and also in stem cell populations.

The aims of this project in detail are:

- To investigate how epigenetic regulation of the gene promoters impacts on *hTR* and *hTERT* gene expression in three different model systems; normal, ALT and tumour cell lines.

- To investigate how epigenetic regulation of the gene promoters impacts on *hTR* and *hTERT* gene expression in other model systems; stem cells and cancer stem cells.

- To investigate how modulation of the chromatin environment can affect the dynamics of the basal transcription machinery and hence transcription in all our model systems.

- To investigate any common features in chromatin based regulatory mechanisms between hTR and hTERT gene regulation.

- To investigate whether chromatin based regulatory mechanisms are conserved between cell types or whether there are cell type differences.

2 Materials and Methods

2.1 Materials

2.1.1 Tissue Culture Reagents and Plasticware

RPMI 1640 growth medium	Invitrogen
Minimum Essential Medium (MEM)	Invitrogen
Dulbecco's modified Eagles (DMEM)	Invitrogen
L-glutamine (200mM)	Invitrogen
Trypsin (2.5%)	Invitrogen
Foetal calf serum	Autogen Bioclear
75cm^2 flasks	Iwaki
25cm^2 flasks	Iwaki

2.1.2 Cell Lines

A2780 ovarian carcinoma	ATCC
C33a cervical carcinoma	ATCC
5637 bladder carcinoma	DSMZ
WI38 Normal Human Lung Fibroblasts	ECACC
WI38-SV40 (ALT)	ECACC
KMST6 (ALT)	ECACC

SKLU (ALT)	ECACC
SUSM1 (ALT)	ECACC
GM847 (ALT)	ECACC

Human Mesenchymal Stem Cells (hMSC)

Normal and CML CD34+ Haematopoietic Stem Cells (hHSCs)

2.1.3 Antibodies

Antibody (and Residues Detected)	**Permissive/ Repressive Modification**	**Company**	**Order Number**
Acetylated H3 (Lysines 9 and 14)	Permissive	Upstate	06-599
Acetylated H4 (Lysines 5, 8, 12 and 16)	Permissive	Upstate	06-866
Acetylated K9 H3	Permissive	Upstate	07-352
Di-Methylated Lysine 4 H3	Permissive	Upstate	07-030
Tri-Methylated Lysine 4 H3	Permissive	Abcam	ab8580
Di-Methylated Lysine 9 H3	Repressive	Abcam	ab7312
Tri-Methylated Lysine 9 H3	Repressive	Abcam	ab8898
Di-Methylated Lysine 20 H4	Repressive	Upstate	07-357
Tri-Methylated Lysine 20 H4	Repressive	Upstate	07-463

Antibody	Company	Order Number
RNA Polymerase II	Abcam	ab5408
Pol II (N-20) rabbit polyclonal IgG	Santa-Cruz Biotechnology	sc-899
TFIIB (C-18) rabbit polyclonal IgG	Santa-Cruz Biotechnology	sc-225
c-Myc	Upstate	06-340

2.1.4 Kits, Reagents and Enzymes

Kit/Reagent	**Supplier**
Nucleospin II RNA extraction kit	Abgene
GeneAmp RNA PCR core kit	Roche
Taq PCR core kit	Qiagen
HotStar Taq DNA polymerase	Qiagen
DyNAmo SYBR Green qPCR kit	Finnzymes
Chromatin Immunoprecipitation assay kit	Upstate Biotechnology
QIAquick PCR purification kit	Qiagen
Glycogen	Roche
Pepstatin	Roche
Aprotinin	Roche

Phenylmethanesulfonyl fluoride (PMSF)	Fluka
DNA molecular markers	Invitrogen
10% SDS solution (sodium dodecyl sulphate)	Invitrogen
RNAseZap	Ambion
DNA 1000 reagents & supplies	Agilent Technologies
DNA Chips	Agilent Technologies

2.1.5 Chemicals

Chemicals were acquired from Sigma or Fisher Scientific, UK.

TRIS-hydrochloride

EDTA (Ethylenediamine tetra-acetic acid)

Absolute ethanol

Propan-2-ol

Sodium hydroxide

Sodium chloride

NP40 (IGEPAL)

Triton-X 100

Sodium Deoxycholate

Lithium Chloride

Agarose

Bromophenol blue

Xylene cyanole

DMSO (Dimethyl sulfoxide)

Ethidium bromide

β-mercaptoethanol

Phenol:chloroform:iso-amyl alcohol

N-Lauroylsarcosine sodium salt

DEPC (diethylpyrocarbonate)

2.1.6 General Laboratory Supplies and Reagents

Provided by Beatson Institute Central Services:

Sterile distilled water

Sterile phosphate buffered saline (PBS)

Sterile PBS + EDTA (PE)

10x TBE electrophoresis buffer (Tris Borate/EDTA)

Sterile glassware and measuring pipettes

General	**Supplier**
Falcon tubes 50ml and 15ml	Becton Dickinson
Universal containers 5ml, 20ml and 100ml	Bibby Sterilin
Micro-centrifuge tubes 1.5ml and 0.5ml	Eppendorf/Abgene

General	**Supplier**
Cell scrapers (rubber policeman)	Corning
Pipette tips	Gilson/Greiner bio-one
Plastic pipettes 1ml, 5ml, 10ml and 25ml	Corning
Polyprep Columns	BioRad

2.1.7 Oligonucleotides for PCR

Primer	Name	Sequence 5' to 3'
hTR Expression	TRC3F	CTA ACC CTA ACT GAG AAG GGC GTA
	TRC3R	GGC GAA CGG GCC AGC AGC TGA CAT T
hTERT Splice Variant	HT2026F	GCC TGA GCT GTA CTT TGT CAA
	HT2482R	CGC AAA CAG CTT GTT CTC CAT GTC
hTR ChIP	HTR29SF	CCC GCC CGA GAG AGT GAC
	HTR5ALTR	AAG TCA GCG AGA AAA ACA GC
hTERT ChIP	TERTFS	TCC CCT TCA CGT CCG GCA TT
	TERTRS	AGC GGA GAG AGG TCG AAT CG
hTERT Exon 12 ChIP	Ex12 FA	CAG GAC AAG GAA GCG GGA GGA A

	Ex12 RAB	CAG CCG CAA GAC CCC AAA GA
GAPDH Expression	GAPDH F	ACC ACA GTC CAT GCC ATC AC
	GAPDH R	CCA CCA CCC TGT TGC TGT A
S15 Expression	S15 F	TTC CGC AAG TTC ACC TAC C
	S15 R	CGG GCC GGC CAT GCT TTA CG

2.1.8 Equipment

Medical Air Technologies Bio-MAT class II microbiological safety cabinet

Scharfe Systems Casy-1 cell counter

Forma Scientific CO_2 H_2O jacketed incubator

Sigma 4K15/ Beckman GS-6R bench top centrifuges

Bio-Rad sub-cell GT electrophoresis gel tank/model 200 power supply

Pharmacia Biotech GeneQuant DNA/RNA calculator

MJ Research PTC-200 Peltier thermal cycler

MJ Research DNA Engine Opticon 2 Real Time PCR Detection System

Beckman J6-MC centrifuge

Beckman Microfuge-R refrigerated micro-centrifuge

Bio-Rad Gel Doc 1000 UV transilluminator

Syngene Gene Genius Bio Imaging system / GeneSnap version 6.03

Agilent 2100 Bioanalyzer, Agilent Technologies

2.2 DNA Techniques

2.2.1 UV Gel Documentation

Analysis and photography of ethidium bromide agarose gels was accomplished using the Bio-Rad gel doc 1000 UV transilluminator with Molecular Analyst software. This system was later replaced by the Syngene Gene Genius Bio Imaging System with GeneSnap version 6.03 software.

2.3 Analysis of Gene Expression

2.3.1 RNA Extraction

Before extraction surfaces and pipettes were wiped with RNaseZap (Ambion) to remove traces of RNases. RNase free pipette tips were used and microcentrifuge tubes were pre-treated with DEPC water (Add 0.1% DEPC to dH_20, bring the DEPC into solution and incubate at 37°C for 12 hours. Finally, autoclave the solution for 15 mins to remove DEPC). Total RNA was isolated from cultured cells using the NucleoSpin RNA II kit as per the manufacturers' instructions. Approximately 1 x 10^6 cells were lysed by addition of 350µl of buffer RA1 and vigorous vortexing. The lysate was homogenised by passing through a 20 gauge needle and was then applied to a NucleoSpin filter which contains the silica membrane. Contaminating DNA, which is also bound to the silica membrane, was removed by treatment with DNase-1 solution for 15 minutes at room temperature. Salts, metabolites and macromolecular cellular components were removed in washing steps and the pure RNA was eluted under low ionic strength conditions with RNase-free water.

2.3.2 cDNA Synthesis

cDNA was prepared using the GeneAmp RNA PCR core kit (Roche). RNA was quantitated by spectrophotometry using the Pharmacia Biotech GeneQuant spectrophotometer and adjusted to 1µg in RNase-free dH_2O. Reactions were prepared in a PCR workstation (Labcaire) using RNase-free tubes and tips which were UV crosslinked prior to use. A reaction mix was made up with 4µl of 10x Buffer, 8.8µl $MgCl_2$, 8 µl dNTPs, 2 µl random hexamers, 0.8µl of RNAse inhibitor, 1µl of reverse transcriptase (RT)

and 1ug of RNA per sample, made up to 40µl with dH_20. For each sample a no RT control mix was also prepared. Samples were then incubated at 25°C for 10 minutes, 48°C for 30 minutes and 95°C for 5 minutes. 1µl of cDNA was used in subsequent PCR reactions. GAPDH specific Q-PCR was performed for RT-PCR reaction products and no RT controls to determine the quality of cDNA produced and to check for DNA contamination of the RNA.

2.3.3 Quantitative Polymerase Chain Reaction (Q-PCR)

Q-PCR master mixes were made using the Finnzymes DyNAmo SYBR green Q-PCR kit. The 2x reaction mix provided contained SYBR green dsDNA binding dye, buffer, Taq polymerase and dNTPs in a pre-mixed form, therefore typical reactions consisted of 1x SYBR green reaction mix, 0.5µM each primer and 1µl DNA made up to a 20µl volume with dH_20. Reaction mixes were prepared in a PCR workstation (Labcaire) and all equipment was UV crosslinked before use. Each reaction is completed in triplicate and standard curve of a known concentration range of human genomic DNA is used to quantify the PCR product levels. Reactions were run on an Opticon 2 DNA Engine from MJ Research according to the cycling conditions detailed below. The average value of the triplicate reactions was taken as the concentration of PCR product. This also allows errors to be generated.

2.3.4 PCR Primers

hTR and hTERT splice variant expression primers were previously established in the laboratory (Anderson *et al.*, 2006). hTR and hTERT ChIP primers were raised against the core promoter sequences of the respective genes. Exon 12 of hTERT primers were raised against the coding sequence of Exon 12 of hTERT coding sequence. Gene products for Q-PCR were designed to be between 100bp and 200bp in length to allow for efficient Q-PCR reactions and promoter specific localisation of the histone modifications detected in each ChIP. Each set of primers gave a single specific product, as visualised by melting curve analysis within the Q-PCR reaction and also by gel electrophoresis. GAPDH primers were also designed, to give a product of approximately 450bp, for Q-PCR reactions for use in normalising hTR and hTERT expression levels. The product of these primers also gave a single specific product as visualised by melting curve analysis within the Q-PCR reaction and also by gel electrophoresis.

2.3.4.1 Cycling Conditions

GAPDH Expression	Step 1: 94 °C for 10 mins; Step 2: 95 °C for 30 secs;
	Step 3: 60°C for 45 secs; Step 4: 72°C for 1 min;
	Step 5: 81°C for 10 secs; Step 6: Plate read;
	Step 7: Goto Step 2, 29 times;
	Step 8: Melting curve 70-90°C.
hTR Expression:	Step 1: 94°C for 10 mins; Step 2: 95°C for 30 secs;
	Step 3: 59.5°C for 45 secs;
	Step 4: 72°C for 30 secs;
	Step 5: 81°C for 10 secs; Step 6: Plate read;
	Step 7: Goto Step 2, 31 times;
	Step 8: Melting curve 70-90°C
hTR and hTERT Promoter ChIP:	Step 1: 94°C for 10 mins; Step 2: 95°C for 30 secs;
	Step 3: 64°C for 45 secs; Step 4: 72°C for 30 secs;
	Step 5: 82°C for 10 secs; Step 6: Plate read;
	Step 7: Goto Step 2, 39 times;
	Step 8: Melting curve 70-90°C
hTERT Exon 12 ChIP	Step 1: 94°C for 15 mins; Step 2: 95°C for 30 secs;
	Step 3: 66°C for 45 secs; Step 4: 72°C for 30 secs;
	Step 5: 82°C for 10 secs; Step 6: Plate read;

	Step 7: Goto Step 2, 39 times;
	Step 8: Melting curve 70-90°C
S15 Expression	Step 1: 94°C for 10 mins; Step 2: 95°C for 30 secs;
	Step 3: 55°C for 30 secs; Step 4: 72°C for 30 secs;
	Step 5: 82°C for 10 secs; Step 6: Plate read;
	Step 7: Goto Step 2, 34 times;
	Step 8: Melting curve 70-90°C

2.3.5 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) master mixes were made using reagents from the Qiagen Taq-core kit. HotStar Taq DNA polymerase was used in place of Taq polymerase for all TERT PCR reactions. Reaction mixes were prepared in a PCR workstation (Labcaire) and all equipment was UV crosslinked before use. Reactions typically contained 1µl template DNA along with final concentrations of master-mix components as follows: 1x PCR buffer; 0.5µM each primer; 0.2mM each of dATP, dTTP, dCTP, dGTP; 1 unit Taq polymerase. Reaction volumes were made up to 25µL with sterile distilled H_20 and run according to the cycling conditions detailed below. PCR products were analysed by agarose gel electrophoresis. Typically 5-10µl was loaded onto a 0.8% agarose gel containing ethidium bromide for UV visualisation of DNA.

2.3.5.1 Cycling conditions

hTERT Splice Variants:	Step 1: 94°C for 15 mins; Step 2: 95°C for 30 secs;
	Step3: 64°C for 45 secs; Step 4: 72°C for 45 secs;
	Step 5: Goto Step 2, 39 times;
	Step 6: 72°C for 5 mins

2.3.6 Quantitation of DNA using the Agilent Bioanalyser

The Agilent 2100 Bioanalyzer LabChip system was also used to visualise and quantify PCR products and was used for the hTERT splice variant PCR. This system utilises LabChips with multiple channels etched into glass. The micro-channels of the chip are filled with a sieving polymer with fluorescent dye and a molecular weight marker is added. PCR products are separated electrophoretically using strategically located electrodes. Sample components are detected by their fluorescence and translated into virtual gel images, sized and quantified in relation to internal standards. The samples and chips were prepared and loaded according to manufacturers' instructions.

2.3.7 Calculation of Expression Levels

hTR levels were established using the specific expression primers and were normalised to the expression of GAPDH levels. GAPDH levels were established using the same amount of cDNA as for hTR expression level analysis and hTR levels were normalised by dividing the hTR figure by the figure for GAPDH expression, and so levels of hTR are expressed as a percentage of GAPDH expression (see formula below).

[Level of hTR expression by Q-PCR for x μl cDNA/Level of GAPDH Expression by Q-PCR for x μl cDNA]*100 = hTR expression levels as percentage of GAPDH.

Expression levels were found to be similar between three different experiments for each cell line and levels shown are from a representative experiment. Means and errors are established from triplication within the Q-PCR experiment of the representative experiment.

hTERT expression levels were established, first by analysing GAPDH levels from cDNA made from 1μg RNA. cDNA amounts used as input in each separate reaction for each cell line were then adjusted to the GAPDH level for the hTERT splice variant PCR reaction so similar concentrations of cDNA were used in each PCR. hTERT expression levels are shown as the sum of all the expressed variants (WT, α, β and αβ). Therefore the hTERT levels are expressed as being normalised to GAPDH expression. Expression levels were found to be similar between three different experiments for each cell lines. Means and errors are established from the average of these Q-PCR experiments.

S15 gene expression was utilised in a similar manner to GAPDH to adjust for hTR and hTERT expression levels in the CD34+ hHSC samples. S15 expression was used as it gave a more robust expression level that for GAPDH for these samples.

Statistical analysis showing any statistical significance levels was established using the ANOVA statistical software program from within the data analysis module of Microsoft Excel.

2.4 Cell Culture

2.4.1 Maintenance and Storage of Mammalian Cell Lines

All cell lines were routinely cultured in 75cm^2 flasks in 20ml of medium supplemented with 10% foetal calf serum (FCS) and 2mM L-Glutamine. A2780 and 5637 cells were grown in RPMI 1640, C33a, SKLU, SUSM1 and GM847 were grown in DMEM and WI38, WI38-SV40 and KMST6 were all grown in MEM. Cells were trypsinised for sub-culturing with 1ml of 0.25% trypsin diluted in PE (PBS + EDTA). All cell lines were regularly tested for mycoplasma contamination.

For long term storage, 1 ml aliquots of 1 x 10^6 cells/ml in growth medium supplemented with 10% FCS and 10% DMSO were cooled to -70°C overnight and then transferred to liquid nitrogen. To recover cells from liquid nitrogen, cryovials were warmed to 37°C. The contents were transferred to 19mL of warm growth medium in a 25cm^2 flask and incubated overnight. The next day, the medium containing DMSO cryopreservant was removed and replaced by normal, complete medium.

2.4.2 Isolation of hMSCs and Cell culture

Isolation of hMSCs, cell culture and treatment was provided by the laboratory of Dr Nedime Serakinci (University of Southern Denmark, Institute of Medical Biology, Department of Anatomy and Neurobiology, DK-5000 Odense C, Denmark). Primary hMSC isolated from bone marrow aspirates by centrifugation (700g for 15 minutes at 4°C) over a Ficoll Hypaque gradient as previously described by Pittenger, et al (Pittenger *et al.*, 1999). The resulting cells were cultivated in high glucose (4.5 g/l) Dulbecco's modified Eagles medium supplemented with 10% fetal bovine serum, 100 U/ml of penicillin and

streptomycin and 2 mM of L-glutamine. Cells were plated out with a confluency of 75% and next day added TSA at final concentration of 350 μM for 17hrs.

2.4.3 hHSC Isolation

hHSCs were provided from the laboratory of Dr Tessa Holyoake (Section of Experimental Haematology and Haematopoietic Stem Cells, Division of Cancer Sciences and Molecular Pathology, University of Glasgow, Glasgow, Scotland, United Kingdom). Fresh leukapheresis products from patients with chronic phase CML or from normal allogeneic donors were enriched for $CD34^+$ cells by either StemSep (Stemcell Technologies, Inc., Vancouver, BC) or Isolex (NEXELL International, Brussels, Belgium) systems. The cells were then cryopreserved in 10% dimethylsulphoxide (DMSO, Sigma Aldrich, UK) in ALBA (4.5% Human Albumin Solution, Scottish National Blood Transfusion Service) and stored in the vapour phase of liquid nitrogen until required. All human cell samples were obtained with informed consent.

2.4.4 Drug Treatment of Cells

ALT and Normal cells were treated with TSA or 5-azadC under the following conditions. Cells utilised were treated twice for 24 hours with 5-azadC to a final concentration of 2.5μM, and then treated for 16 hours with Trichostatin A (TSA) at a final concentration of 350nM. Control cells were treated over the same time course but with 100% ethanol instead of TSA and 50% acetic acid instead of 5-azadC.

2.5 Chromatin Immunoprecipitation Assay (ChIP)

During the course of this study 2 ChIP protocols were used. Most of the antibodies used worked well with the Upstate ChIP kit protocol (ChIP Assay I), RNA polymerase II (Santa-Cruz) and TFIIB a modified protocol was found to be more effective (ChIP Assay II).

2.5.1 Chromatin Immunoprecipitation Assay I

Cells were grown in 75 cm^2 flasks and harvested before reaching confluence with around 1 x 10^6 cells used per IP. Neutral buffered formalin (NBF) was added to 1% in the media within each flask in a fume hood to crosslink proteins and DNA. Flasks were sealed and incubated for 10 minutes at 37°C. NBF was removed and cells rinsed twice in cold PBS

(4°C). 3ml cold PBS containing protease inhibitors (1mM phenylmethlysulfonyl (PMSF), 1μg/ml aprotinin and 1ug/ml pepstatin A) were added to each flask. Cells were scraped and transferred to 15ml falcon tubes then centrifuged at 4000rpm for 10 minutes at 4°C and the supernatant removed. The cell pellets were re-suspended in 200 μl of SDS lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl, pH 8.1) with protease inhibitors and incubated on ice for 10 minutes. Samples were sonicated on ice for 6 x 10 second pulses using an MSE Soniprep 150, sonicator at amplitude 5 microns, optimised to give chromatin fragments of around 500bp.

The sonicated samples were centrifuged at 13000 rpm for 10 minutes at 4°C to remove debris and supernatant transferred to Eppendorf tubes. Chromatin was collected and diluted 10 fold with ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCL, pH 8.1, 167mM NaCl) containing protease inhibitors (1mM PMSF, 1μg/ml aprotinin and 1μg/ml pepstatin A). A 200μl sample was removed and stored at -20°C as the Input sample. 40μl of salmon sperm/agarose A protein slurry per ml of diluted chromatin was added to each tube of the remaining chromatin and rotated at 4°C for 30 minutes. Samples were spun at 1000 rpm for 2 minutes at 4°C and the supernatant from each was split into ~1ml aliquots. Antibodies were added to each aliquot, leaving one without antibody as a negative control and the samples were left to rotate overnight at 4°C.

80μl of salmon sperm/agarose A protein slurry was added to each sample and samples rotated at 4°C for 1 hour. The samples were spun at 1000 rpm at 4°C for 2 minutes to pellet the agarose beads and the supernatant removed, taking care not to remove any beads. 1ml low salt immune complex wash (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 150mM NaCl) was added to the beads, mixed by rotation at room temperature for 4 minutes and centrifuged at 1000 rpm for 2 minutes at 4°C. The supernatant was removed and the above step repeated with 1ml high salt immune complex (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 500mM NaCl), 1ml LiCl immune complex (0.25M LiCl, 1% NP40, 1% deoxycholate, 1mM EDTA, 10mM Tris-HCl, pH 8.1), and twice with 1ml TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0). The immune precipitate was then eluted by adding 250μl of elution buffer (0.1M $NaHCO_3$, 1% SDS), mixing for 15 minutes by rotation at room temperature and centrifugation at 1000 rpm for 2 minutes at room temperature. This was repeated and supernatants pooled to give 500μl. The Input sample (200μl) was diluted with 300μl H_20 to give 500μl. 20μl of 5M NaCl was added to each sample and these were incubated for 4 hours at 65°C to reverse the cross-linking.

Proteins were degraded by adding 10µl of 0.5 M EDTA, 20µl 1M Tris-HCl and 2µl proteinase K to each sample and incubating for 1 hour at 45°C, followed by phenol/chloroform/isoamylalcohol extraction. The DNA was precipitated by addition of 1ml 100% ethanol and 1µl of glycogen and incubated at -20 °C overnight. The samples were spun at 13000 rpm for 10 minutes and the supernatant removed carefully. The DNA pellet was washed in 500µl 70% ethanol, spun at 13000 rpm for 5 minutes and supernatant removed. The pellet was air dried for 15 minutes and then resuspended in 50µl sterile water. DNA was further purified using the Qiagen PCR purification kit following manufacturers' instructions and DNA was eluted in 50µl sterile water.

2.5.2 Chromatin Immunoprecipitation Assay II

Chromatin immunoprecipitation for RNA polymerase II (Santa-Cruz) and TFIIB were carried out using a protocol from Prof. R.J. White's laboratory at the University of Glasgow (Gomez-Roman *et al.*, 2003) for the hMSC study. Cells were grown in 75 cm^2 flasks and harvested before reaching confluence. Cells were cross-linked for 10 minutes at 37°C with 1% neutral buffered formaldehyde (NBF) (final concentration) added directly into the growth medium. Cross-linked cells were washed in PBS then scraped in 3ml PBS/NP40 (0.5%) and transferred to falcon tubes on ice. Cells were centrifuged at 1500rpm for 5 minutes at 4°C and the supernatant discarded.

Pellets were subjected to hypertonic lysis by incubation in 40ml high salt buffer (0.5% NP40, PBS, 1M NaCl) for 30 minutes on ice. After centrifugation at 1500rpm for 5 minutes at 4°C, pellets were washed with PBS/NP40 (0.5%) and subjected to hypotonic lysis by incubation in 40ml low salt buffer (0.5% NP40, 10mM Tris-HCl, pH 8.0, 1mM EDTA, 0.1M NaCl) for 30 minutes on ice. After centrifugation at 1500rpm for 5 minutes at 4°C, pellets were again washed with PBS/NP40 (0.5%) and resuspended in 1ml low salt buffer. Nuclei were extracted by homogenising the pellet by passing through a 26-gauge needle, and a further 1.7ml low salt buffer was added, to a final volume of 2.7ml. 300µl 20% sarcosyl was added and each 3ml sample was transferred slowly in 1ml fractions to a sucrose gradient (100mM sucrose in 40ml low salt buffer) in 50ml falcon tubes. Samples were centrifuged at 4000rpm for 10 minutes at 4°C and the supernatant discarded. After resuspension of the pellet in 3ml TE buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA) the lysate was transferred to a second sucrose gradient and again centrifuged at 4000rpm for 10 minutes at 4°C. Pellets were resuspended in a suitable volume of TE (typically around 200ul per 5 x 10^6 cells and sonicated on ice for 12 x 10 second pulses using an MSE

Soniprep 150, sonicator at amplitude 7 microns, to give chromatin fragments of around 500bp.

After sonication samples were centrifuged at 13000rpm for 5 minutes at 4°C to remove cellular debris. Supernatants were adjusted with 1/10th volume 11x NET buffer (1.65 M NaCl, 5.5mM EDTA, 5.5% NP40, 550mM Tris HCl, pH 7.4) and diluted to a total volume of 1ml/sample in TE buffer. 200μl was removed as the input fraction then the remaining sample was immunoprecipitated with the appropriate antibody at 4°C on a rotator overnight.

100μl protein G agarose beads in 1x NET buffer were added to each sample and incubated at 4°C for 2 hours on a rotator and then transferred to polyprep columns. Beads were washed twice with 10ml RIPA buffer (150mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% NP40, 50mM Tris-HCl, ph 8.0), twice with 10ml LiCl buffer (250mM LiCl, 0.5% NP40, 0.5% deoxycholate, 1mM EDTA, 10mM Tris-HCl, pH 8.0) and twice with 10ml TE all at 4°C. After washing, beads were resuspended in 1ml TE, transferred to 1.5ml tubes and centrifuged at 8000rpm for 1 minute at 4°C. Immunoprecipitated chromatin was eluted from the beads twice in 200μl TE/1% SDS and pooled to 400μl final volume. Input fractions were also made up to 400μl in TE/1% SDS. Protein was degraded by incubation with 5μl Proteinase K at 56°C for 4 hours or 42°C overnight. DNA was extracted twice with 400μl phenol/chloroform/isoamylalcohol and once with 400μl chloroform and then precipitated with 1ml 100% ethanol and 40μl 3M sodium acetate at -20°C overnight. The samples were spun at 13000 rpm for 20 minutes and the supernatant removed carefully. The glassy DNA pellet was washed in 500μl 70% ethanol, spun at 13000 rpm for 5 minutes and supernatant removed. The pellet was air dried for 15 minutes and then resuspended in 50μl sterile water.

2.5.3 Calculation of ChIP Results

In each ChIP experiment an Input sample (200ul) is taken before the antibody-IP incubation and is a 20% fraction of the chromatin used in each antibody-IP. After Q-PCR, the results for each antibody-IP are then related back to the Input sample in order to normalise the data between experiments. Therefore the results for each antibody-IP are expressed as being a percentage of the Input fraction.

As a negative control, an IP to which no antibody is added is included (IP-) is used. This method of normalisation has been previously used and shown to be reproducible in

independent experiments from our laboratory (Anderson *et al.*, 2006). Throughout the ChIP experiments within these studies the IP- is zero or negligible and this lack of background was reproducible between multiple experiments of each different set of experiments and between the different primers sets. The use of this control will establish any background signal present and can be subtracted from the results to control for background in each experiment.

Each ChIP experiment was repeated in triplicate and was found to be similar between experiments. Results shown are for a representative experiment and means and standard errors are generated from Q-PCR reactions by reading each sample in triplicate.

Statistical analysis showing any statistical significance levels was established using the ANOVA statistical software program from within the data analysis module of Microsoft Excel.

3 Epigenetic Regulation of hTR Expression in Normal, Tumour and ALT Cell Lines

3.1 Introduction

Reactivation of telomerase activity in cancer cells is often attributed to the re-expression or an increase in the levels of hTERT. However, telomerase activity is minimally reconstituted by hTERT *and* hTR (Autexier *et al.*, 1996; Weinrich *et al.*, 1997) and so the expression of hTR may also be important in the regulation of telomerase activity. hTR is the RNA template component of the telomerase holoenzyme and was first cloned in the ciliate *Tetrahymena* and was found to contain a sequence complimentary to that of telomeric repeats and is the template for telomere elongation (Greider and Blackburn, 1989; Shippen-Lentz and Blackburn, 1990).

Although expression of hTR is evident in some normal tissues (Feng *et al.*, 1995) levels of expression of hTR are increased relative to the levels observed in normal tissues in human tumours (Downey *et al.*, 2001; Soder *et al.*, 1998; Avilion *et al.*, 1996; Guilleret *et al.*, 2002b). Upregulation of hTR expression in tumour cell lines derived from multiple cancer tissues relative to primary mortal cell lines has also been observed (Feng *et al.*, 1995). Studies in mice have also shown that mTR is essential for telomere length maintenance (Blasco *et al.*, 1997; Hathcock *et al.*, 2002; Chiang *et al.*, 2004) while increases in mTR expression have also been observed in the early pre-neoplastic stages and further increased during the progression of certain carcinomas (Blasco *et al.*, 1996). Overall, these studies indicate an underlying mechanism for transcriptional upregulation of hTR during tumourigenesis. Interestingly, hTERT activation has also been shown to occur concomitant with elevation of hTR transcription suggesting partially overlapping regulatory mechanisms (Yi *et al.*, 1999; Stanta *et al.*, 1999).

Some interesting studies have also indicated a new role for hTR. Depletion of hTR has been shown to lead to rapid growth inhibition of human cancer cells independently of p53 or telomere length (Li *et al.*, 2004). This was due to changes in the global gene expression profile of the cells and a response pathway to hTR depletion was proposed which was distinct from the response to uncapping of telomeres (Li *et al.*, 2005). All telomerase positive tumour cell lines tested showed a similar response to hTR depletion but this was

not observed in the WI38-SV40 cell line which maintains its telomeres by utilising the ALT mechanism.

Cloning and characterisation of the hTR promoter sequence aided investigations into how hTR expression is regulated at the transcriptional level (Zhao *et al.*, 1998) which has been identified as an important level of control in the regulation of hTR expression (Yi *et al.*, 1999). The hTR gene was found to lie within a CpG island 733bp in length (Zhao *et al.*, 1998) suggesting that DNA methylation could be a possible regulatory mechanism for hTR expression. However, initial studies found no correlation between hTR expression and DNA methylation of the promoter sequence (Guilleret *et al.*, 2002b; Nakamura *et al.*, 2004) but further studies showed that the lack of hTR expression in some ALT cell lines was found to be tightly correlated to DNA methylation of the hTR promoter (Hoare *et al.*, 2001).

Analysis of the hTR promoter uncovered binding sites for many transcription factors including Sp1 sites, a CCAAT box, a binding site for the hetero-trimeric NF-Y complex and a Hypoxia Response Element (HRE) (Anderson *et al.*, 2006). Studies showed that Sp1 and NF-Y binding could activate the hTR promoter, while Sp3 and MDM2 could mediate repression (Zhao *et al.*, 2003; Zhao *et al.*, 2000; Zhao *et al.*, 2005). Upregulation of hTR gene expression under hypoxic conditions was also shown to occur, mediated through the binding of Hypoxia Inducible Factor 1α (HIF1α) to an HRE (Anderson *et al.*, 2006). Unexpectedly pRB was also found to activate the hTR promoter through an E2F-independent mechanism (Zhao *et al.*, 2000) while the adenoviral E1A protein was also found to activate the hTR promoter by sequestering the co-repressor CtBP which is found in histone modifying complexes which mediate gene repression (Shi *et al.*, 2003; Glasspool *et al.*, 2005).

These studies showing the role of DNA methylation and transcriptional regulation suggest that the chromatin environment could have a role to play in the regulation of hTR expression. To this end, the chromatin environment surrounding the hTR promoter was studied in telomerase positive tumour, telomerase negative ALT and normal cell lines to establish any links between hTR expression and the endogenous chromatin environment.

3.2 Results

3.2.1 Expression of hTR in Tumour, Normal and ALT Cell Lines

To understand the relationship between expression levels of hTR and chromatin remodelling at the promoter, we first examined hTR gene expression levels from each cell line. Three tumour cell lines (A2780; ovarian carcinoma, 5637; bladder carcinoma and C33a; cervical carcinoma), one normal (WI38; normal human foetal lung fibroblast) and five ALT cell lines (SUSM-1, KMST-6, GM847, WI38-SV40 and SKLU) were utilised. Firstly, total RNA was extracted and cDNA synthesised in order to quantitate hTR expression. Semi-quantitative PCR was used to analyse GAPDH expression as a loading control for subsequent PCR reactions and semi-quantitative PCR for hTR was then used to evaluate the levels of hTR in each cell line.

Figure 3.1 demonstrates that hTR is expressed in the tumour cell lines, the normal cell line and two of the ALT cell lines. The ALT cell line GM847 expresses the highest levels of hTR with 5637, A2780, SKLU, C33a and WI38 also expressing hTR, although WI38 expresses very low levels. hTR expression levels were negligible in SUSM-1, KMST-6 and WI38-SV40. This range of cell lines with differing hTR expression levels allows the study of the chromatin environments in relation to various levels of expression.

3.2.2 Profile of Repressive Histone Lysine Methylation Modifications at the hTR Promoter

In order to examine the relationship between hTR expression and histone modifications present at the promoter sequences, ChIP assays using antibodies against specific histone modifications were used to generate a profile of the chromatin environment surrounding the hTR promoter in each cell line. Using the ChIP assay, a high definition *in vivo* snapshot of the chromatin environment of the endogenous hTR promoter can be constructed (Eberhardy *et al.*, 2000; Orlando, 2000; Kuo and Allis, 1999).

The cells lines are ranked in respect to their hTR expression levels in order to aid establishment of any trends. Modifications linked to repression of gene transcription were studied first and so methylation of K9 H3 and methylation of K20 H4 were studied. Antibodies recognising the di- and tri-methylated forms of K9 H3 and K20 H4 were used and the respective totals for the two antibodies compared between cell lines. Figure 3.2A

Figure 3.1 hTR Expression in Normal, Tumour ALT and Normal Cells

Cell lines from which hTR levels are established are ALT (A), telomerase positive tumour (T) or normal (N) cell lines, as noted after each cell line name. RNA was extracted from each cell line and cDNA synthesised using 1μg RNA. hTR levels were detected using specific primers by Quantitative PCR (Q-PCR) using specific primers to the hTR coding sequence. hTR levels were normalised to the expression of GAPDH levels established using the same amount of cDNA as for hTR. Therefore, levels of hTR are expressed as a percentage of GAPDH expression. Similar expression levels were established from at least 3 different experiments for each cell line and levels shown are from a representative experiment. Means and errors are established from triplication within the Q-PCR experiment.

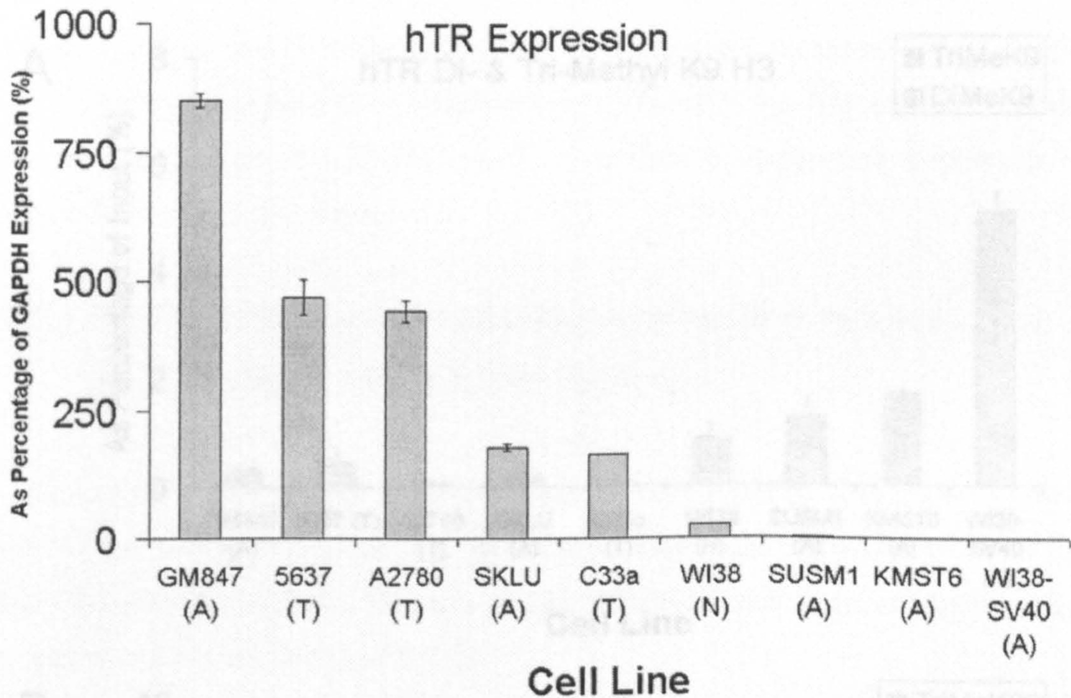
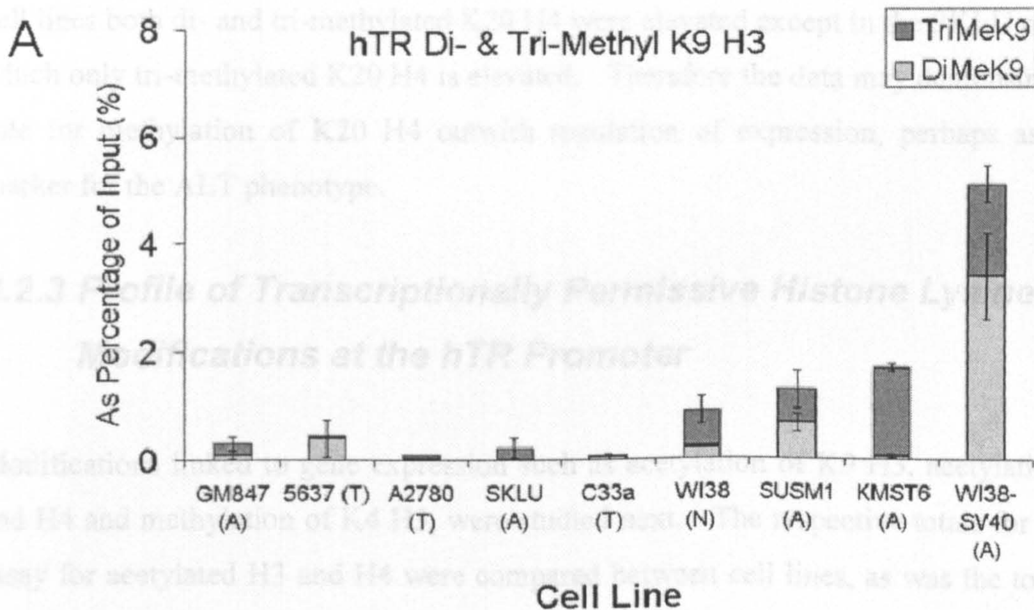
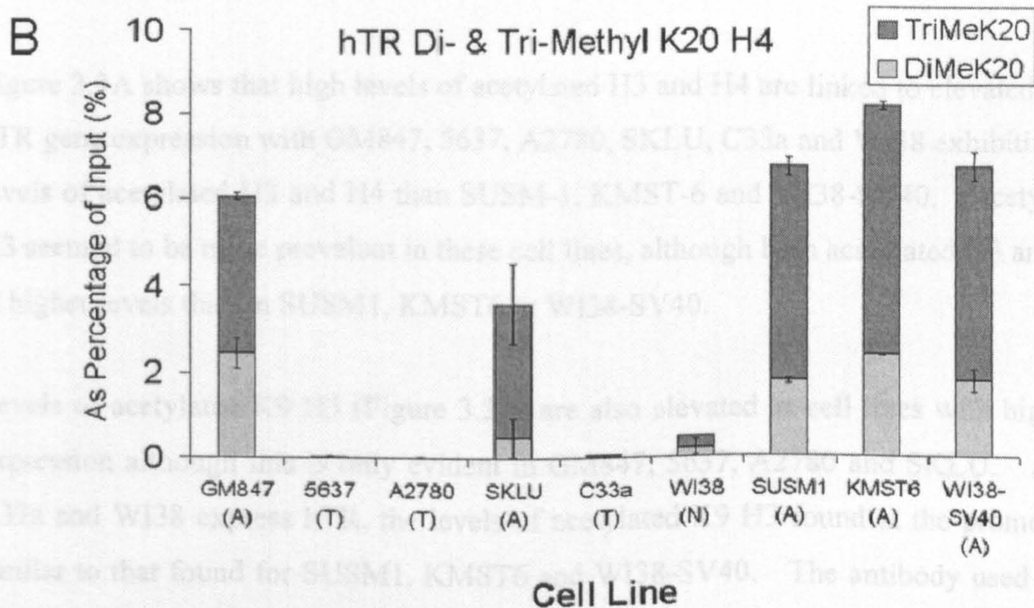


Figure 3.2 Modifications Linked to Repression of hTR Transcription

Levels of **A** Di- and tri-methylated K9 H3 and **B** di- and tri-methylated K20 H4 present at the hTR promoter in ALT (A), telomerase positive tumour (T) or normal (N) cell lines, detected by Q-PCR using primers which detect the hTR core promoter sequence, from products of chromatin immunoprecipitation assays. Results are expressed as the total IP amount for both the antibodies and are expressed as a percentage of an Input sample, which is a 20% fraction of chromatin utilised for each IP experiment, and so normalises the data. Each IP is also corrected for a control IP experiment, established in parallel with other IP's, in which no antibody was added to control for any background. Each IP value was established in at least three separate experiments and found to be similar and the IP figure shown is of a representative experiment. Means and standard errors are from triplication within the Q-PCR reactions.





shows that total methylated K9 H3 is elevated in cells with low or no hTR expression (WI38, SUSM-1, KMST-6 and WI38-SV40) when compared to cells expressing relatively high levels of hTR (GM847, 5637, A2780, SKLU and C33a), which were nearly devoid of methylated K9 H3. Levels of both di- and tri-methylated K9 H3 were elevated in WI38, SUSM1 and WI38-SV40, while in the KMST6 cell line only tri-methylated K9 H3 is elevated. This demonstrates that increased levels of methylated K9 H3 at the hTR promoter are linked to lower hTR gene expression.

Figure 3.2B indicates that there is no clear link between methylation of K20 H4 and hTR expression in any of the cell lines. Indeed, methylation of K20 H4 seems only to be elevated in the ALT cell lines (GM847, SKLU, SUSM-1, KMST-6 and WI38-SV40) which could indicate a novel role for this modification. Interestingly the total levels of methylated K20 H4 are similar between these cell lines at approximately 4-8%. In these cell lines both di- and tri-methylated K20 H4 were elevated except in the SKLU cell line in which only tri-methylated K20 H4 is elevated. Therefore the data may demonstrate a new role for methylation of K20 H4 outwith regulation of expression, perhaps as a novel marker for the ALT phenotype.

3.2.3 Profile of Transcriptionally Permissive Histone Lysine Modifications at the hTR Promoter

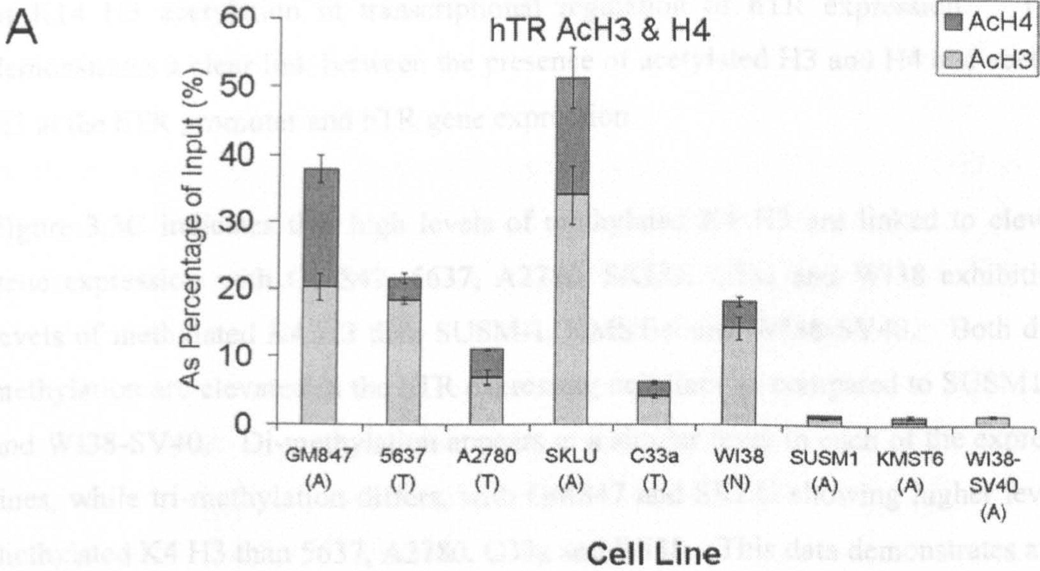
Modifications linked to gene expression such as acetylation of K9 H3, acetylation of H3 and H4 and methylation of K4 H3, were studied next. The respective totals for the ChIP assay for acetylated H3 and H4 were compared between cell lines, as was the total of the di- and tri-methylated forms of K4 H3.

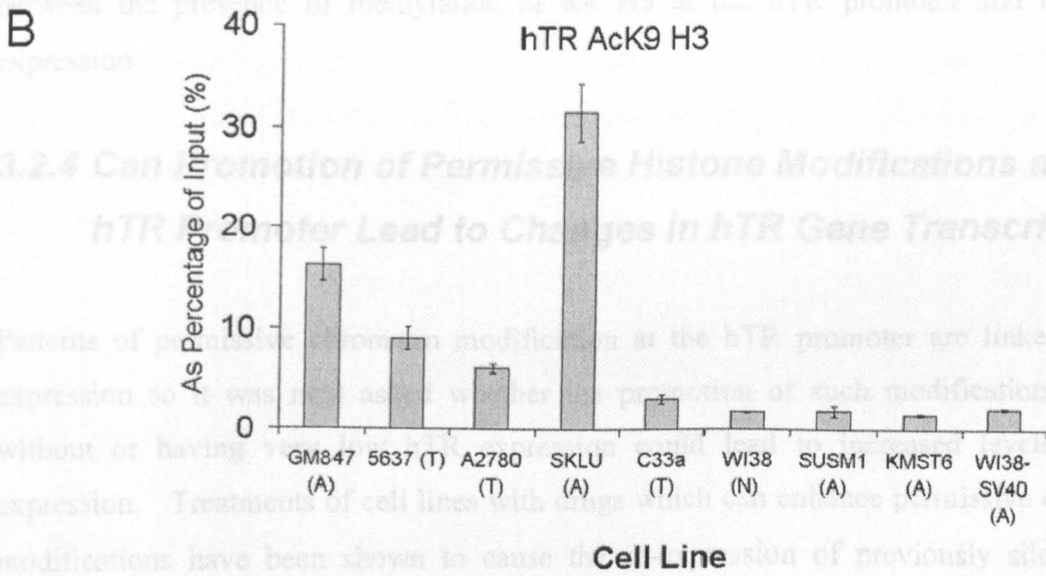
Figure 3.3A shows that high levels of acetylated H3 and H4 are linked to elevated levels of hTR gene expression with GM847, 5637, A2780, SKLU, C33a and WI38 exhibiting higher levels of acetylated H3 and H4 than SUSM-1, KMST-6 and WI38-SV40. Acetylation of H3 seemed to be more prevalent in these cell lines, although both acetylated H3 and H4 are at higher levels than in SUSM1, KMST6 or WI38-SV40.

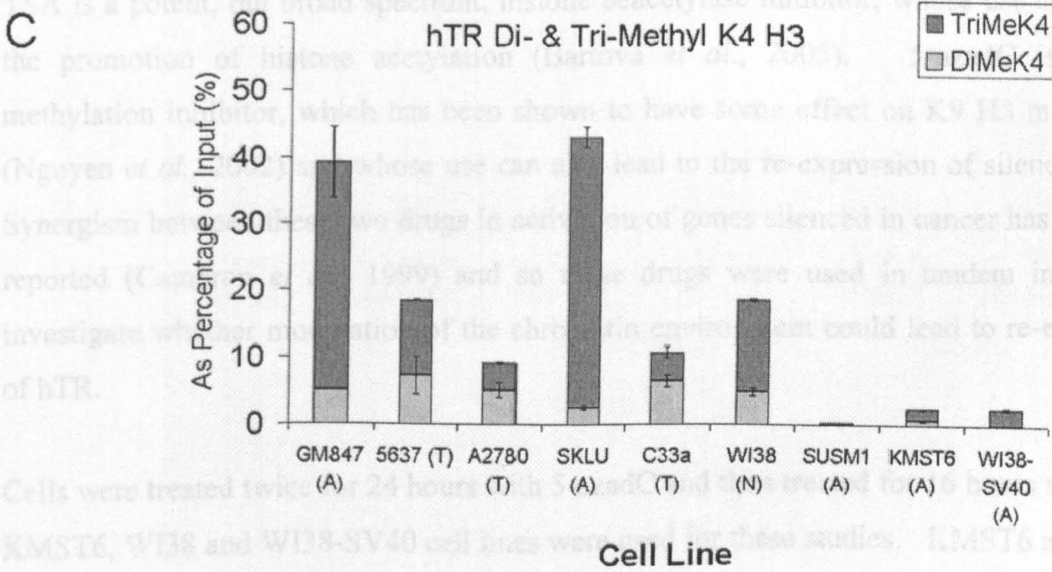
Levels of acetylated K9 H3 (Figure 3.3B) are also elevated in cell lines with higher hTR expression although this is only evident in GM847, 5637, A2780 and SKLU. Although C33a and WI38 express hTR, the levels of acetylated K9 H3 found at the promoter were similar to that found for SUSM1, KMST6 and WI38-SV40. The antibody used to detect acetylated H3 recognises acetylated K9 H3 and K14 H3 so may also reflect the importance

Figure 3.3 Modifications Linked to Permissiveness of hTR Transcription

Levels of **A** Acetylated H3 and H4, **B** Acetylated K9 H3 and **C** Di- and Tri-methylated K4 H3 present at the hTR promoter in ALT (A), telomerase positive tumour (T) or normal (N) cell lines, detected by Q-PCR using primers which detect the hTR core promoter sequence. from products of chromatin immunoprecipitation assays. Results are expressed as the total IP amount for both the antibodies (apart from acetylated K9 H3) and are expressed as a percentage of an Input sample, which is a 20% fraction of chromatin utilised for each IP experiment, and so normalises the data. Each IP is also corrected for a control IP experiment, established in parallel with other IP's, in which no antibody was added to control for any background. Each IP value was established in at least three separate experiments and found to be similar and the IP figure shown is of a representative experiment. Means and standard errors are from triplication within the Q-PCR reactions.


A
hTR AcH3 & H4
AcH4
AcH3
As Percentage of Input (%)
0
10
20
30
40
50
60
GM847 (A)
5637 (T)
A2780 (T)
SKLU (A)
C33a (T)
WI38 (N)
SUSM1 (A)
KMST6 (A)
WI38-SV40 (A)
Cell Line


B
hTR AcK9 H3
As Percentage of Input (%)
0
10
20
30
40
GM847 (A)
5637 (T)
A2780 (T)
SKLU (A)
C33a (T)
WI38 (N)
SUSM1 (A)
KMST6 (A)
WI38-SV40 (A)
Cell Line


C
hTR Di- & Tri-Methyl K4 H3
TriMeK4
DiMeK4
As Percentage of Input (%)
0
10
20
30
40
50
60
GM847 (A)
5637 (T)
A2780 (T)
SKLU (A)
C33a (T)
WI38 (N)
SUSM1 (A)
KMST6 (A)
WI38-SV40 (A)
Cell Line

of K14 H3 acetylation in transcriptional regulation of hTR expression. These data demonstrates a clear link between the presence of acetylated H3 and H4 and acetylated K9 H3 at the hTR promoter and hTR gene expression.

Figure 3.3C indicates that high levels of methylated K4 H3 are linked to elevated hTR gene expression with GM847, 5637, A2780, SKLU, C33a and WI38 exhibiting higher levels of methylated K4 H3 than SUSM-1, KMST-6 and WI38-SV40. Both di- and tri-methylation are elevated in the hTR expressing cell lines as compared to SUSM1, KMST6 and WI38-SV40. Di-methylation appears at a similar level in each of the expressing cell lines, while tri-methylation differs, with GM847 and SKLU showing higher levels of tri-methylated K4 H3 than 5637, A2780, C33a and WI38. This data demonstrates a clear link between the presence of methylation of K4 H3 at the hTR promoter and hTR gene expression.

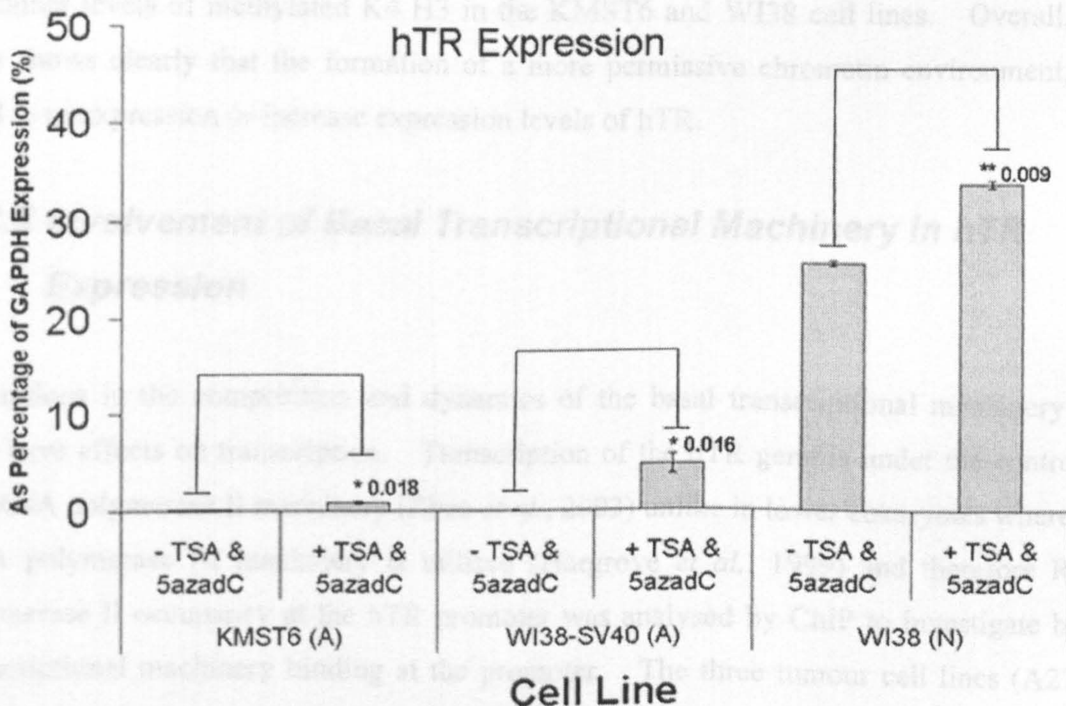
3.2.4 Can Promotion of Permissive Histone Modifications at the hTR Promoter Lead to Changes in hTR Gene Transcription?

Patterns of permissive chromatin modification at the hTR promoter are linked to hTR expression so it was next asked whether the promotion of such modifications in cells without or having very low hTR expression could lead to increased levels of hTR expression. Treatments of cell lines with drugs which can enhance permissive epigenetic modifications have been shown to cause the re-expression of previously silent genes. TSA is a potent, but broad spectrum, histone deacetylase inhibitor, whose use can lead to the promotion of histone acetylation (Bartova *et al.*, 2005). 5-azadC is a DNA methylation inhibitor, which has been shown to have some effect on K9 H3 methylation (Nguyen *et al.*, 2002) and whose use can also lead to the re-expression of silenced genes. Synergism between these two drugs in activation of genes silenced in cancer has also been reported (Cameron *et al.*, 1999) and so these drugs were used in tandem in order to investigate whether modulation of the chromatin environment could lead to re-expression of hTR.

Cells were treated twice for 24 hours with 5-azadC and then treated for 16 hours with TSA. KMST6, WI38 and WI38-SV40 cell lines were used for these studies. KMST6 and WI38-SV40 were studied as they do not express hTR (or hTERT) and WI38, a normal cell line, was also studied, although it does express hTR. Treatment of KMST-6, WI38 and WI38-SV40 with TSA and 5-azadC in combination resulted in significantly increased hTR gene expression (Figure 3.4).

Figure 3.4 hTR Expression Following Treatment with TSA and 5-azadC

Cell lines from which hTR levels are established are ALT (A) or normal (N) cell lines, as noted after each cell line name. Cells were treated twice for 24 hours with 5-azadC to a final concentration of 2.5mM, and then treated for 16 hours with Trichostatin A (TSA) at a final concentration of 350nM (+). Control cells were treated over the same time course but with 50% acetic acid instead of 5-azadC and 100% ethanol instead of TSA (-). RNA was extracted from each cell line and cDNA synthesised using 1μg RNA. hTR levels were detected using specific primers by Quantitative PCR (Q-PCR). hTR levels were normalised to the expression of GAPDH levels established using the same amount of cDNA as for hTR. Therefore, levels of hTR are expressed as a percentage of GAPDH expression. Similar expression levels were established from at least 3 different experiments for each cell line and levels shown are from a representative experiment. Means and errors are established from triplication within the Q-PCR experiment. Statistics were analysed using ANOVA software from the Microsoft Excel data analysis tool pack. Asterisks denote a statistically significant difference between untreated and treated; $*p < 0.05$; $**p < 0.01$.



Histone modifications known to allow for a transcriptionally permissive chromatin environment were studied to allow us to link increases in expression with increases in such modifications. Acetylation of H3 and H4 were studied (Figure 3.5A), and in the KMST6 and WI38-SV40 cell line, overall acetylation increased after treatment. Acetylated H4 levels increased dramatically, while increases in acetylated H3 were less obvious. In the WI38 cell line, total acetylation increased after treatment, but while acetylated H3 increased, there was a noticeable decrease in acetylated H4. Acetylated K9 H3 was also studied, and Figure 3.5B shows that significant increases in acetylation at this residue were observed for each cell line tested.

Levels of methylated K4 H3 were studied next (Figure 3.5C) and increases in total methylated K4 H3 were observed in KMST6 and WI38 but unexpectedly, overall levels decreased slightly in WI38-SV40. Further analysis shows that di-methylation of K4 H3 is absent in both the KMST6 and WI38-SV40 cell lines and only tri-methylation of K4 H3 shows any changes. In the WI38 cell line, both di- and tri-methylation of K4 H3 showed significant increases upon treatment. Again, some synergism is observed between H3 and H4 acetylation and methylated K4 H3, with higher levels of acetylation generally linking to higher levels of methylated K4 H3 in the KMST6 and WI38 cell lines. Overall, this data shows clearly that the formation of a more permissive chromatin environment, can lead to re-expression or increase expression levels of hTR.

3.2.5 Involvement of Basal Transcriptional Machinery in hTR Expression

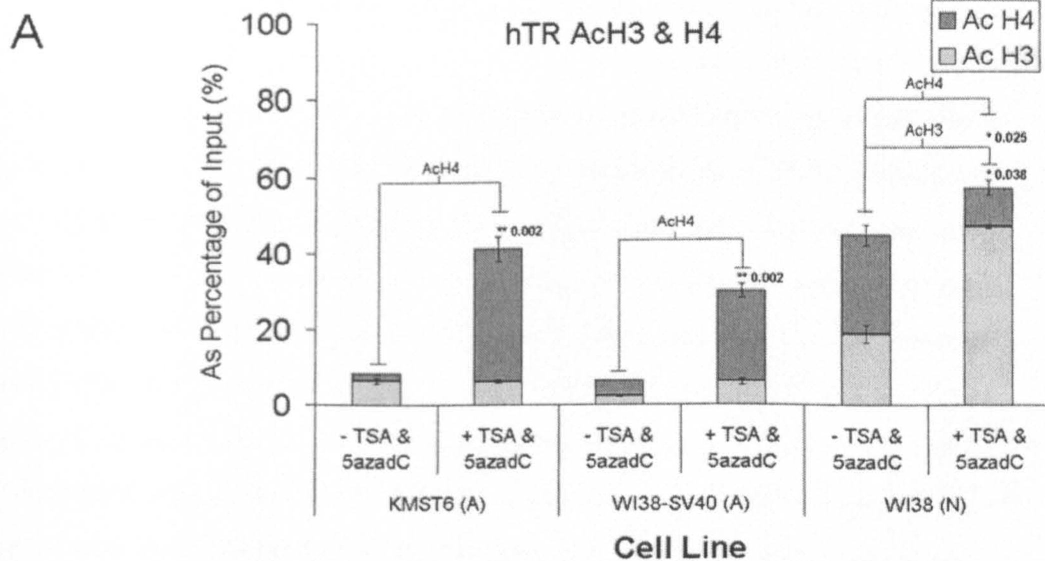
Alterations in the composition and dynamics of the basal transcriptional machinery can also have effects on transcription. Transcription of the hTR gene is under the control of the RNA polymerase II machinery (Zhao *et al.*, 2003) unlike in lower eukaryotes where the RNA polymerase III machinery is utilised (Hargrove *et al.*, 1999) and therefore RNA polymerase II occupancy at the hTR promoter was analysed by ChIP to investigate basal transcriptional machinery binding at the promoter. The three tumour cell lines (A2780, C33a and 5637) and a normal cell line (WI38) were used in this experiment.

Analysis of RNA polymerase II occupancy at the hTR promoter shows a link between expression and promoter occupancy as Figure 3.6A shows that RNA polymerase II occupancy at the promoter is higher in cells with higher expression. 5637 and A2780 show higher expression and have higher amounts of RNA polymerase II at the promoter

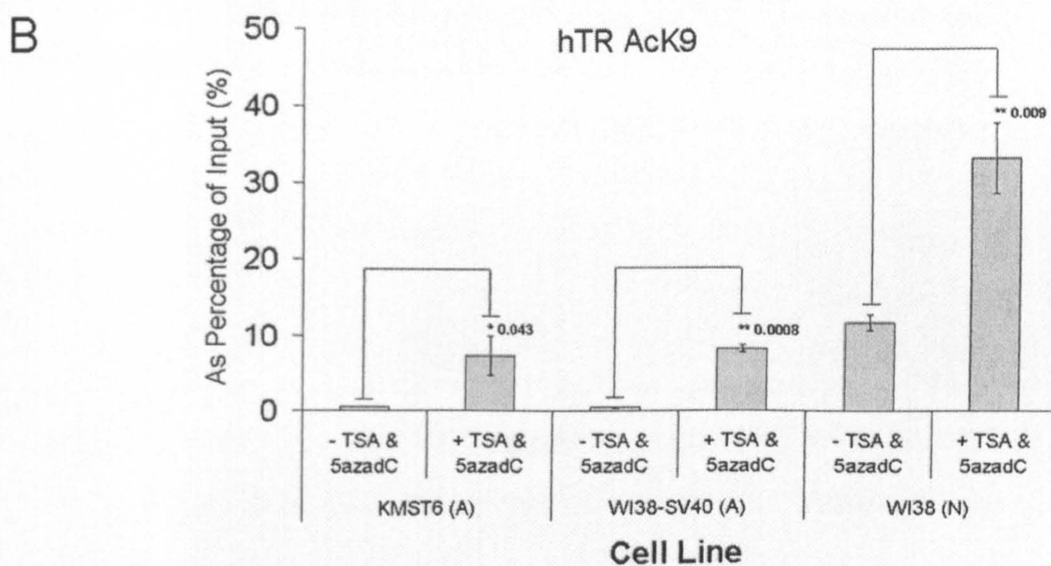
Figure 3.5 Changes in Histone Modifications at the hTR Promoter Following Treatment with TSA and 5-azadC

Q-PCR results of the ChIP using antibodies against **A** Acetylated H3 and H4, **B** Acetylated K9 H3 and **C** Di- and Tri-methylated K4 H3 detected by Q-PCR using primers which detect the hTR core promoter sequence. Cell lines used are ALT (A) or normal (N) cell lines, as noted after each cell line name. Cells were treated twice for 24 hours with 5-azadC to a final concentration of 2.5mM, and then treated for 16 hours with Trichostatin A (TSA) at a final concentration of 350nM (+). Control cells were treated over the same time course but with 50% acetic acid instead of 5-azadC and 100% ethanol instead of TSA (-). Results are expressed as the total IP amount for both the antibodies (apart from acetylated K9 H3) and are expressed as a percentage of an Input sample, which is a 20% fraction of chromatin utilised for each IP experiment, and so normalises the data. Each IP is also corrected for a control IP experiment, done in parallel with other IP's, in which no antibody was added to control for any background. Each IP value was established in at least three separate experiments and the IP figure shown is of a representative experiment. Means and standard errors are from triplication within the Q-PCR reactions. Statistics were analysed using ANOVA software from the Microsoft Excel data analysis tool pack. Asterisks denote a statistically significant difference between untreated and treated; *$p < 0.05$; **$p < 0.01$.

A



B



C

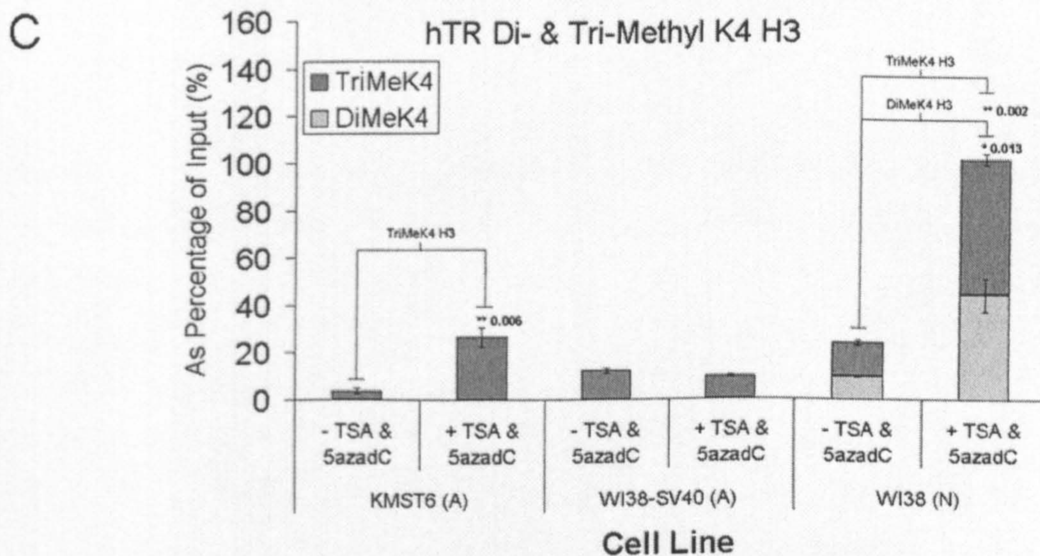
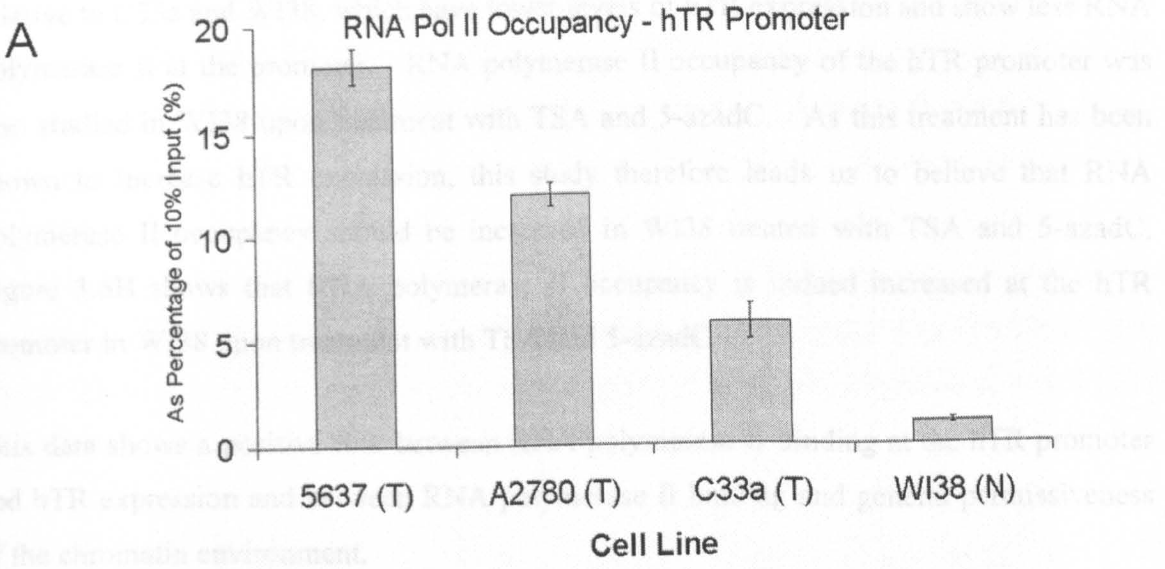
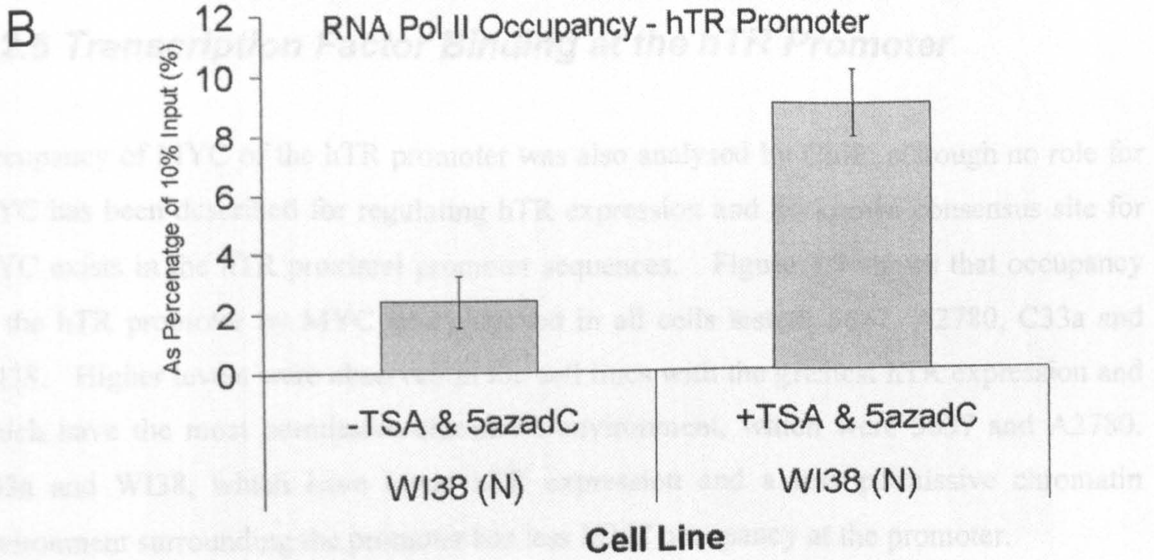


Figure 3.6 RNA Polymerase II Binding at the hTR Promoter

Q-PCR results of the ChIP for **A** RNA Pol II in tumour (T) and normal (N) cells and **B** RNA Pol II following treatment of the normal (N) cell line WI38 with 5-azadC and TSA detected by Q-PCR using primers which detect the hTR core promoter sequence. WI38 cells were treated twice for 24 hours with 5-azadC to a final concentration of 2.5mM, and then treated for 16 hours with Trichostatin A (TSA) at a final concentration of 350nM (+). Control cells were treated over the same time course but with 50% acetic acid instead of 5-azadC and 100% ethanol instead of TSA (-). Results are expressed as a percentage of 10% of the Input sample, with the Input fraction being a 20% fraction of chromatin utilised for each IP experiment, and so normalises the data. Each IP is also corrected for a control IP experiment, done in parallel with other IP's, in which no antibody was added to control for any background. Each IP value was established in at least three separate experiments and the IP figure shown is of a representative experiment. Means and standard errors are from triplication within the Q-PCR reactions.


A
RNA Pol II Occupancy - hTR Promoter
As Percentage of 10% Input (%)
20
15
10
5
0
5637 (T)
A2780 (T)
C33a (T)
WI38 (N)
Cell Line


B
RNA Pol II Occupancy - hTR Promoter
As Percentage of 10% Input (%)
12
10
8
6
4
2
0
-TSA & 5azadC
+TSA & 5azadC
WI38 (N)
WI38 (N)
Cell Line

relative to C33a and WI38, which have lower levels of hTR expression and show less RNA polymerase II at the promoter. RNA polymerase II occupancy of the hTR promoter was also studied in WI38 upon treatment with TSA and 5-azadC. As this treatment has been shown to increase hTR expression, this study therefore leads us to believe that RNA polymerase II occupancy should be increased in WI38 treated with TSA and 5-azadC. Figure 3.6B shows that RNA polymerase II occupancy is indeed increased at the hTR promoter in WI38 upon treatment with TSA and 5-azadC.

This data shows a positive link between RNA polymerase II binding at the hTR promoter and hTR expression and between RNA polymerase II binding and general permissiveness of the chromatin environment.

3.2.6 Transcription Factor Binding at the hTR Promoter

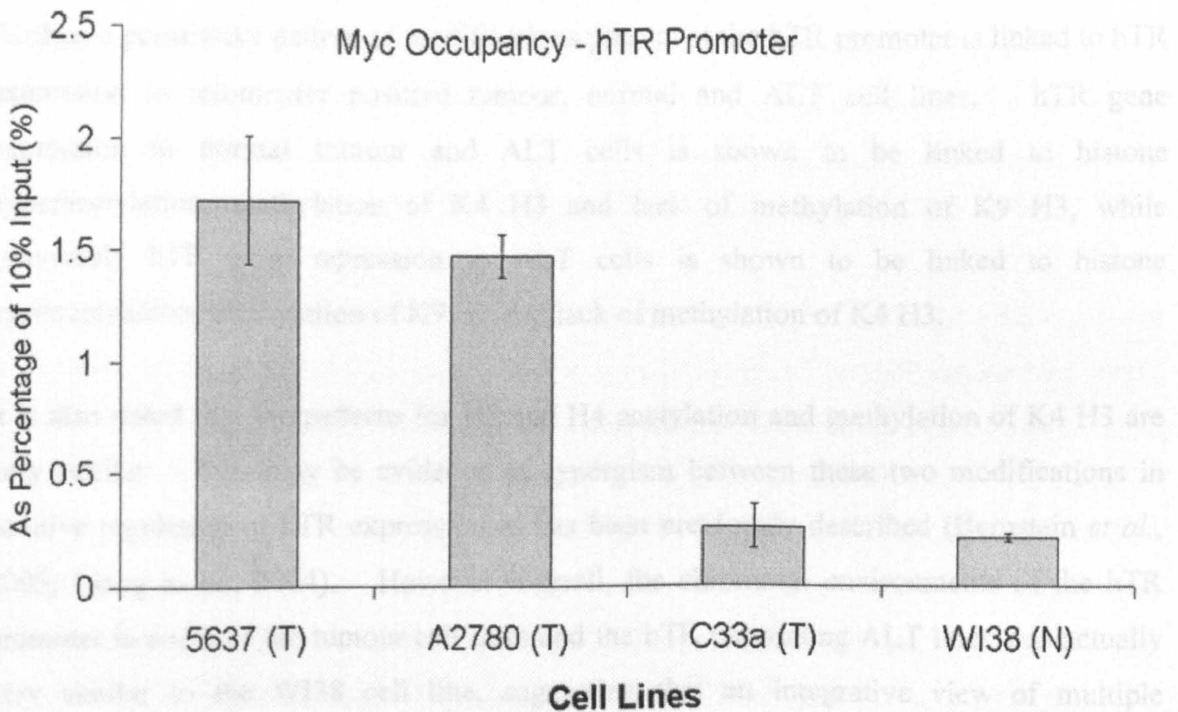
Occupancy of MYC of the hTR promoter was also analysed by ChIP, although no role for MYC has been described for regulating hTR expression and no known consensus site for MYC exists in the hTR proximal promoter sequences. Figure 3.7 shows that occupancy of the hTR promoter by MYC was observed in all cells tested; 5637, A2780, C33a and WI38. Higher levels were observed in the cell lines with the greatest hTR expression and which have the most permissive chromatin environment, which were 5637 and A2780. C33a and WI38, which have lower hTR expression and a less permissive chromatin environment surrounding the promoter has less MYC occupancy at the promoter.

3.3 Discussion

Classically, cancer is viewed as a set of diseases that are driven by progressive genetic abnormalities that include mutations in tumour-suppressor genes and oncogenes, and chromosomal abnormalities. However, it is now becoming apparent that cancer is also a disease that is driven by "epigenetic changes" — patterns of altered gene expression that are mediated by mechanisms that do not affect the primary DNA sequence. Extensive alterations to chromatin in cancer cells in comparison with the normal cells from which they are derived are observed. These involve both losses and gains of DNA methylation as well as altered patterns of histone modifications (Jones and Baylin, 2002; Fraga *et al.*, 2005; Seligson *et al.*, 2005; Herman and Baylin, 2003). However, how these changes affect senescence and tumourigenesis is relatively unknown.

Figure 3.7 MYC Occupancy of the hTR promoter

Q-PCR results of the ChIP for Myc in tumour (T) and normal (N) cells detected by Q-PCR using primers which detect the hTR core promoter sequence. Results are expressed as a percentage of 10% of the Input sample, which is a 20% fraction of chromatin utilised for each IP experiment, and so normalises the data. Each IP is also corrected for a control IP experiment, done in parallel with other IP's, in which no antibody was added to control for any background. Each IP value was established in at least three separate experiments and the IP figure shown is of a representative experiment. Means and standard errors are from triplication within the Q-PCR reactions.



hTR overexpression has been observed in tumourigenesis (Avilion *et al.*, 1996; Soder *et al.*, 1998; Downey *et al.*, 2001; Wisman *et al.*, 2000; Feng *et al.*, 1995) and consistent with this was the increased expression of hTR in the three telomerase positive tumour cell lines (Figure 3.1), and in two ALT cell lines (GM847 and SKLU) relative to the normal WI38 cell line. The data shows that hTR is expressed in a range of cells and the range of expression in these is useful in studying the regulation of hTR expression. Further it is observed that the tumour cells lines and some of the ALT cell lines show increased levels of hTR expression linking increased hTR expression with tumourigenesis. Overall, the data shows that a repressive pattern of modifications present at the hTR promoter sequences is associated with the repression of transcription in the ALT cell lines.

Further, a permissive pattern of modifications present at the hTR promoter is linked to hTR expression in telomerase positive tumour, normal and ALT cell lines. hTR gene expression in normal tumour and ALT cells is shown to be linked to histone hyperacetylation, methylation of K4 H3 and lack of methylation of K9 H3, while conversely hTR gene repression in ALT cells is shown to be linked to histone hypoacetylation, methylation of K9 H3 and lack of methylation of K4 H3.

It is also noted that the patterns for H3 and H4 acetylation and methylation of K4 H3 are very similar. This may be evidence of synergism between these two modifications in positive regulation of hTR expression as has been previously described (Bernstein *et al.*, 2005; Liang *et al.*, 2004). However, overall, the chromatin environments of the hTR promoter in some of the tumour cell lines and the hTR expressing ALT lines was actually very similar to the WI38 cell line, suggesting that an integrative view of multiple regulatory mechanisms is needed in order to fully comprehend the transcriptional regulation of hTR and also other genes. The chromatin environment however is very important in expression as shown by the repressive chromatin environment of the hTR promoter associated with the ALT cell lines which lacked hTR expression.

As well as uncovering a link between hTR transcription and the chromatin environment of the promoter, understanding promoter specific chromatin changes upon the bypass of senescence is also permitted. As has been discussed previously, changes in chromatin structure are observed upon senescence or senescence-like arrest leading to the repression and activation of genes important in controlling proliferation and growth (Narita *et al.*, 2003). It is possible that dysregulation of pathways controlling histone modifications could lead to the reactivation of genes needed for cell proliferation and growth permitting the bypass of senescence and may also allow the gain of a tumourigenic phenotype.

The data presented allows a comparison to be drawn between a normal presenescent cell and a transformed cell of the same cell type and can allow us to ascertain whether or not chromatin changes occur upon the bypass of senescence and the gain of an immortal phenotype, at least for the two genes studied here; hTR and as will be studied in the next chapter, hTERT. Compared to WI38, expression of hTR is further repressed in the WI38-SV40 cell line, which exhibits increased methylation of K9 H3 and K20 H4 at the hTR promoter compared to the WI38 cell line. Acetylation of H3 and H4 and methylation of K4 H3 at the promoter is also decreased in the WI38-SV40 cell line when compared to the WI38 cell line. So, the bypass of senescence in the normal WI38 cell line leads to a decrease in hTR expression with an increase in repressive chromatin modifications and a decrease in permissive modifications at the promoter, which may be why the WI38-SV40 cell line uses the ALT mechanism to mediate telomere maintenance. The formation of heterochromatin at the hTR promoter will lead to the heavy repression of hTR expression and therefore repression of telomerase activity in these cells and in turn could lead to selective pressure to activate the ALT mechanisms in this cell line.

Also, the WI38 cell line is a fibroblastic cell line and therefore mesenchymal in origin and it is understood that telomerase expression is tightly repressed in such tissues and so the repression of hTR in this cell line and the utilisation of ALT are understandable. The relatively low hTR expression in the WI38 cell line may be part of the repressive nature of the cell type utilised. This study indicates that the bypass of senescence can lead to changes in the chromatin environment at the promoter sequences and will possibly have global effects as modulation of the chromatin environment can have genome wide effects. So, overall the transformation process in WI38 which has lead to the use of the ALT telomere maintenance mechanism is linked to the downregulation of hTR expression by the formation of a repressive chromatin environment at the hTR promoter.

The treatment of hTR negative ALT cell lines and a normal cell line with low hTR expression with drugs capable of modulating the chromatin environment have shown that the promotion of permissive histone modifications at the promoter sequence can allow re-expression or increased levels of expression. Lack of acetylated H3 and H4 and methylated K4 H3 has been previously linked to gene silencing and indeed loss of these modifications has been proposed to be the primary event in gene silencing (Mutskov and Felsenfeld, 2004) and so the promotion of these modifications could lead to re-expression of previously silenced genes. On treatment with the DNA-methylation inhibitor 5-azadC and the HDAC-inhibitor TSA, higher levels of acetylated H3 and H4 were present and some increases in methylated K4 H3 were apparent at the hTR promoter, linked to the

observed increase in hTR expression. This data is in agreement with previous studies on the synergistic effect of TSA and 5-azadC treatment showing increases in acetylated K9 H3 and methylated K4 H3 at previously silenced gene promoters (Kondo *et al.*, 2003).

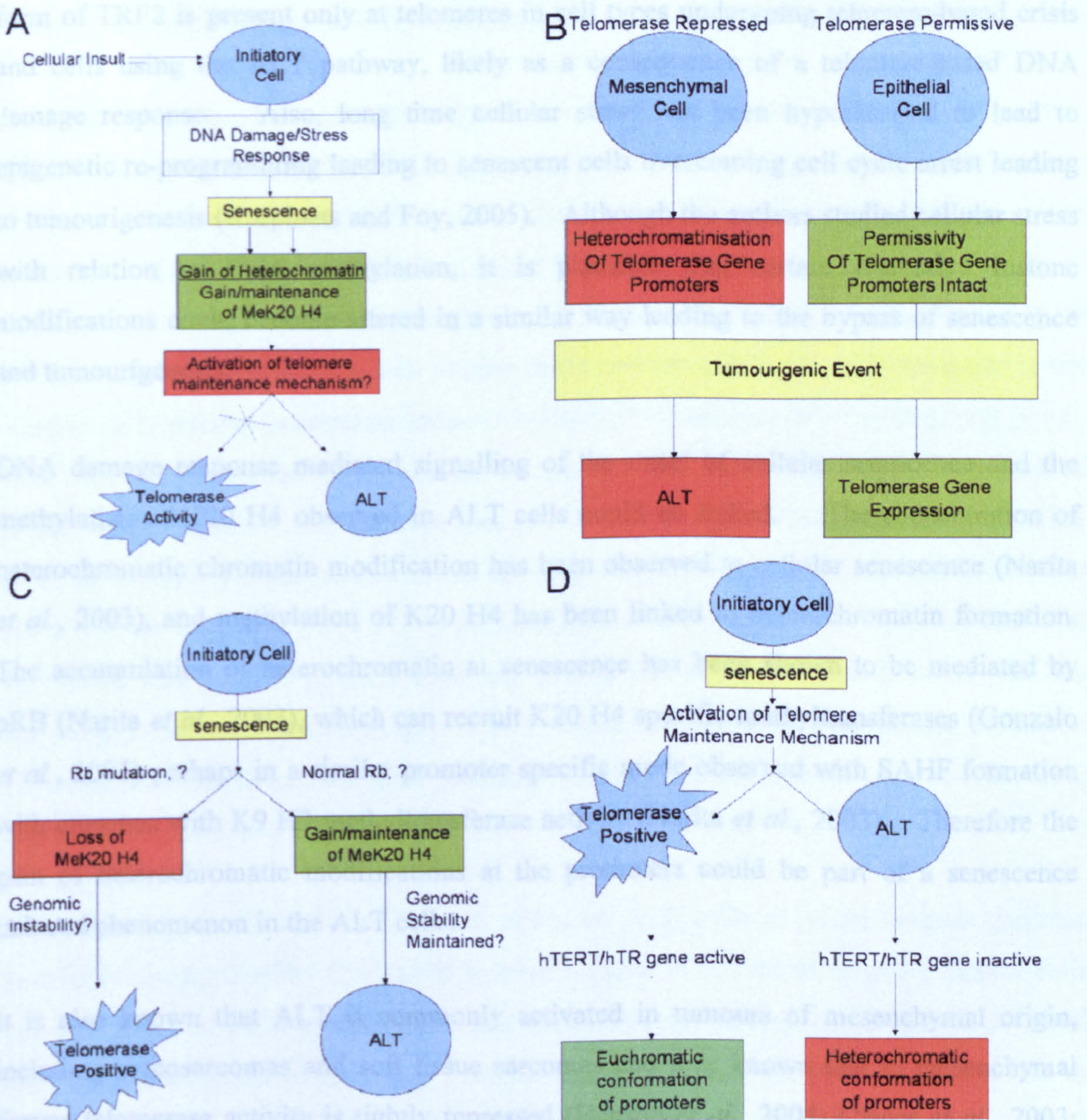
This data further shows that the role of the chromatin environment is key to the regulation of hTR expression. Whether this increase is enough to allow telomerase activity (also allowing for hTERT expression) in the cells in question is unknown but the low levels of expression obtained suggest that if telomerase activity is present, it would be at very low levels. The increased levels of hTR in the hTR negative cell lines were much less than that of even the levels of expression in any of the tumour cell lines, indicating that although the chromatin environment has an important role to play, other mechanisms must also act to allow high hTR expression. A recent study also showed that treatment of ALT cell lines with 5-azadC and TSA leads to the enhancement of hTR expression although the chromatin environment of the hTR promoter was not studied (Kumakura *et al.*, 2005). This study is in agreement with this previous data and strengthens the point by studying changes in chromatin modifications.

Although general patterns of histone modifications have now been linked to hTR expression and repression, the data attained for methylation of K20 H4 did not show any correlations to activation or repression. As mentioned, methylation of K20 H4 has been previously linked to gene repression but the data suggests that methylation of K20 H4 may have another interesting role as of the cell lines examined only the ALT cell lines had high levels of methylated K20 H4 which suggests that this modification may mark for the ALT phenotype rather than having a role in silencing of hTR expression. Regardless of hTR expression levels, methylation of K20 H4 is present in each ALT cell line at similar levels (approximately 4-8%).

Other than being linked to transcriptional repression, methylation of K20 H4 has also been previously linked with the sensing of DNA double stranded breaks in yeast by the binding of Crb2 to this modified lysine residue (Sanders *et al.*, 2004b). Constitutively high levels of methylated K20 H4 are needed for the Crb2 mediated DNA damage response so the highly methylated K20 H4 observed in the ALT cell lines might be explained as a DNA damage or stress response (Figure 3.8A) perhaps as a response to telomere dysfunction due to the heterogeneous telomere length observed in ALT cell lines. However, the human homologue of Crb2, 53BP1, is known to bind methylated K79 H3 in double-stranded break response instead (Huyen *et al.*, 2004). Further studies into the role of methylated K79 H3

Figure 3.8 Possible Roles of K20 Methylation in Telomerase Gene Expression and ALT

A Methylation of K20 H4 as a DNA damage response could lead to the activation of ALT over telomerase activity. **B** Tight repression of telomerase activity due to the formation of heterochromatin at the telomerase gene promoters in mesenchymal tissues could lead to the preferential activation of ALT. **C** Active Rb could lead to the maintenance of methylation of K20 H4 favouring ALT activation. **D** Lack of telomerase gene activity due to activation of ALT could lead to the formation of heterochromatin at the telomerase gene promoters.



could be of interest and could aid in the elucidation of the role for methylation of K20 H4. Investigating factors which are able to bind methylated K20 H4 in ALT cells and cells undergoing various forms of cellular stresses could further uncover the role of methylation of K20 H4 in mammals.

Interestingly, the phosphorylated form of TRF2, which is present after DNA damage and is known to be found in ALT-associated PML bodies (Yeager *et al.*, 1999), is present at telomeres in ALT cells (Tanaka *et al.*, 2005). The authors report that the phosphorylated form of TRF2 is present only at telomeres in cell types undergoing telomere-based crisis and cells using the ALT pathway, likely as a consequence of a telomere-based DNA damage response. Also, long time cellular stress has been hypothesised to lead to epigenetic re-programming leading to senescent cells overcoming cell cycle arrest leading to tumourigenesis (Karpinets and Foy, 2005). Although the authors studied cellular stress with relation to DNA methylation, it is plausible that certain repressive histone modifications could become altered in a similar way leading to the bypass of senescence and tumourigenesis.

DNA damage response mediated signalling of the onset of cellular senescence and the methylation of K20 H4 observed in ALT cells could be linked. The concentration of heterochromatic chromatin modification has been observed at cellular senescence (Narita *et al.*, 2003), and methylation of K20 H4 has been linked to heterochromatin formation. The accumulation of heterochromatin at senescence has been shown to be mediated by pRB (Narita *et al.*, 2003), which can recruit K20 H4 specific methyltransferases (Gonzalo *et al.*, 2005) perhaps in a similar promoter specific mode observed with SAHF formation with enzymes with K9 H3 methyltransferase activity (Narita *et al.*, 2003). Therefore the gain of heterochromatic modifications at the promoters could be part of a senescence induced phenomenon in the ALT cells.

It is also known that ALT is commonly activated in tumours of mesenchymal origin, including osteosarcomas and soft tissue sarcomas and it is known that in mesenchymal tissues telomerase activity is tightly repressed (Henson *et al.*, 2005; Ulaner *et al.*, 2003; Hakin-Smith *et al.*, 2003; Johnson *et al.*, 2005). Mesenchymal tissues have a slower turnover and less telomere shortening than in many epithelial type cells and this is possibly why they may repress telomerase more tightly. Three of the ALT cell lines investigated within this study tend to show a more heterochromatic environment at the hTR promoter (as well as hTERT as will be discussed in the next chapter), with hTERT and in some cases hTR, being transcriptionally silenced. Therefore, methylation of K20 H4 may be a

specific cellular response of the mesenchymal type cells to repress telomerase activity (Figure 3.8B) and further ALT may be utilised over telomerase activity due to this repression of telomerase gene expression. However, the fact that two of the ALT cells express hTR and the WI38 cell line has little K20 H4 methylation seems to argue against this point. Cells undergoing a transforming event which would require telomerase activity could perhaps be "forced" to utilise the ALT pathway as hTR expression, needed for telomerase activity, would be tightly repressed due to the heterochromatic nature of the hTR promoter sequence. This may also occur for hTERT, in the context of telomerase activity, as will be discussed in the next chapter.

A recent study has shown that globally, chromatin in cancer cells is less trimethylated at K20 H4 (Fraga *et al.*, 2005), and in agreement with this, the telomerase positive tumour cell lines (C33a, A2780 and 5637) show a distinct lack of any methylated K20 H4. The normal cell line, WI38, also shows minimal K20 H4 methylation, but the ALT cells all show comparatively high levels of methylated K20 H4. The loss of trimethylation of K20 H4 has also been observed at both preneoplastic and tumour stages of liver cancer in the context of hepatocarcinogenesis induced by methyl deficiency in rats (Pogribny *et al.*, 2006) and corresponded to a decrease in the K20 H4 HMT SUV4-20H2. Loss of methylation of K20 H4 and therefore loss of heterochromatin formation could account for genomic instability observed in cancers, although mechanisms on how methylation of K20 H4 is lost are unknown. The SUV4-20H enzymes directly interact with pRB (Gonzalo *et al.*, 2005), and the loss of pRB could the lead to the loss of this modification and the silencing properties it entails (Figure 3.8C). This could lead to a permissive chromatin environment at the telomerase gene promoters perhaps leading to telomerase activity being more likely to be activated over ALT.

If methylation of K20 H4 is a global effect in ALT cells, it could counter genomic instability encountered by the loss of heterochromatin in telomerase positive tumour cells and could lead to ALT tumours being less aggressive and having less neoplastic potential than telomerase positive tumours. Indeed ALT tumours have these characteristics as indicated by laboratory studies (Stewart *et al.*, 2002; Chang *et al.*, 2003). In one study of osteosarcoma, progression free and overall survival was observed to be much better in the hTERT negative ALT primary tumour samples than the hTERT positive primary tumour samples, linking ALT to better overall survival (Sanders *et al.*, 2004a). Another study studying the prevalence of telomerase activity in osteosarcoma and Ewing's sarcoma primary tumour and metastasis samples showed that telomerase activity was far more prevalent than ALT in the metastasis when compared to the primary tumour samples in

which few samples showed telomerase activity (Sotillo-Pineiro *et al.*, 2004). The presence of the ALT telomere maintenance mechanism is also linked to a better prognosis in glioblastoma multiforme (Hakin-Smith *et al.*, 2003).

However several studies have found different outcomes. A study of osteosarcoma tumour samples found that the *absence* of telomere maintenance mechanisms was linked to better survival (90%) (Ulaner *et al.*, 2003) and another study also showed no correlation between telomere maintenance mechanism and patient outcome or tumour aggressiveness in soft tissue sarcomas and osteosarcoma (Henson *et al.*, 2005). Correlating with these studies, were the findings that the ALT phenotype was associated with complex karyotypes in sarcomas (Montgomery *et al.*, 2004) and the presence of ALT associated with chromosomal instability in osteosarcomas (Scheel *et al.*, 2001). A recent study in mouse embryonic fibroblasts has also shown that chromosomal instability upon loss of hTERT and the WRN helicase leads to the activation of ALT (Laud *et al.*, 2005).

It may also be possible that the telomerase gene promoters become heterochromatinised because they are not active. In this model, the lack of activating transcription factors binding at the telomerase gene promoters will lead to the accumulation of repressive histone modifications. So the repressive chromatin environment could be due to telomerase gene repression and not telomerase gene repression due to the accumulation of repressive histone modifications (Figure 3.8D). All of the above hypotheses are summarised in Figure 3.8. Overall, this and other studies suggest an interesting role for methylation of K20 H4 in regulation of telomerase gene control and a possible marker for the ALT mechanism. Further work studying the effects of methylated K20 H4 globally and in the context of ALT could uncover some very interesting molecular pathways towards controlling telomere maintenance mechanisms.

Overall, this data provides evidence that the expression of hTR is linked to the state of the chromatin environment. To better understand expression of hTR, transcriptional dynamics were investigated by studying RNA polymerase II occupancy of the promoter, to investigate how the chromatin environment may be directly linked to gene repression/expression. The data shows that the more permissive the chromatin environment is, as in 5637 and A2780, higher binding of RNA polymerase II at the promoter sequence is observed and the less permissive the chromatin environment is, as with C33a and WI38, lower binding of RNA polymerase II at the hTR promoter sequence is observed. Also by modulating the chromatin environment to promote the formation of a

more permissive chromatin environment in the normal cell WI38, we observe an increase in RNA polymerase II binding at the hTR promoter.

RNA polymerase II has been shown to associate with various proteins which mediate K4 H3 methylation (Ng *et al.*, 2003), K36 H3 methylation (Kizer *et al.*, 2005) and histone acetylation (von Mikecz *et al.*, 2000). K4 H3 and K36 H3 methylation has been associated with transcriptional elongation (Kizer *et al.*, 2005; Ng *et al.*, 2003) and it is shown in this study that a more permissive chromatin landscape at promoter sequences is associated with higher RNA polymerase II binding and increased transcription.

A recent study has also highlighted the role of a RNA polymerase II associated protein in tumourigenesis (Moniaux *et al.*, 2006). The protein in question, hPAF1 is the human homologue of yeast pafl which is proposed to promote gene transcription by allowing the formation of a permissive chromatin environment through tri-methylation of K4 H3 (Adelman *et al.*, 2006). Therefore the overexpression of hPAF1 in human cancer could allow increased transcription of genes involved in tumourigenesis by facilitating RNA polymerase II mediated gene transcription. Each of the cell lines studied shows a degree of RNA polymerase II binding to the promoter and correspondingly, each cell line does show some trimethylation of K4 H3. Increased permissivity of the chromatin environment may make the DNA more accessible to RNA polymerase II binding or certain histone modifications, such as acetylation, may promote the binding of members of the basal transcriptional machinery (such as TAF(II)250 binding through bromodomains).

However, basal transcriptional machinery has also been shown to bind silenced chromatin (Sekinger and Gross, 2001) suggesting that modulation of the chromatin environment may allow promoter clearance and elongation rather than enhancing actual binding. Changes in the chromatin environment of promoter sequences may therefore drive changes in the dynamics of the basal transcription machinery and complex interactions between transcriptional complexes and chromatin may therefore be important in mediating transcriptional activity.

Also studied was the role of MYC in telomerase gene regulation in the context of the chromatin environment. MYC genes are key regulators of cell proliferation, and their deregulation contributes to the genesis of most human tumours (Adhikary and Eilers, 2005). The role of MYC in hTERT transcription is well documented (Xu *et al.*, 2001; Oh *et al.*, 1999b; Kyo *et al.*, 2000; Wu *et al.*, 1999) but any role for MYC in the regulation of hTR expression has not been reported and no consensus binding site for MYC exists in the

basal hTR promoter. The data presented here provides some evidence that MYC has a positive link with hTR expression, although further studies will need to be undertaken to uncover the full influence of MYC on the regulation of hTR expression. MYC binding is observed to be increased in cell lines with a more permissive chromatin environment (5637 and A2780) and MYC binding is decreased in cell lines with a less permissive chromatin environment (C33a and WI38).

MYC binding to promoters without consensus binding sites has been observed (Fernandez *et al.*, 2003) and MYC has been observed to bind to variants of the consensus sequence *in vitro* and such variants can also be high-affinity binding sites *in vivo* (Grandori *et al.*, 2003). Another possibility is that MYC may be recruited to non-consensus binding sites through the interaction with other transcription factors to modulate transcription (Mao *et al.*, 2003). Further to MYCs role in the modulation of the chromatin environment, MYC has been shown to bind to histone acetyltransferases (Liu *et al.*, 2003; Frank *et al.*, 2003; Faiola *et al.*, 2005) although one study has shown that the chromatin around the MYC-bound loci was highly acetylated, even before MYC binding (Fernandez *et al.*, 2003) suggesting that the permissivity of the chromatin environment is needed to allow MYC firstly to bind at promoter sequences.

Evidence for a role for MYC in regulating hTR expression is however lacking and so further studies will be needed to ascertain MYCs role on hTR expression. Transient transfection studies utilising an hTR promoter driven reporter gene with MYC protein co-transfection may shed some light on the roles of MYC on hTR expression by allowing us to study if different amounts of transfected MYC can lead to increases in hTR expression.

4 Epigenetic Regulation of hTERT Expression in Normal, Tumour and ALT Cell Lines

4.1 Introduction

hTERT is a specialised reverse transcriptase initially purified from the ciliate *Euplotes aediculatus* as a protein homologous to the yeast protein Est2p (Lingner *et al.*, 1997) and is one of the core components required for reconstitution of telomerase activity (Weinrich *et al.*, 1997). While being absent or at extremely low levels in normal human cells, expression is observed to be reactivated or greatly increased in cancer cell lines and tumours (Ramakrishnan *et al.*, 1998; Meyerson *et al.*, 1997) demonstrating that tight regulation of hTERT expression in normal cells is of great importance and so the elucidation of regulatory mechanisms in normal and cancer cells would be also of great importance and interest.

Cloning and characterisation of the hTERT gene and promoter (Horikawa *et al.*, 1999; Takakura *et al.*, 1999; Cong *et al.*, 1999; Wick *et al.*, 1999) has allowed detailed analysis of hTERT regulation and has revealed regulation at many levels. Initial analysis of the hTERT gene revealed complex splicing patterns in different cell types (Kilian *et al.*, 1997) and modulation of telomerase activity by splice variant expression has now been shown to be an important regulator of telomerase activity in both development (Ulaner *et al.*, 1998) and cancer progression (Ulaner *et al.*, 2000; Drummond *et al.*, 2005; Anderson *et al.*, 2006). While the full length wild type variant is the only transcript with reverse transcriptase activity, three main deletions variants are also usually expressed. These are the α, β and γ splice variants and importantly the α-deletion variant has been shown to be a dominant negative inhibitor of telomerase activity (Colgin *et al.*, 2000; Yi *et al.*, 2000).

Evaluation of the promoter sequences established that the hTERT gene promoter contains a large CpG island although the correlation between CpG methylation and hTERT expression remains unclear. Initial studies found no clear link between methylation and expression (Dessain *et al.*, 2000; Devereux *et al.*, 1999), however some studies have shown positive (Guilleret and Benhattar, 2003; Guilleret *et al.*, 2002a) and also negative correlations (Bechter *et al.*, 2002; Shin *et al.*, 2003). These data suggest that methylation may play an important but complex role in the regulation of hTERT expression but that other mechanisms are also crucial.

Transcriptional regulation of hTERT is generally understood to be greatly important in mediating hTERT expression. Characterisation of the promoter sequence revealed binding sites for multiple transcription factors (Horikawa *et al.*, 1999; Takakura *et al.*, 1999; Cong *et al.*, 1999; Wick *et al.*, 1999). The core promoter sequences contain two E-box sequences to which MYC/MAX heterodimers are known to bind and transactivate the promoter (Wu *et al.*, 1999) while MAD/MAX heterodimers can bind the same sites to repress the promoter (Oh *et al.*, 2000).

Several other factors can compete with MYC for binding to E-boxes including Upstream Stimulatory Factor (USF) which can activate the hTERT promoter (Goueli and Janknecht, 2003) and the HTLV-1 oncoprotein Tax (Gabet *et al.*, 2003) which represses the promoter. The tumour suppressor protein BRCA1 (Xiong *et al.*, 2003) also mediates repression by binding to and repressing MYCs activating properties. Sp1 sites are also evident in the promoter and Sp1 and Sp3 can activate and repress the hTERT promoter respectively while Sp1 can synergise with MYC to activate hTERT transcription (Kyo *et al.*, 2000). The human Papillomavirus type 16 E6 protein can also activate hTERT expression seemingly through binding to MYC and Sp1 (Oh *et al.*, 2001), while p53 and p73 seemingly inhibit hTERT expression by sequestering Sp1 away from the promoter (Xu *et al.*, 2000; Racek *et al.*, 2005). There also exists two sites in the core hTERT promoter sequence to which the oestrogen receptor (ER) can bind, one being a half site next to a canonical recognition sequence for Sp1, allowing synergism between the ER and Sp1 (Krishnan *et al.*, 1994) and oestrogen has been shown to activate the hTERT promoter (Kyo *et al.*, 1999).

HREs have also been discovered in the hTERT promoter and HIF1α has recently been shown to regulate hTERT splice variant expression under hypoxic conditions in human cancer cells (Anderson *et al.*, 2006). HIF1α binding correlated to higher expression of the full length active form being transcribed, at the expense of the other inactive deletion variants. Other factors which can activate the hTERT promoter include the adenoviral E1A protein (Glasspool *et al.*, 2005) possibly acting by sequestering the co-repressor CtBP away from the promoter sequences. CtBP can be found in histone modifying complexes which can mediate heterochromatin formation (Shi *et al.*, 2003) and so the removal of CtBP may allow for the formation of a more permissive chromatin environment allowing transcription. Two consensus binding sites for the ETS transcription factor are also evident in the hTERT promoter sequence, which can directly mediate EGF-induced transactivation of hTERT (Maida *et al.*, 2002).

Negative regulation of hTERT can be mediated by several factors through binding sites within the hTERT promoter. These include MZF2 (Fujimoto *et al.*, 2000), which represses hTERT transcription through several sites in the promoter and the Wilms' Tumour 1 (WT1) tumour suppressor gene (Oh *et al.*, 1999a). E2F1 can also repress transcription of hTERT possibly through the binding of pRB leading to possible heterochromatin formation and silencing of the promoters sequence (Crowe *et al.*, 2001). There also exists a Vitamin D3 receptor (VDR) binding site, through which VDR and the retinoid x receptor (RXR) can bind and inhibit hTERT expression (Ikeda *et al.*, 2003; Pendino *et al.*, 2003).

Interestingly many of the transcription factors discussed above have been shown to interact with chromatin modifying enzymes. Differentiation of HL-60 cells leads to decreases in histone acetylation at the hTERT promoter. This is concomitant with a change from MYC/MAX binding to MAD/MAX binding and decreases in hTERT transcription and indicates that the MYC/MAX and MAD/MAX complexes recruit HATs and HDACs respectively (Xu *et al.*, 2001). Sp1 and Sp3 have been shown to be able to recruit HDAC activity to the hTERT promoter to repress transcription in normal human somatic cells (Won *et al.*, 2002). E2F1 and pRB generally bind and repress promoters and pRB has been shown to recruit repressive histone modifying activities which could alter the chromatin environment leading to the repression of transcription (Brehm *et al.*, 1998; Gonzalo *et al.*, 2005; Nielsen *et al.*, 2001; Nguyen and Crowe, 1999; Won *et al.*, 2004; Narita *et al.*, 2003).

Data showing the presence of DNaseI hypersensitivity sites at the hTERT regulatory sequences in hTERT expressing cells further strengthens the hypothesis that the chromatin environment plays a role in the regulation of hTERT expression (Wang and Zhu, 2003; Wang and Zhu, 2004; Szutorisz *et al.*, 2003). Other studies have shown that down-regulation of hTERT expression during differentiation is concomitant with histone deacetylation and the accumulation of CpG methylation at the hTERT promoter (Liu *et al.*, 2004; Lopatina *et al.*, 2003) and upon screening for proteins involved in hTERT transcriptional regulation, a novel human gene encoding a HAT-like protein was discovered by one group (Lv *et al.*, 2003).

Recently regulation of hTERT transcription by histone phosphorylation has also been demonstrated (Ge *et al.*, 2006). A recent study has also shown that phosphorylation of S10 H3 is known to enhance histone acetylation (Lo *et al.*, 2000) and so may aid transcription of hTERT. The use of drugs which can target histone modifying enzymes,

notably Trichostatin A (TSA), have been shown to cause increases in transcription of hTERT in normal human cells (Takakura *et al.*, 2001; Mukhopadhyay *et al.*, 2005; Cong and Bacchetti, 2000; Hou *et al.*, 2002). This has been shown to be due to the acetylation of promoter proximal histones possibly by inhibiting the interactions between negative regulatory factors and HDACs or by inhibiting their actions at the hTERT promoter (Hou *et al.*, 2002).

These findings, showing the role of DNA methylation and chromatin modifications suggest that the chromatin environment could have a role to play in the regulation of hTERT expression. To this end, the chromatin environment surrounding the hTERT promoter was studied in tumour, ALT and normal cell lines to establish any links between hTERT expression and the endogenous chromatin environment.

4.2 Results

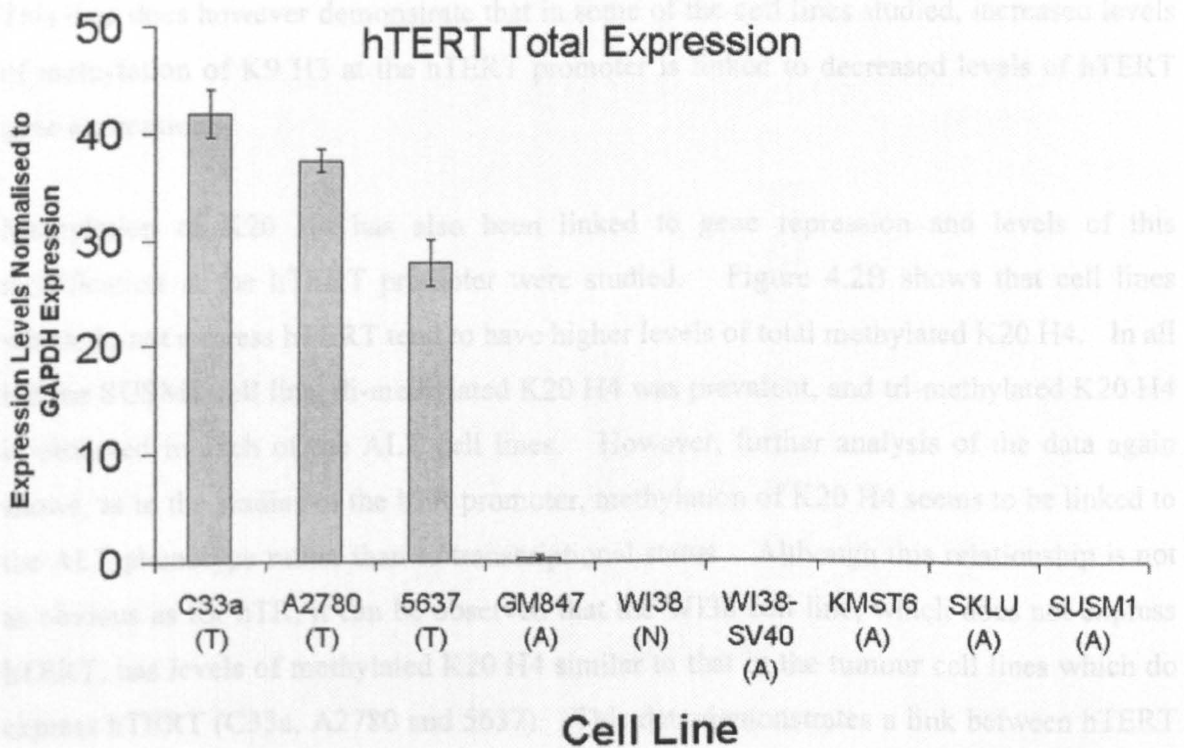
4.2.1 Expression of hTERT in Tumour, Normal and ALT Cell Lines

As for hTR we first established hTERT gene expression levels from each cell line. Semi-quantitative PCR was used to analyse GAPDH expression to normalise for subsequent PCR reactions. Primers that recognise the main splice variants of hTERT were used and total amounts for each splice variant summed and expressed as total hTERT expression. As Figure 4.1 shows, of the cell lines studied only the tumour cell lines (C33a, A2780 and 5637) have appreciable levels of hTERT gene expression. Apart from the GM847 cell line which expresses very low amounts of hTERT (only the β deletion variant, data not shown), the ALT and normal cell lines exhibited no hTERT expression. The profile of the chromatin environment surrounding the hTERT promoter was then established and any relationship to expression was examined.

4.2.2 Profile of Transcriptionally Repressive Histone Modifications at the hTERT Promoter

In order to examine the relationship between hTERT expression and histone modifications present at the promoter sequences, chromatin immunoprecipitation (ChIP) assays using antibodies against specific histone modifications were used to generate a profile of the chromatin environment surrounding the hTERT promoter in each cell line.

Figure 4.1 hTERT Expression in Normal, Telomerase Negative Tumour and ALT Cell Lines
Cell lines from which hTERT expression levels are established are ALT (A), telomerase positive tumour (T) or normal (N) cell lines, as noted after each cell line name. RNA was extracted from each cell line and cDNA synthesised using 1μg RNA. hTERT expression levels were established, first by analysing GAPDH levels. cDNA amounts used as input in each separate hTERT splice variant PCR reaction for each cell line were then adjusted so similar concentrations of cDNA were used in each PCR reaction. hTERT expression levels are shown as the sum of all the expressed variants (WT, α, β and αβ). Therefore total hTERT levels are expressed as being normalised to GAPDH expression. Expression levels were found to be similar between three different experiments for each cell lines. Means and errors are established from the average of these Q-PCR experiments



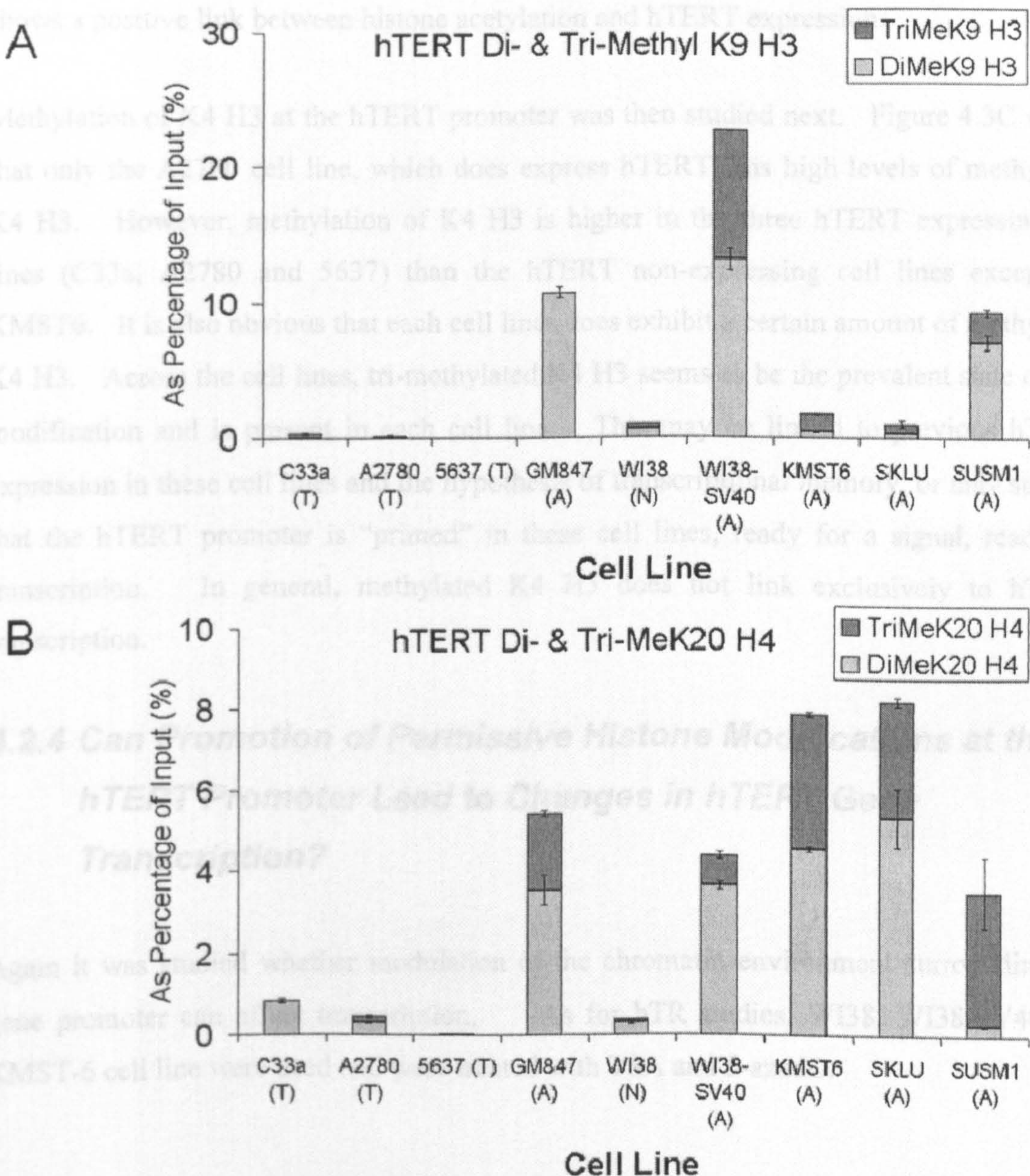
Modifications linked to repression of gene transcription were studied first and to this end methylation of K9 H3 and methylation of K20 H4 were studied. Figure 4.2A shows that overall levels of methylated K9 H3 are negligible at the hTERT promoter in the cell lines expressing high levels of hTERT (C33a, A2780 and 5637) but high levels of methylated K9 H3 are observed in GM847, WI38-SV40 and SUSM1 and lower levels of this modification are present in other cell lines with no hTERT expression (WI38, KMST6 and SKLU). Cell lines which exhibited high total levels of methylated K9 H3 tended to have more di-methylated than the tri-methylated form (GM847, WI38-SV40 and SUSM1), although the dimethylated form was still present at high levels in some cell lines (WI38-SV40 and SUSM1). A low level of a repressive modification in a cell line with no hTERT expression underlies the principle that multiple modifications need to be studied in order to understand fully the interplay between the chromatin environment and gene transcription. This data does however demonstrate that in some of the cell lines studied, increased levels of methylation of K9 H3 at the hTERT promoter is linked to decreased levels of hTERT gene expression.

Methylation of K20 H4 has also been linked to gene repression and levels of this modification at the hTERT promoter were studied. Figure 4.2B shows that cell lines which do not express hTERT tend to have higher levels of total methylated K20 H4. In all but the SUSM1 cell line, di-methylated K20 H4 was prevalent, and tri-methylated K20 H4 is observed in each of the ALT cell lines. However, further analysis of the data again shows, as in the studies of the hTR promoter, methylation of K20 H4 seems to be linked to the ALT phenotype rather than to transcriptional status. Although this relationship is not as obvious as for hTR, it can be observed that the WI38 cell line, which does not express hTERT, has levels of methylated K20 H4 similar to that in the tumour cell lines which do express hTERT (C33a, A2780 and 5637). This data demonstrates a link between hTERT gene repression and methylation of K20 H4 at the hTERT gene promoter although the presence of methylated K20 H4 in the 5 ALT cell lines and absence in the normal cell line may indicate another role for this modification as we observed for hTR.

4.2.3 Profile of Transcriptionally Permissive Histone Modifications at the hTERT Promoter

Modifications linked to gene expression were then studied. The modifications studied were acetylation of K9 H3, acetylated histones H3 and H4 and methylation of K4 H3.

Figure 4.2 Modifications Linked to Repression of hTERT Transcription
Levels of **A** Di- and tri-methylated K9 H3 and **B** di- and tri-methylated K20 H4 present at the hTERT promoter in ALT (A), telomerase positive tumour (T) or normal (N) cell lines, detected by Q-PCR using primers which detect the hTERT core promoter sequence, from products of chromatin immunoprecipitation assays. Results are expressed as the total IP amount for both the antibodies and are expressed as a percentage of an Input sample, which is a 20% fraction of chromatin utilised for each IP experiment, and so normalises the data. Each IP is also corrected for a control IP experiment, done in parallel with other IP's, in which no antibody was added to control for any background. Each IP value was established in at least three separate experiments and the IP figure shown is of a representative experiment. Means and standard errors are from triplication within the Q-PCR reactions.



Histone acetylation of promoters is linked to gene expression and so the acetylation status of the hTERT promoter was established. Figure 4.3A shows that in the two cell lines expressing the highest levels of hTERT (C33a and A2780), total acetylation was also highest. Levels of acetylated H3 and H4 were very similar between these two cell lines (approximately 20% total and 10% each for acetylation of H3 and H4 for both cell lines) and this links well to hTERT expression. Acetylation in those cell lines with little or no hTERT expression (GM847, WI38, WI38-SV40, KMST-6, SKLU and SUSM-1) was much lower than in A2780 and C33a. However the 5637 cell line which expresses hTERT at a relatively high level does not have levels of total acetylation any higher than those cell lines which do not express hTERT. Studies of acetylated K9 H3 at the hTERT promoter (Figure 4.3B) shows similar results as those seen for acetylated H3 and H4 with only C33a and A2780 showing high levels of acetylation while the other cell lines, including the 5637 cell line, show very low or no acetylated K9 H3. In general this data shows a positive link between histone acetylation and hTERT expression.

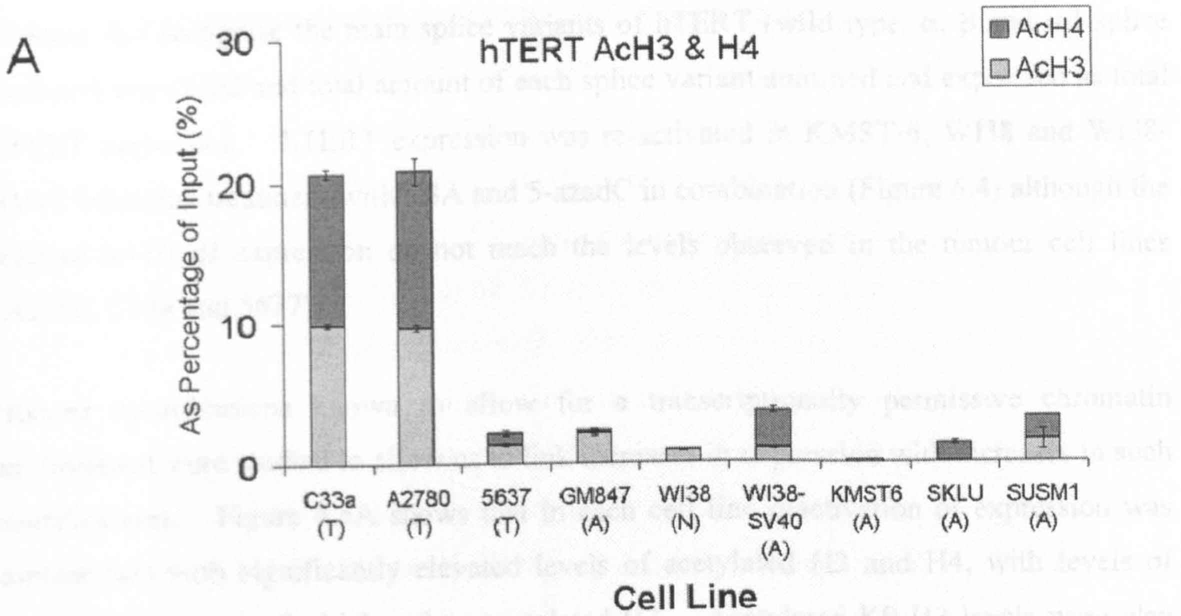
Methylation of K4 H3 at the hTERT promoter was then studied next. Figure 4.3C shows that only the A2780 cell line, which does express hTERT, has high levels of methylated K4 H3. However, methylation of K4 H3 is higher in the three hTERT expressing cell lines (C33a, A2780 and 5637) than the hTERT non-expressing cell lines except for KMST6. It is also obvious that each cell lines does exhibit a certain amount of methylated K4 H3. Across the cell lines, tri-methylated K4 H3 seems to be the prevalent state of this modification and is present in each cell line. This may be linked to previous hTERT expression in these cell lines and the hypothesis of transcriptional memory, or may suggest that the hTERT promoter is "primed" in these cell lines, ready for a signal, ready for transcription. In general, methylated K4 H3 does not link exclusively to hTERT transcription.

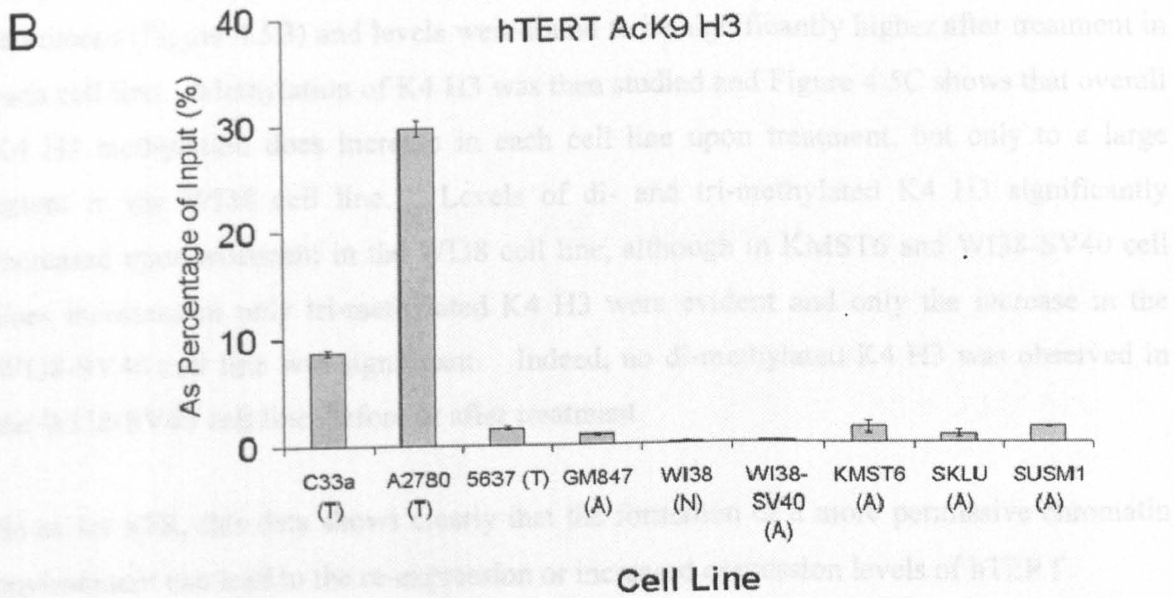
4.2.4 Can Promotion of Permissive Histone Modifications at the hTERT Promoter Lead to Changes in hTERT Gene Transcription?

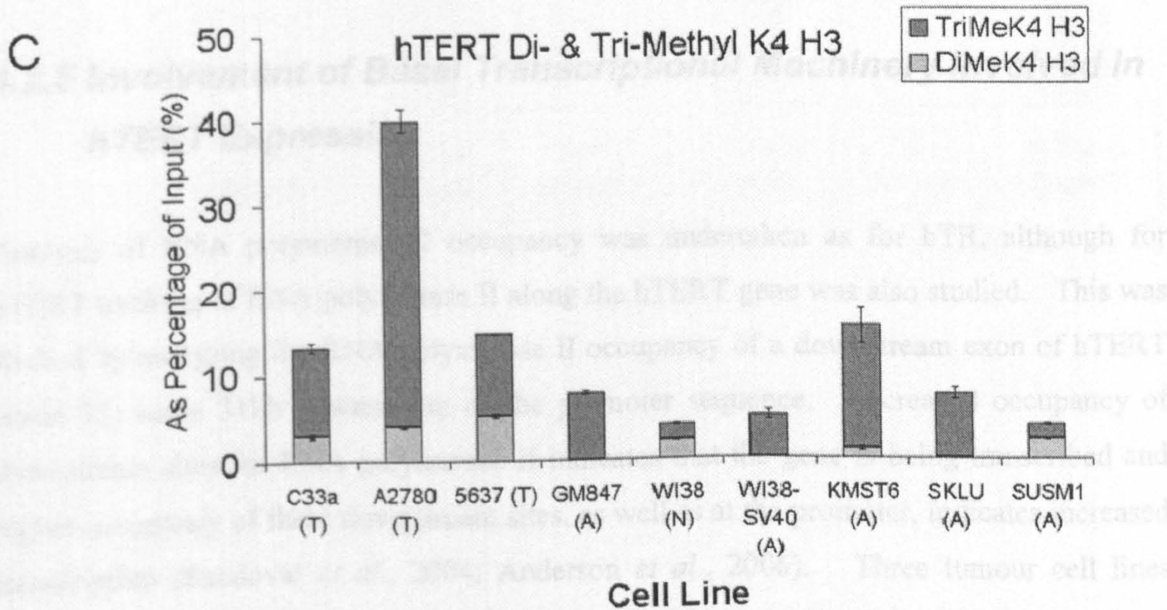
Again it was studied whether modulation of the chromatin environment surrounding the gene promoter can affect transcription. As for hTR studies, WI38, WI38-SV40 and KMST-6 cell line were used and were treated with TSA and 5-azadC.

Figure 4.3 Modifications Linked to Permissiveness of hTERT Transcription

Levels of **A** Acetylated H3 and H4, **B** Acetylated K9 H3 and **C** Di- and Tri-methylated K4 H3. present at the hTERT promoter in ALT (A), telomerase positive tumour (T) or normal (N) cell lines, detected by Q-PCR using primers which detect the hTERT core promoter sequence, from products of chromatin immunoprecipitation assays. Results are expressed as the total IP amount for both the antibodies (apart from acetylated K9 H3) and are expressed as a percentage of an Input sample, which is a 20% fraction of chromatin utilised for each IP experiment, and so normalises the data. Each IP is also corrected for a control IP experiment, done in parallel with other IP's, in which no antibody was added to control for any background. Each IP value was established in at least three separate experiments and the IP figure shown is of a representative experiment. Means and standard errors are from triplication within the Q-PCR reactions.


A
hTERT AcH3 & H4
AcH4
AcH3
As Percentage of Input (%)
30
20
10
0
C33a (T)
A2780 (T)
5637 (T)
GM847 (A)
WI38 (N)
WI38-SV40 (A)
KMST6 (A)
SKLU (A)
SUSM1 (A)
Cell Line


B
hTERT AcK9 H3
As Percentage of Input (%)
40
30
20
10
0
C33a (T)
A2780 (T)
5637 (T)
GM847 (A)
WI38 (N)
WI38-SV40 (A)
KMST6 (A)
SKLU (A)
SUSM1 (A)
Cell Line


C
hTERT Di- & Tri-Methyl K4 H3
TriMeK4 H3
DiMeK4 H3
As Percentage of Input (%)
50
40
30
20
10
0
C33a (T)
A2780 (T)
5637 (T)
GM847 (A)
WI38 (N)
WI38-SV40 (A)
KMST6 (A)
SKLU (A)
SUSM1 (A)
Cell Line

Primers that recognise the main splice variants of hTERT (wild type, α, β and αβ splice variants) were used and total amount of each splice variant summed and expressed as total hTERT expression. hTERT expression was re-activated in KMST-6, WI38 and WI38-SV40 following treatment with TSA and 5-azadC in combination (Figure 4.4) although the relative levels of expression do not reach the levels observed in the tumour cell lines (A2780, C33a and 5637).

Histone modifications known to allow for a transcriptionally permissive chromatin environment were studied to allow us to link increases in expression with increases in such modifications. Figure 4.5A shows that in each cell line reactivation of expression was concomitant with significantly elevated levels of acetylated H3 and H4, with levels of acetylated H4 generally higher than acetylated H3. Acetylated K9 H3 levels were also monitored (Figure 4.5B) and levels were found to be significantly higher after treatment in each cell line. Methylation of K4 H3 was then studied and Figure 4.5C shows that overall K4 H3 methylation does increase in each cell line upon treatment, but only to a large extent in the WI38 cell line. Levels of di- and tri-methylated K4 H3 significantly increased upon treatment in the WI38 cell line, although in KMST6 and WI38-SV40 cell lines increases in only tri-methylated K4 H3 were evident and only the increase in the WI38-SV40 cell line was significant. Indeed, no di-methylated K4 H3 was observed in the WI38-SV40 cell line, before or after treatment.

So as for hTR, this data shows clearly that the formation of a more permissive chromatin environment can lead to the re-expression or increased expression levels of hTERT.

4.2.5 Involvement of Basal Transcriptional Machinery Involved in hTERT Expression

Analysis of RNA polymerase II occupancy was undertaken as for hTR, although for hTERT tracking of RNA polymerase II along the hTERT gene was also studied. This was studied by analysing the RNA polymerase II occupancy of a downstream exon of hTERT (exon 12) some 31kb downstream of the promoter sequence. Increased occupancy of downstream sites for RNA polymerase II indicates that the gene is being transcribed and higher occupancy of these downstream sites, as well as at the promoter, indicates increased transcription (Sandoval *et al.*, 2004; Anderson *et al.*, 2006). Three tumour cell lines (A2780, C33a and 5637) and a normal cell line (WI38) were utilised for this assay.

Figure 4.4 hTERT Expression Following Treatment with TSA and 5-azadC

Cell lines from which hTERT levels are established are ALT (A) or normal (N) cell lines, as noted after each cell line name. Cells were treated twice for 24 hours with 5-azadC to a final concentration of 2.5mM, and then treated for 16 hours with Trichostatin A (TSA) at a final concentration of 350nM (+). Control cells were treated over the same time course but with 50% acetic acid instead of 5-azadC and 100% ethanol instead of TSA (-). hTERT expression levels were established, first by analysing GAPDH levels from cDNA made from 1μg RNA. cDNA amounts used as input in each separate reaction for each cell line were then adjusted for the hTERT splice variant PCR reaction so similar concentrations of cDNA were used in each PCR. hTERT expression levels are shown as the sum of all the expressed variants (WT, α, β and αβ). Therefore the hTERT levels are expressed as being normalised to GAPDH expression. Expression levels were found to be similar between three different experiments for each cell lines. Means and errors are established from the average of these Q-PCR experiments.

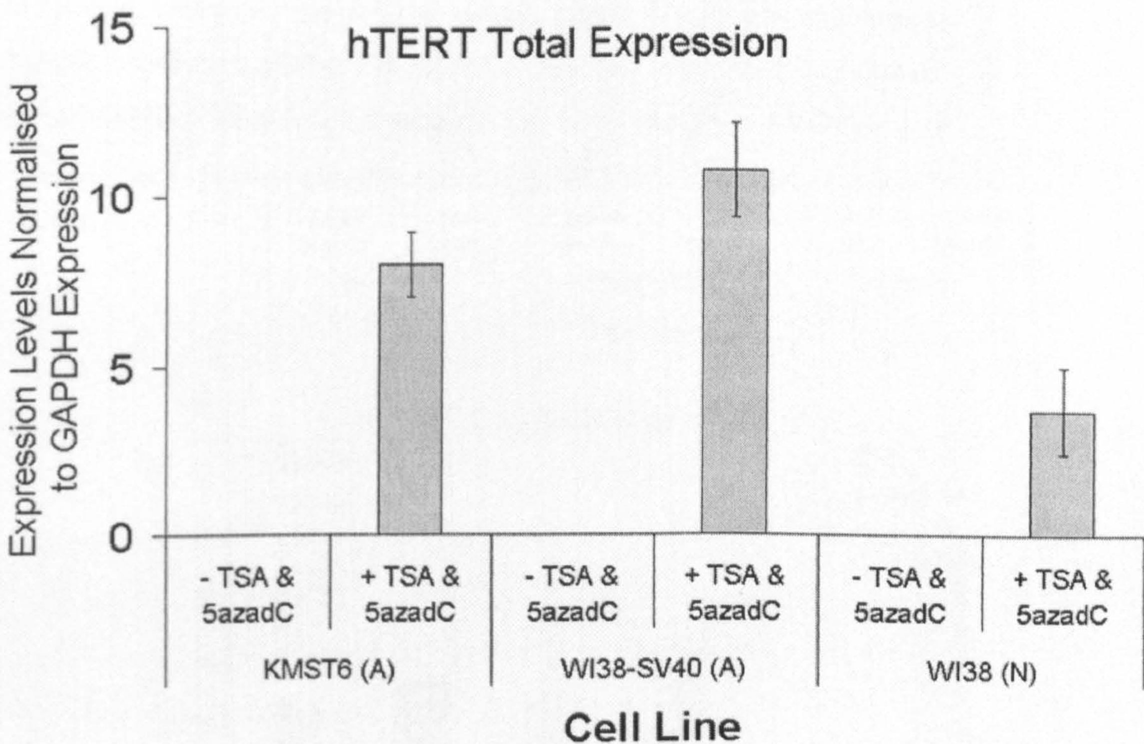
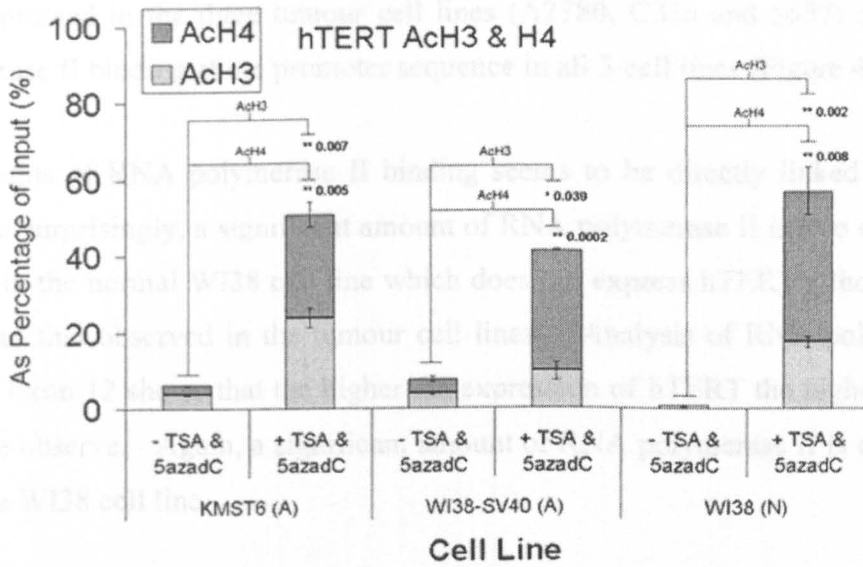
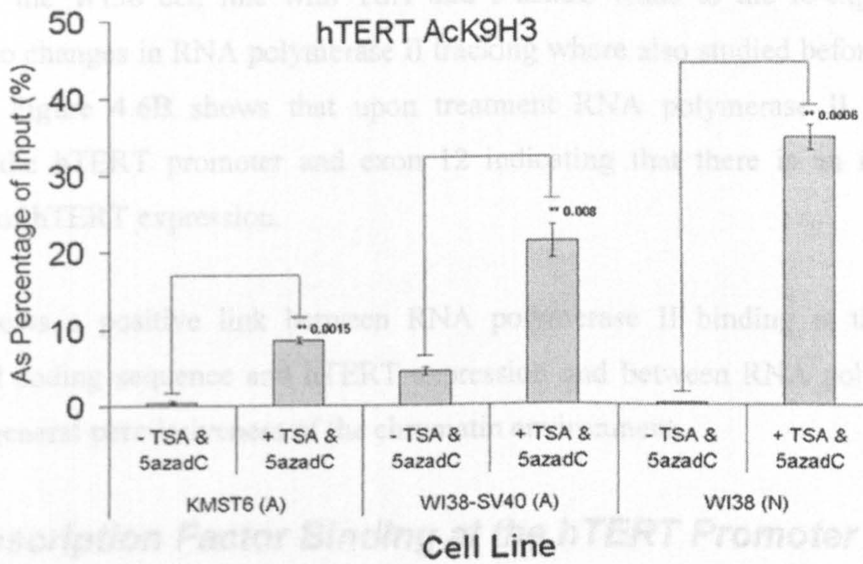
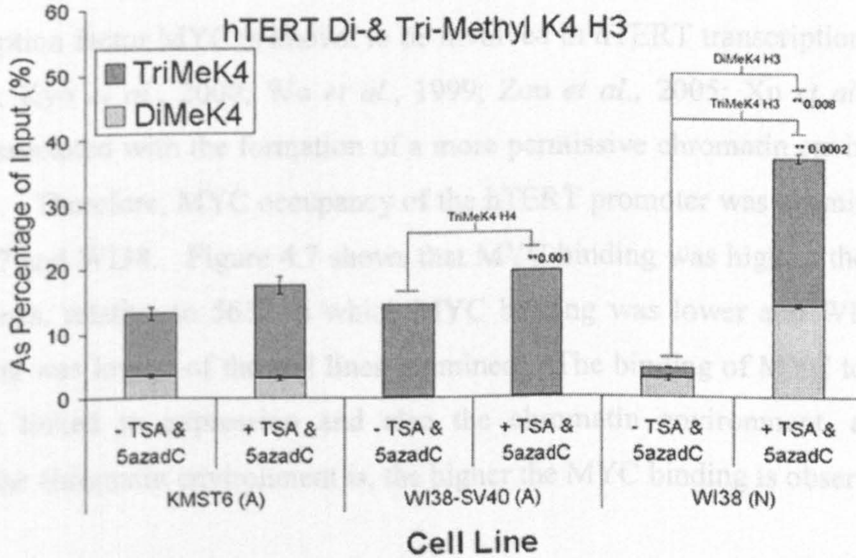


Figure 4.5 Changes in Levels of Histone Modifications at the hTERT Promoter Following Treatment with TSA and 5-azadC

Q-PCR results of the ChIP using antibodies against **A** Acetylated H3 and H4, **B** Acetylated K9 H3 and **C** Di- and Tri-methylated K4 H3 detected by Q-PCR using primers which detect the hTERT core promoter sequence, from products of chromatin immunoprecipitation assays. Cell lines used are ALT (A) or normal (N) cell lines, as noted after each cell line name. Cells were treated twice for 24 hours with 5-azadC to a final concentration of 2.5mM, and then treated for 16 hours with Trichostatin A (TSA) at a final concentration of 350nM (+). Control cells were treated over the same time course but with 50% acetic acid instead of 5-azadC and 100% ethanol instead of TSA (-). Results are expressed as the total IP amount for both the antibodies (apart from acetylated K9 H3) and are expressed as a percentage of an Input sample, which is a 20% fraction of chromatin utilised for each IP experiment, and so normalises the data. Each IP is also corrected for a control IP experiment, done in parallel with other IP's, in which no antibody was added to control for any background. Each IP value was established in at least three separate experiments and the IP figure shown is of a representative experiment. Means and standard errors are from triplication within the Q-PCR reactions. Statistics were analysed using ANOVA software from the Microsoft Excel data analysis tool pack. Asterisks denote a statistically significant difference between untreated and treated; *$p < 0.05$; **$p < 0.01$.


A
hTERT AcH3 & H4
AcH4
AcH3
As Percentage of Input (%)
100
80
60
40
20
0
AcH3
AcH4
** 0.007
** 0.005
AcH3
AcH4
* 0.039
** 0.0002
AcH3
AcH4
** 0.002
** 0.008
- TSA & 5azadC
+ TSA & 5azadC
- TSA & 5azadC
+ TSA & 5azadC
- TSA & 5azadC
+ TSA & 5azadC
KMST6 (A)
WI38-SV40 (A)
WI38 (N)
Cell Line


B
hTERT AcK9H3
As Percentage of Input (%)
50
40
30
20
10
0
** 0.0015
** 0.008
** 0.0006
- TSA & 5azadC
+ TSA & 5azadC
- TSA & 5azadC
+ TSA & 5azadC
- TSA & 5azadC
+ TSA & 5azadC
KMST6 (A)
WI38-SV40 (A)
WI38 (N)
Cell Line


C
hTERT Di-& Tri-Methyl K4 H3
TriMeK4
DiMeK4
As Percentage of Input (%)
60
50
40
30
20
10
0
TriMeK4 H4
**0.001
DiMeK4 H3
TriMeK4 H3
**0.008
** 0.0002
- TSA & 5azadC
+ TSA & 5azadC
- TSA & 5azadC
+ TSA & 5azadC
- TSA & 5azadC
+ TSA & 5azadC
KMST6 (A)
WI38-SV40 (A)
WI38 (N)
Cell Line

hTERT is expressed in the three tumour cell lines (A2780, C33a and 5637) and we see RNA polymerase II binding at the promoter sequence in all 3 cell lines (Figure 4.6A).

Relative amounts of RNA polymerase II binding seems to be directly linked to hTERT expression but surprisingly, a significant amount of RNA polymerase II is also observed at the promoter in the normal WI38 cell line which does not express hTERT although this is much less than that observed in the tumour cell lines. Analysis of RNA polymerase II occupancy at Exon 12 shows that the higher the expression of hTERT the higher levels of occupancy we observe. Again, a significant amount of RNA polymerase II is observed at exon 12 in the WI38 cell line.

Treatment of the WI38 cell line with TSA and 5-azadC leads to the re-expression of hTERT and so changes in RNA polymerase II tracking where also studied before and after treatment. Figure 4.6B shows that upon treatment RNA polymerase II occupancy increases at the hTERT promoter and exon 12 indicating that there is an increase in transcription of hTERT expression.

This data shows a positive link between RNA polymerase II binding at the hTERT promoter and coding sequence and hTERT expression and between RNA polymerase II binding and general permissiveness of the chromatin environment.

4.2.6 Transcription Factor Binding at the hTERT Promoter

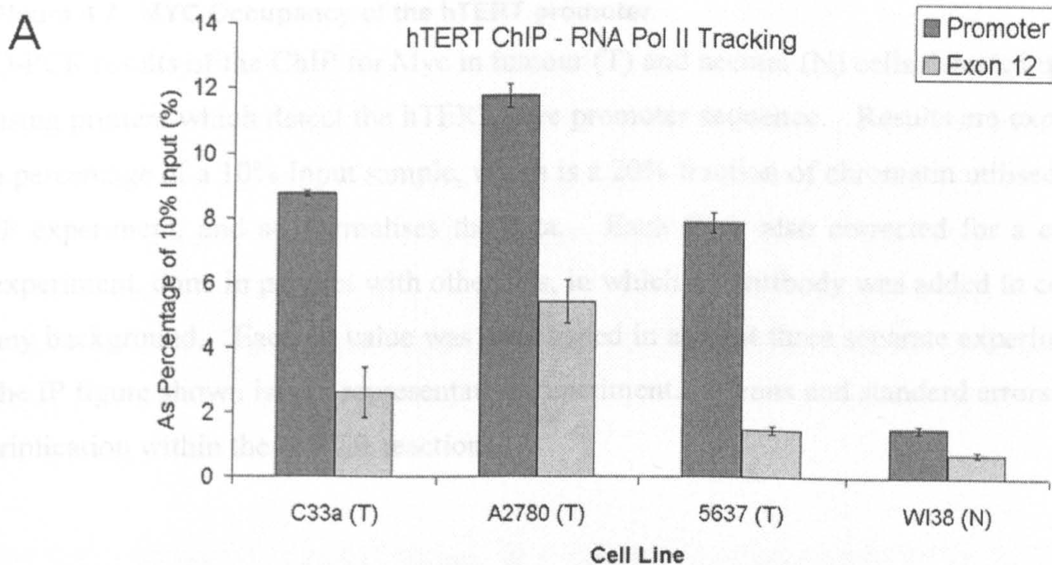
The transcription factor MYC is known to be involved in hTERT transcription (Greenberg *et al.*, 1999; Kyo *et al.*, 2000; Wu *et al.*, 1999; Zou *et al.*, 2005; Xu *et al.*, 2001) and binding is associated with the formation of a more permissive chromatin environment (Xu *et al.*, 2001). Therefore, MYC occupancy of the hTERT promoter was examined in C33a, A2780, 5637 and WI38. Figure 4.7 shows that MYC binding was high in the A2780 and C33a cell lines, relative to 5637 in which MYC binding was lower and WI38 in which MYC binding was lowest of the cell lines examined. The binding of MYC to the hTERT promoter is linked to expression and also the chromatin environment, as the more permissive the chromatin environment is, the higher the MYC binding is observed to be.

4.3 Discussion

These studies demonstrate that changes in the chromatin environment of the hTERT promoter in normal and telomerase positive tumour and telomerase negative ALT cell lines

Figure 4.6 RNA Polymerase II Binding at the hTERT promoter and Exon 12 of hTERT

Q-PCR results of the ChIP for **A** RNA Pol II in tumour (T) and normal (N) cells and **B** RNA Pol II following treatment of the normal (N) cell line WI38 with 5-azadC and TSA detected by Q-PCR using primers which detect the hTERT core promoter sequence and Exon 12 of hTERT. WI38 cells were treated twice for 24 hours with 5-azadC to a final concentration of 2.5mM, and then treated for 16 hours with Trichostatin A (TSA) at a final concentration of 350nM (+). Control cells were treated over the same time course but with 100% ethanol instead of TSA and 50% acetic acid instead of 5-azadC (-). Results are expressed as a percentage of a 10% Input sample, with the Input fraction being a 20% fraction of chromatin utilised for each IP experiment, and so normalises the data. Each IP is also corrected for a control IP experiment, done in parallel with other IP's, in which no antibody was added to control for any background. Each IP value was established in at least three separate experiments and the IP figure shown is of a representative experiment. Means and standard errors are from triplication within the Q-PCR reactions.


A
hTERT ChIP - RNA Pol II Tracking
Promoter
Exon 12
As Percentage of 10% Input (%)
14
12
10
8
6
4
2
0
C33a (T)
A2780 (T)
5637 (T)
WI38 (N)
Cell Line

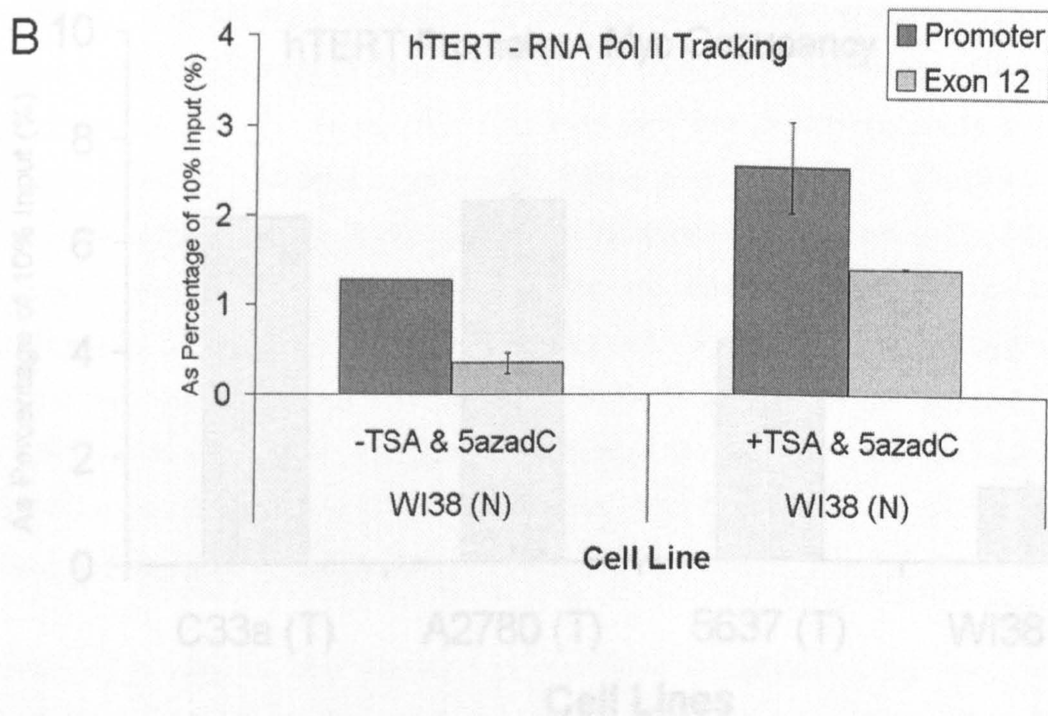
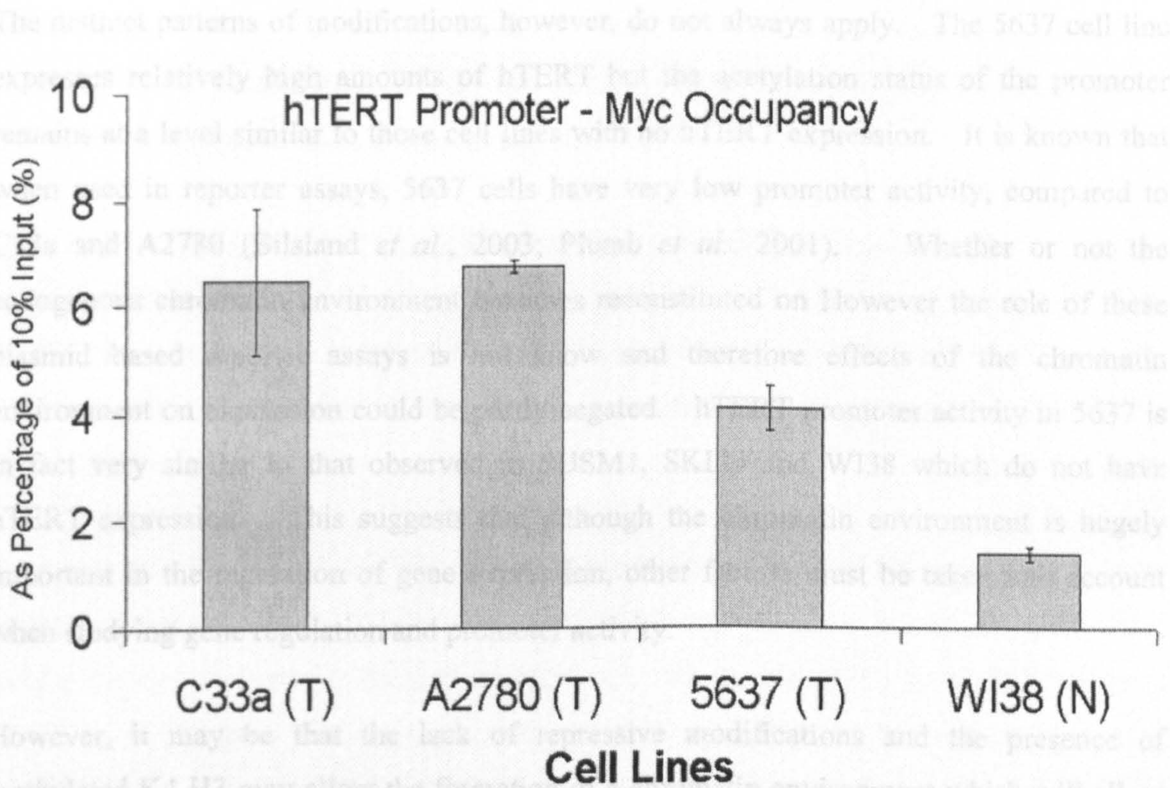

B
hTERT - RNA Pol II Tracking
Promoter
Exon 12
As Percentage of 10% Input (%)
4
3
2
1
0
-TSA & 5azadC
WI38 (N)
+TSA & 5azadC
WI38 (N)
Cell Line

Figure 4.7 MYC Occupancy of the hTERT promoter

Q-PCR results of the ChIP for Myc in tumour (T) and normal (N) cells detected by Q-PCR using primers which detect the hTERT core promoter sequence. Results are expressed as a percentage of a 10% Input sample, which is a 20% fraction of chromatin utilised for each IP experiment, and so normalises the data. Each IP is also corrected for a control IP experiment, done in parallel with other IP's, in which no antibody was added to control for any background. Each IP value was established in at least three separate experiments and the IP figure shown is of a representative experiment. Means and standard errors are from triplication within the Q-PCR reactions.



is different and these changes correlates with expression. hTERT expression is repressed in the normal and ALT cell lines and data shows that a repressive pattern of modifications present at the hTERT promoter sequences is associated with the repression of hTERT transcription in the ALT and normal cell lines. Further, a permissive pattern of modifications present at the hTERT promoter is linked to hTERT expression in telomerase positive tumour cell lines. hTERT gene expression in tumour cells is linked to histone hyperacetylation, methylation of K4 H3 and lack of methylation of K9 H3 while hTERT gene repression in normal and ALT cell lines is linked to histone hypoacetylation and methylation of K9 H3.

The distinct patterns of modifications, however, do not always apply. The 5637 cell line expresses relatively high amounts of hTERT but the acetylation status of the promoter remains at a level similar to those cell lines with no hTERT expression. It is known that when used in reporter assays, 5637 cells have very low promoter activity, compared to C33a and A2780 (Bilsland *et al.*, 2003; Plumb *et al.*, 2001). Whether or not the endogenous chromatin environment becomes reconstituted on However the role of these plasmid based reporter assays is not know and therefore effects of the chromatin environment on expression could be partly negated. hTERT promoter activity in 5637 is in fact very similar to that observed in SUSM1, SKLU and WI38 which do not have hTERT expression. This suggests that although the chromatin environment is hugely important in the regulation of gene expression, other factors must be taken into account when studying gene regulation and promoter activity.

However, it may be that the lack of repressive modifications and the presence of methylated K4 H3 may allow the formation of a chromatin environment which will allow transcription to occur at a low level, for example in the 5637 cell line. Similarly, WI38, KMST6 and SKLU cell lines have relatively low levels of methylated K9 H3 at the promoter but still do not express hTERT. This may be due to the fact that these cell lines have promoters which lack high levels of acetylation of histones H3 and H4. Therefore the lack of permissive modifications at the hTERT promoter in these cell lines may be enough to repress transcription.

hTERT plays an important role in senescence and immortalisation and so allows an excellent opportunity to study modes of gene regulation in senescence bypass and immortalisation. This study also allows a comparison to be drawn between a normal presenescent cell and a transformed cell of the same cell type and to ascertain whether or not chromatin changes occur upon the bypass of senescence and the gain of an immortal

phenotype. When comparing the WI38 and its transformed cell line WI38-SV40 some interesting differences are observed. Upon transformation the WI38-SV40 cell line utilises the ALT pathway to maintain telomeres instead of the activation of telomerase activity. Neither cell line expresses hTERT but distinct differences in the chromatin environment are observed when comparing the two cell lines. In the WI38 cell line the chromatin environment seems to be a "blank canvas" or an environment in that no great amounts of permissive or repressive modifications are present at the promoter. In comparison, the WI38-SV40 cell line has high levels of methylated K9 H3 and K20 H4 while acetylation levels and methylation of K4 H3 remain similar. The lack of these permissive modifications in the WI38 cell line but the lack of many repressive modifications may represent a "primed" state for hTERT expression while the accumulation of methylated K9 H3 and K20 H4 may represent heavy transcriptional repression and link to the ALT mechanism, as for hTR.

This further indicates that the transformation process and therefore the bypass of senescence is associated with changes in the chromatin environment. Again, as for hTR, the formation of a heavy repressive heterochromatic environment at the hTERT promoter may not allow the expression of hTERT, repress telomerase activation and may "force" the cell to utilise the ALT mechanism for telomere maintenance.

Comparisons of the WI38 cell line with the telomerase positive cell lines also lead to some interesting conclusions. It is shown that hTERT expression is increased in the three tumour cells as compared to the normal WI38 cell with the upregulation of hTERT activity in C33a, A2780 and 5637 is associated with the upregulation of a similar pattern of modifications, such as methylation of K4 H3 and acetylation of H3 and H4 while lacking repressive modifications. This suggests that the mode of hTERT upregulation may be conserved in these tumour cell lines and that a common mechanism may be regulating hTERT expression in these different tumour cell lines; namely the modulation of the chromatin environment of the gene promoters.

Similar data for two genes (hTR and hTERT) with direct links to immortalisation and the bypass of senescence, showing upregulation of gene expression linked to permissive modification patterns at promoter sequences, enhances the claim that the observed changes in the chromatin environment on the bypass of senescence are a global effect rather than specific for one gene. These data suggest further analysis of the chromatin environment and the role it plays in transcriptional control of the telomerase genes and also of the

chromatin environment on multiple genes in the control of senescence and in immortalisation.

The role of methylation of K20 H4 in regulation of hTERT shows some similarity to data gained for hTR. Methylated K20 H4 again seems to mark strongly for the ALT phenotype rather than being linked to gene repression. However, the modification is present at high levels in 5 of the 6 cell lines showing no or negligible hTERT expression, which would indicate that methylation of K20 H4 does have a role in the repression of transcription of hTERT. However, alternate roles, highlighted by the studies of hTR, can not be ignored.

Similarly to data collected for hTR it is shown that if levels of permissive modifications (methylated K4 H3 and acetylation of histones H3 and H4) are enhanced by the use of TSA and 5-azadC, hTERT can become re-expressed. However expression does not increase to levels similar to that in the three tumour cells, exemplifying that although the modulation of the chromatin environment is important, other mechanisms must be involved in mediating transcriptional activation. Whether this increase is enough to allow telomerase activity in the cells in question (also allowing for hTR expression) is unknown but the low levels of expression obtained suggest that if telomerase activity is present, it would be at low levels.

Prior studies have suggested that TSA alone can induce hTERT expression in normal human cells but not in telomerase positive tumour cells, except for in a few cell lines which have very low levels of telomerase activity (Hou *et al.*, 2002; Cong and Bacchetti, 2000; Mukhopadhyay *et al.*, 2005; Takakura *et al.*, 2001). This may indicate that the formation of a permissive chromatin environment may be an early event in transcriptional activation, being the "priming" event allowing other factors further involved in transcriptional activation play a role. Transient transfection assays with the proximal hTERT promoter sequences (Hou *et al.*, 2002; Cong and Bacchetti, 2000; Takakura *et al.*, 2001) and the lack of the effect on hTERT transcription of the protein synthesis inhibitor cyclohexamide (CHX) upon TSA treatment (Mukhopadhyay *et al.*, 2005) suggest that TSA directly affects the hTERT promoter, although TSA is a broad-spectrum HDAC inhibitor and indirect effects cannot be ruled out. The latter study also showed that treatment with 5-azadC had no effect on hTERT expression although various other studies have shown 5-azadC has a positive effect on hTERT transcription (Kumakura *et al.*, 2005; Dessain *et al.*, 2000; Shin *et al.*, 2003).

Dual use of TSA and 5-azadC has also been observed to be needed for hTERT re-expression (Devereux *et al.*, 1999) agreeing with this study and again this data is also in agreement with previous studies on the synergistic effect of TSA and 5-azadC treatment showing increases in acetylated K9 H3 and methylated K4 H3 at previously silenced gene promoters (Kondo *et al.*, 2003).

The recent study by Kumakura *et al* (Kumakura *et al.*, 2005) also studied the chromatin environment surrounding the hTERT promoter. Ratio's of methylation of K9 H3 to permissive modifications such as methylation of K4 and acetylation of K9 and K14 H3, where observed to be higher in telomerase negative ALT cells with no hTERT expression as compared to hTERT expressing telomerase positive cells, in which the ratios where much higher. TSA or 5-azadC treatment alone was shown to be able to promote the presence of permissive chromatin modifications at a previously repressive chromatin domain at the hTERT promoter. These changes were shown to lead to hTERT re-expression and telomerase activity, with 5-azadC having a greater effect on hTERT re-expression than TSA in some cell lines. TSA alone was unable to induce telomerase activity, whereas 5-azadC could induce telomerase activity but treatment with both TSA and 5-azadC led to more robust levels of activity. This study further indicates that epigenetic control of hTERT is important in transcriptional regulation and is in agreement with the data in this study showing that hTERT re-expression only occurred with the treatment of both TSA and 5-azadC (Kumakura *et al.*, 2005).

Also studied was the role of the basal transcriptional machinery in promoter clearance and elongation in the context of chromatin remodelling. As for hTR, RNA polymerase II binding was linked to the chromatin environment of the promoter, with RNA polymerase II binding at the promoter being higher in those cell lines with a more permissive chromatin environment. Also studied was RNA polymerase II trafficking by investigating RNA polymerase II occupancy at hTERT exon 12. Higher binding at hTERT exon 12 has been previously linked to higher gene expression (Anderson *et al.*, 2006) and correspondingly RNA polymerase II binding at exon 12 was higher in those cell lines with higher hTERT expression linking the permissivity of the chromatin environment at the promoter with increased transcription.

Creation of a more permissive chromatin environment by treatment of WI38 with TSA and 5-azadC, led to increased RNA polymerase II binding and trafficking to hTERT exon 12, likely as a result of the permissive chromatin environment allowing a more open chromatin structure therefore allowing increased binding and competence for transcriptional

machinery complex assembly. As noted for hTR, the RNA polymerase II molecule is known to co-localise with chromatin modifying enzymes capable of generating a permissive chromatin environment for the passage of the RNA polymerase II molecule. This study indicates that increased RNA polymerase II binding at the hTERT promoter is linked to a more transcriptionally permissive chromatin environment with increased levels of permissive modifications present with higher levels of RNA polymerase II binding. However, RNA polymerase II binding was still apparent in the WI38 cell line which does not exhibit a permissive chromatin environment and does not show any hTERT expression. This binding of RNA polymerase II at the promoter and exon 12 in the WI38 cell line could be explained by the presence of paused RNA polymerase II molecule being present at the promoter, perhaps indicating that the hTERT gene may be permissive for transcription, only needing changes in the chromatin environment of the promoter to allow transcription.

Basal transcriptional machinery has been shown to bind silenced chromatin (Sekinger and Gross, 2001) suggesting that modulation of the chromatin environment may allow promoter clearance and elongation rather than enhancing actual binding. Appreciable levels of RNA polymerase II occupancy at the hTERT promoter in WI38 suggest that RNA polymerase II binding does occur at silent chromatin domains and that the permissivity of the chromatin environment of the promoter sequences is important in enhanced RNA polymerase II binding at the promoter and gene transcription. Changes in the chromatin environment of promoter sequences may therefore drive changes in the dynamics of the basal transcription machinery and complex interactions between transcriptional complexes and chromatin may therefore be important in mediating transcriptional activity.

MYC has been linked to hTERT transcription in many different studies (Xu *et al.*, 2001; Oh *et al.*, 1999b; Kyo *et al.*, 2000; Wu *et al.*, 1999) and so MYC binding was interrogated at the hTERT promoter. MYC binding was found to be increased in the tumour cells expressing higher levels of hTERT (C33a, A2780 and 5637) when compared to the normal WI38 cell line which does not express hTERT. Expression of C33a and A2780 is higher than in the 5637 cell line and this is reflected in MYC binding. The permissiveness of the chromatin environment is therefore linked to MYC binding, although the nature of this link is not known, as MYC binding could be causing increased histone acetylation (Frank *et al.*, 2001; Frank *et al.*, 2003; Xu *et al.*, 2001) or the permissiveness of the chromatin environment could allow increased MYC binding although high levels of acetylation have been shown to exist at a promoter sequence even before MYC binding (Fernandez *et al.*, 2003).

5 Epigenetic Regulation of the Telomerase Genes in Stem Cell Populations

5.1 Introduction

Stem cells are unique in their ability to both self renew and to give rise to differentiated tissues. The self-renewal capacity of stem cells has been linked to neoplastic growth potential (Reya *et al.*, 2001; Pardal *et al.*, 2003) and studies into stem cells as the originator cells for cancer are of great interest (Marx, 2003). Strong links between normal stem cells and cancer stem cells in many tissues (Al-Hajj *et al.*, 2003; Perez-Losada and Balmain, 2003; Collins *et al.*, 2005; Singh *et al.*, 2004; Kim *et al.*, 2005; Gibbs *et al.*, 2005) suggest that stem cells are targets for neoplastic transformation (Keith, 2004).

Further lending support to the cancer stem cell hypothesis is the recent hypothesis that epigenetic disruption of stem cells may be a common unifying theme in cancer aetiology (Feinberg *et al.*, 2006). This so called "epigenetic progenitor model" may help to explain many different aspects about cancer. In this model, cancer arises in three steps; first is an epigenetic alteration of stem/progenitor cells within a given tissue, second is a gatekeeper mutation (GKM) and third is genetic and epigenetic instability, which leads to increased tumour evolution. Also noted is the correlation between the properties of advanced tumours and the inherent properties of the progenitor cells that give rise to the primary tumour which include invasion, metastasis and drug resistance, further highlighting the importance of epigenetic factors in tumour progression. Therefore, studying the epigenetic regulation of gene expression in normal and cancer stem cells is an exciting opportunity.

Human mesenchymal stem cells (hMSCs) are multipotential cells and have the ability both *in vivo* and *in vitro* to differentiate into a variety of adult mesenchymal tissues, such as bone, cartilage, adipose and muscle (Pittenger *et al.*, 1999). Unusually for a stem cell, hMSCs lack telomerase activity (Zimmermann *et al.*, 2003) and when passaged in culture, hMSCs gradually reduce their proliferative rate and enter replicative senescence (Simonsen *et al.*, 2002; Stenderup *et al.*, 2003). Ectopic expression of hTERT in the hMSC leads to reactivation of telomerase activity and maintenance of telomere length, abolishes the senescence-associated phenotype and can lead to extension of lifespan and maintenance of proliferative capacity (Simonsen *et al.*, 2002; Shi *et al.*, 2002). This has consequences for the therapeutic potential of the hMSC in tissue engineering as such cells may only have a

limited lifespan. However, the re-expression of telomerase by the ectopic expression of hTERT in the hMSC has been shown to allow the accumulation of neoplastic changes (Serakinci *et al.*, 2004; Burns *et al.*, 2005). Recent studies have also shown that the long term passaging of hMSCs without hTERT transduction can also lead to the formation of tumourigenic mesenchymal type cells (Wang *et al.*, 2005; Rubio *et al.*, 2005). These studies suggest that the adult hMSC can be a target for neoplastic transformation.

The lack of hTERT expression in the hMSC provides an ideal model to study telomerase gene regulation in the normal adult stem cell. A key issue in telomere biology is how the complex pattern of telomerase gene expression is maintained in the adult stem cell and the hMSC can allow us to study hTR and hTERT transcriptional control. As discussed previously, the histone deacetylase inhibitor TSA has previously been shown to cause an increase in histone acetylation at the hTERT promoter and cause re-expression of the hTERT gene. To this end, hMSCs were studied before and after treatment with TSA to investigate possible mechanisms behind the silencing of hTERT expression and possible pathways controlling hTR and hTERT expression in the hMSC. This includes all stages of promoter function including the key steps of transcription pre-initiation complex formation, promoter clearance and elongation in the context of chromatin remodelling. hTERT and hTR expression was analysed and ChIP assays were utilised in order to study the acetylation status of the hTR and hTERT promoters and RNA polymerase II and TFIIB dynamics.

Also studied were human haematopoietic stem cells (hHSCs), a stem cell population in which cancer stem cells were first identified and studied. The haematopoietic system is organized in a hierarchical manner, in which rare haematopoietic stem cells (hHSCs) initiate the hierarchy. They have the ability to self-renew, proliferate and differentiate into different lineages of peripheral blood cells though intermediating haematopoietic progenitor cells (HPCs) (Reya, 2003). hHSC self-renewal is driven by both intrinsic and extrinsic factors but serial transplantation studies have clearly indicated that self-renewal potential of hHSCs is impaired after replicative stress (Kamminga *et al.*, 2005b). hHSC activity may be irreversibly lost in a single cell division (Nakauchi *et al.*, 2001; Takano *et al.*, 2004) indicating that the epigenetic regulation of gene expression may play an important role. Indeed expression of PcG proteins BMI1 and EZH2, which are found in complexes and can repress target gene expression by modification of histones, allows the counteraction of senescence and allows self-renewal of hHSCs (Kamminga *et al.*, 2005a; Park *et al.*, 2003; Lessard and Sauvageau, 2003). Loss of BMI1 has also been noted to cause a decrease in hHSC number (Park *et al.*, 2003) and limits proliferative capacity in

normal and leukaemic haematopoietic stem cells (Lessard and Sauvageau, 2003) and inhibition of HDACs is also known to promote haematopoietic stem cell self-renewal (Young *et al.*, 2004; Bug *et al.*, 2005).

It is also known that in a number of haematological malignancies that arise in hHSCs, the functions of chromatin modifiers can become altered due to chromosomal rearrangements and translocations. Many chromosomal translocations associated with acute leukaemias disrupt genes either encoding histone modifying factors or express transcription factors that recruit histone-modifying complexes. Enzymes implicated include the histone methyltransferase MLL, histone acetyltransferases CBP/p300, MOZ, MOF, MORF and histone deacetylases (Linggi *et al.*, 2005). Therefore, modulations in the chromatin environment may be involved in modulating growth potential in the haematopoietic system in a similar way to that the regulation of chromatin environment can affect senescence and differentiation in many normal and tumour cell lines.

With regards to telomerase activity, it is known that hHSCs express telomerase only at a low level which is not enough to fully counteract telomere shortening with replication and age (Vaziri *et al.*, 1994; Broccoli *et al.*, 1995; Chiu *et al.*, 1996) but is enough to allow hHSCs to function properly throughout a normal lifespan (Engelhardt *et al.*, 1997). The telomere loss is represented by about 4kb shorter telomeres in the adult bone morrow compared to foetal liver hHSCs (Vaziri *et al.*, 1994). However, dysfunction of hHSCs has been observed during normal aging (Kamminga *et al.*, 2005b) and ex-vivo cultures of hHSCs undergo a replicative stress that causes accelerated senescence due to rapid telomere shortening (Piacibello *et al.*, 2005).

Chronic Myeloid Leukaemia (CML) is a clonal myeloproliferative disorder that arises in an hHSC. Leukaemic cells are characterised by the presence of the Philadelphia chromosome and the BCR-ABL oncogene (Sawyers, 1999) and the disease typically follows three distinct phases: chronic phase (CP) which lasts around 5 years, accelerated phase (AP) lasting 6-12 months and an aggressive blast phase (BP) which is invariably fatal. Proliferation of CML CD34+ hHSCs is increased compared to normal CD34+ hHSCs and this is thought to lead to shortening of telomere length observed in CML (Brummendorf *et al.*, 2000), with the rate and degree of telomere shortening offering some prognostic information regarding time to disease progression and correlates with the Hasford Risk score (Drummond *et al.*, 2004). In peripheral blood leukocytes, the rate of telomere loss in CP CML was 10 to 20 times the rate observed in normal granulocytes and in BP CML 30-60 additional divisions were observed (Drummond *et al.*, 2005). Telomere

shortening is linked to disease evolution in CML (Boultwood *et al.*, 1999) as extended amounts of telomere erosion can increase genetic instability leading to additional mutations and defects can accumulate. Telomere shortening in CML suggests that the telomerase genes expression may become dysregulated and links between accelerated telomere loss and dysregulation of the telomerase genes in the CML CD34+ hHSCs have recently been uncovered (Drummond *et al.*, 2005; Campbell *et al.*, 2006).

In the first study, telomerase activity was first observed to be increased when comparing CML CD34+ cells to normal CD34+ in the chronic phase of CML, but this was actually correlated to increased cell cycling within the CML CD34+ hHSCs. Expression of hTR and hTERT were analysed to understand whether dysregulation of either of these genes could be contributing to the enhanced telomere loss observed in CML CD34+ hHSCs. Although total amounts of hTERT expression did not change, a switch in splicing was observed with more full-length transcript being present at the expense of the β-deletion variant. In addition, hTR expression was observed to be decreased 5-fold in the BCR-ABL+ CD34+ hHSCs when compared to the normal CD34+ hHSCs. This dysregulation in hTR expression in CP CML CD34+ cells, in conjunction with increased cell cycle activity, may contribute to the rapid telomere loss seen in early stages of CML and during disease progression from CP through AP to BP. The second study also analysed expression of hTR and hTERT in CP CML and demonstrated that hTERT expression decreased in contradiction with the first study, but splicing still favoured the wild type hTERT transcript as was found in the first study (Campbell *et al.*, 2006) and also found that hTR levels were increased in CP CML, again in contradiction with the first study. This study suggests that reduction of hTERT expression is causally linked to the decrease in telomere length in the CP of CML, leading to genomic instability and disease progression.

Overall, studies suggest that dysregulation of telomerase gene expression may lead to telomere shortening and disease progression due to genomic instability observed in CML. Dysregulation of telomerase gene expression in CML CD34+ hHSCs provides an excellent opportunity to dissect regulatory pathways affecting telomerase gene expression between a normal stem cell population and a cancer stem cell population within the same cell type. To this end we studied hTR and hTERT expression in respect to any changes in the chromatin environment of the hTR and hTERT gene promoters in normal CD34+ hHSCs and CML CD34+ hHSCs establishing whether modulations in the chromatin environment surrounding the telomerase gene promoters can be linked to changes in transcription of hTR and hTERT.

5.2 Results

5.2.1 hTERT Gene Expression, Chromatin Remodelling and Basal Transcriptional Machinery Dynamics at the hTERT Promoter in the hMSC

Isolation of hMSCs, cell culture and treatment was undertaken by the laboratory of Dr Nedime Serakinci (University of Southern Denmark, Institute of Medical Biology, Department of Anatomy and Neurobiology, DK-5000 Odense C, Denmark). hTERT expression was studied before and after treatment with TSA and histone acetylation at the promoter was also analysed by ChIP assays in order to link changes in the chromatin environment with any changes in gene expression.

In control (untreated) hMSCs, there was a lack of expression of any hTERT transcripts (Figure 5.1). Acetylation of H3 was not detected and acetylation of H4 was low (Figure 5.2), linking the lack of expression of hTERT with low levels of promoter proximal histone acetylation. However, upon treatment with TSA, hMSCs showed re-expression of full-length hTERT transcripts (Figure 5.1) and this was associated with increased levels of acetylated H3 and acetylated H4 (Figure 5.2), linking remodelling of the chromatin environment of the hTERT promoter with re-expression of hTERT. However, although increases are observed for both acetylated H3 and acetylated H4, only the increase in acetylated H4 was statistically significant.

As well as monitoring acetylation, RNA polymerase II and TFIIB occupancy at the hTERT promoter and exon 12 of hTERT were studied to investigate the trafficking of the basal transcription machinery along the hTERT gene. Exon 12 is located some 31kb downstream from the promoter and therefore can give an insight into the extent of basal transcription machinery tracking along the gene coding sequence. RNA polymerase II levels were low at the hTERT promoter before treatment with TSA (Figure 5.3A) perhaps signifying that any RNA polymerase II that was present at the promoter was not engaged in transcription. The decreased levels of RNA polymerase II observed at the hTERT promoter after treatment might be explained by increased movement of RNA polymerase II from the promoter onto the coding sequence of the gene (Figure 5.3A). Levels of RNA polymerase

Figure 5.1 hTERT Splice Variant Expression in hMSC

hTERT spice variant expression analysis of hMSC before and after treatment with TSA. hMSCs were treated with 350 μM TSA for 17hrs, while the control hMSC were treated with 100% Ethanol, after which RNA was extracted. hTERT expression levels were established, first by analysing GAPDH levels from cDNA made from 1μg RNA. cDNA amounts used as Input in each separate reaction were then adjusted for the hTERT splice variant PCR reaction so similar concentrations of cDNA were used in each PCR. Splice Variant expression was then visualised using the Agilent Bioanalyser (Agilent Technologies). Lane 1 shows size markers, lane 2, the negative control for the PCR reaction, lane 3, hTERT expression for the hMSC, lane 4, hTERT expression for the hMSC treated with TSA, and lane 5, hTERT expression in the MCF7 cell line as comparison. Splice variants are labelled on the right side of the gel, while the size of these products are shown on the left (in bp). The results are shown from a single experiment.

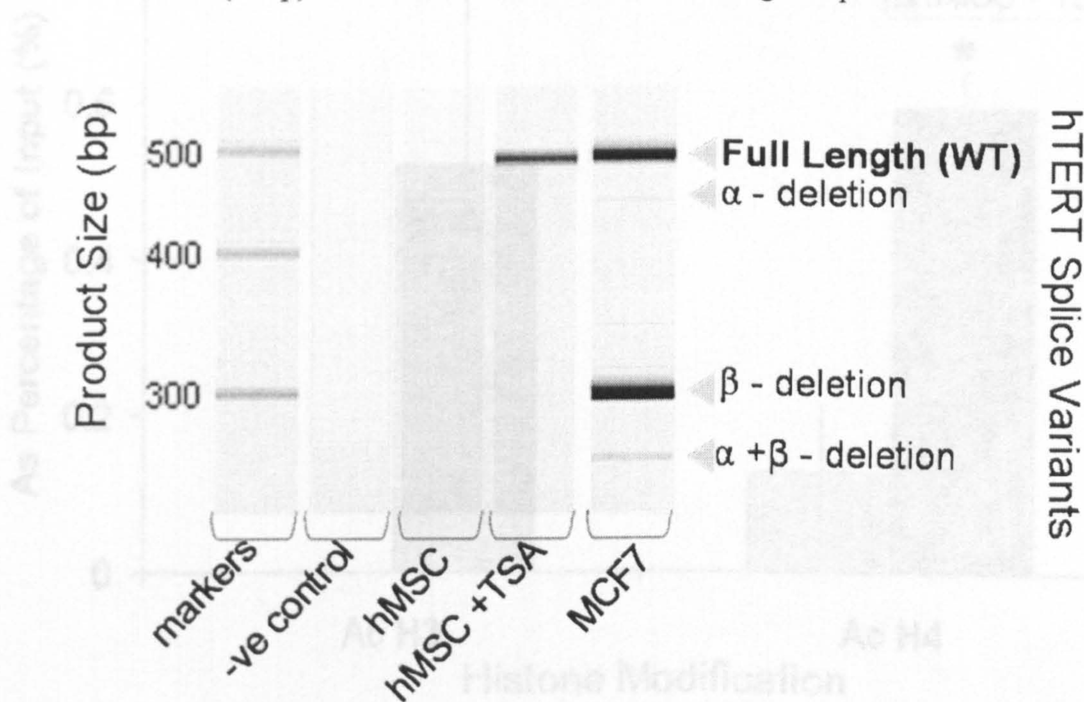


Figure 5.2 Acetylation Status of the hTERT Promoter in hMSCs

Q-PCR results of the ChIP using antibodies against Acetylated H3 and H4, analysed by Q-PCR using primers which detect the hTERT core promoter sequence before and after treatment with TSA (350 μM TSA for 17hrs, while the control hMSC were treated with 100% Ethanol). Results are expressed as a percentage of an Input sample, which is a 20% fraction of chromatin utilised for each IP experiment, and so normalises the data. Each IP is also corrected for a control IP experiment, done in parallel with other IP's, in which no antibody was added to control for any background (IP-). Means and standard errors are from triplication within a Q-PCR reaction from a single experiment. Asterisks denote a statistically significant difference between untreated and treated (ANOVA): $*p < 0.05$.

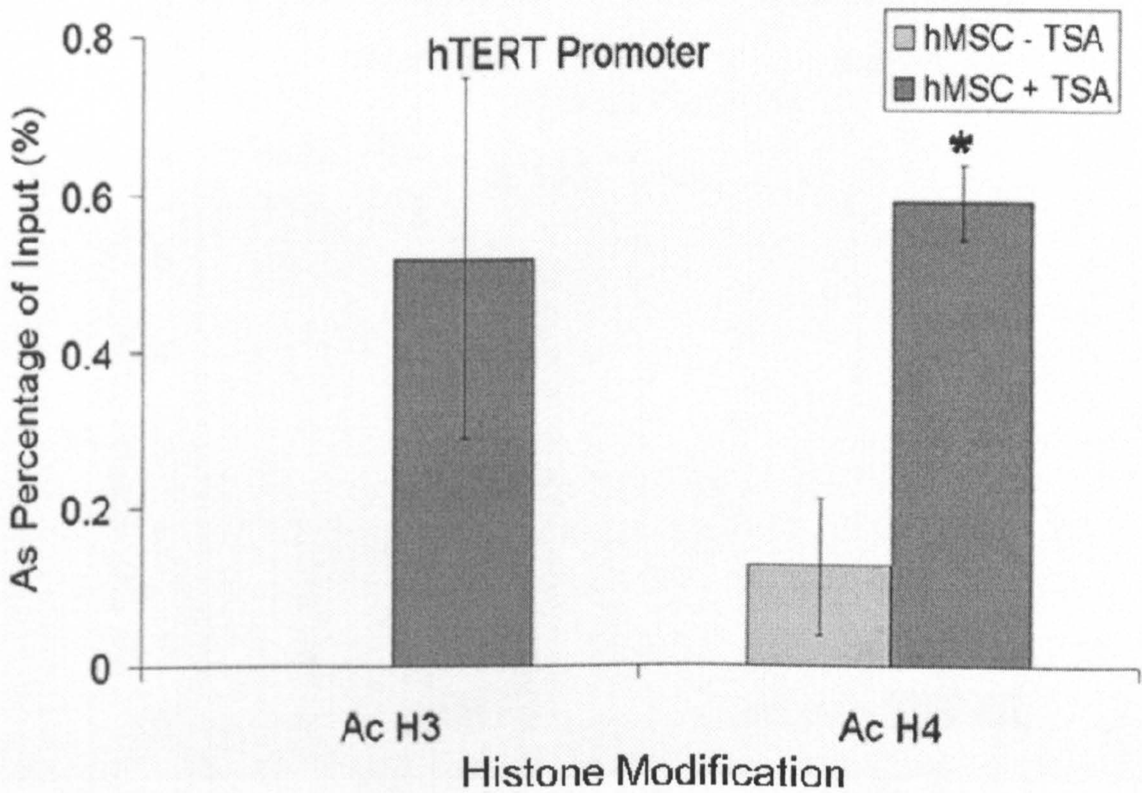
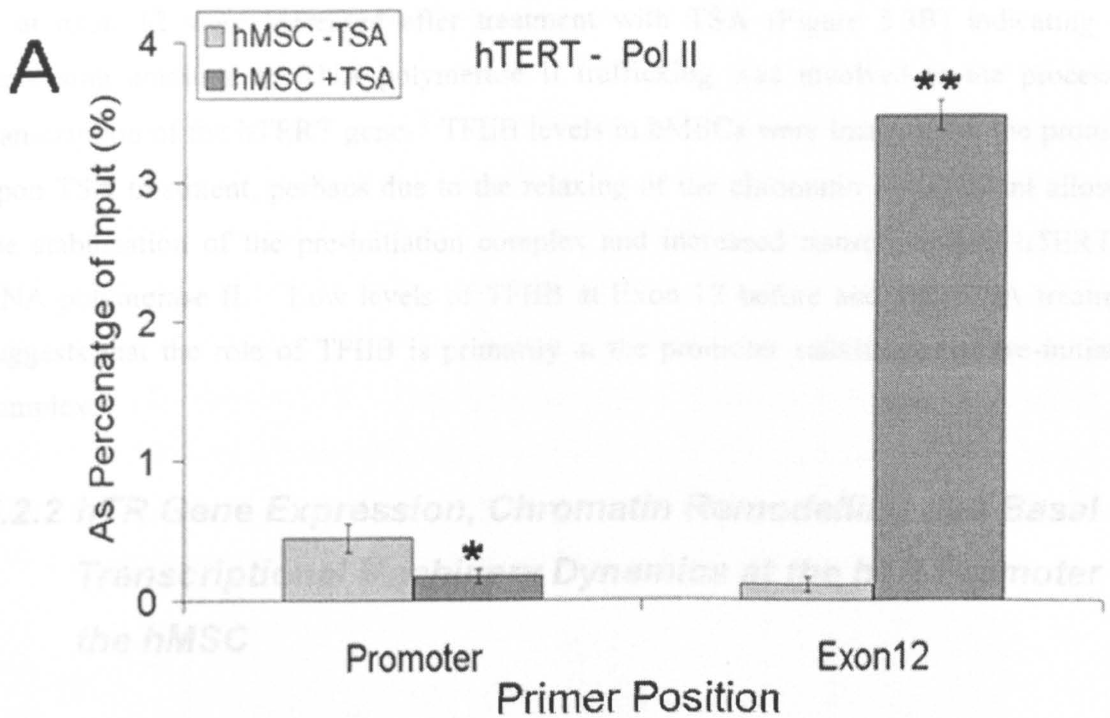
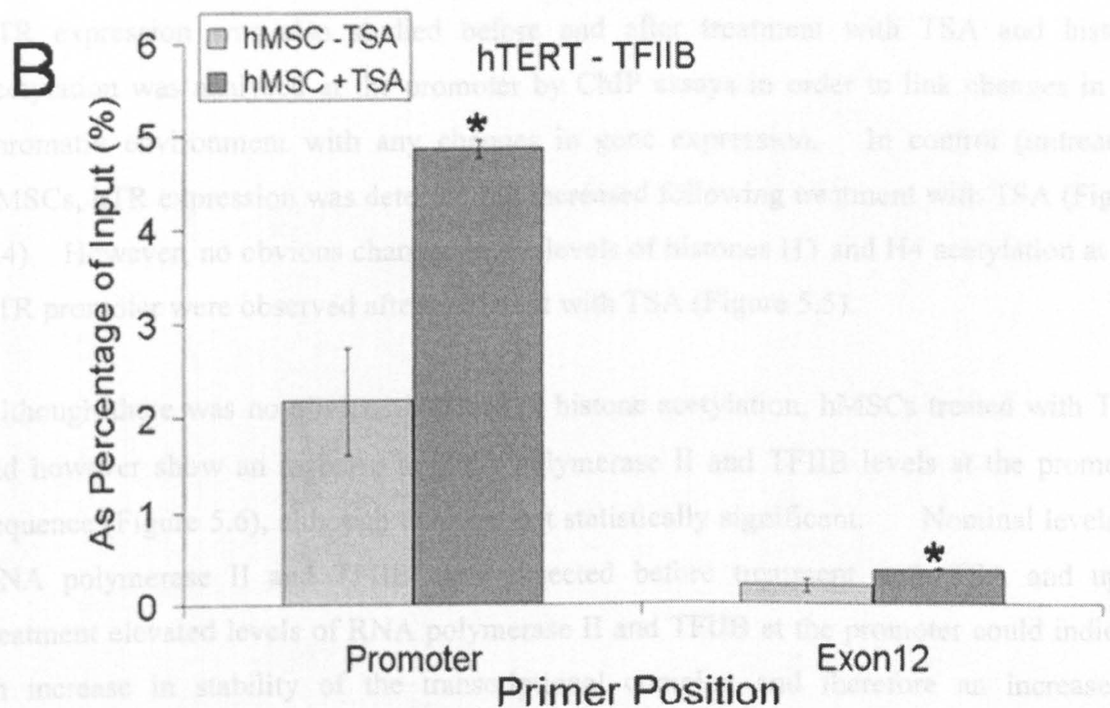


Figure 5.3 RNA polymerase II and TFIIB Tracking on hTERT Promoter and Exon 12 in the hMSC

ChIP assays were performed for **A,** RNA Pol II occupancy and **B,** TFIIB occupancy of the promoter and exon 12 of hTERT before and after treatment of hMSCs with TSA (350 μM TSA for 17hrs, while the control hMSC were treated with 100% Ethanol). Results are expressed as a percentage of an Input sample, which is a 20% fraction of chromatin utilised for each IP experiment, and so normalises the data. Each IP is also corrected for a control IP experiment, done in parallel with other IP's, in which no antibody was added to control for any background (IP-). Means and standard errors are from triplication within a Q-PCR reaction from a single experiment. Asterisks denote a statistically significant difference between untreated and treated (ANOVA): $*p < 0.05$; $**p < 0.01$.


A
hMSC -TSA
hMSC +TSA
hTERT - Pol II
As Percenatge of Input (%)
4
3
2
1
0
**
*
Promoter
Exon12
Primer Position


B
hMSC -TSA
hMSC +TSA
hTERT - TFIIB
As Percentage of Input (%)
6
5
4
3
2
1
0
*
*
Promoter
Exon12
Primer Position

II at exon 12 were increased after treatment with TSA (Figure 5.3B) indicating that increasing amounts of RNA polymerase II trafficking was involved in the process of transcription of the hTERT gene. TFIIB levels in hMSCs were increased at the promoter upon TSA treatment, perhaps due to the relaxing of the chromatin environment allowing the stabilisation of the pre-initiation complex and increased transcription of hTERT by RNA polymerase II. Low levels of TFIIB at Exon 12 before and after TSA treatment suggests that the role of TFIIB is primarily at the promoter stabilising the pre-initiation complex.

5.2.2 hTR Gene Expression, Chromatin Remodelling and Basal Transcriptional Machinery Dynamics at the hTR Promoter in the hMSC

hTR expression was also studied before and after treatment with TSA and histone acetylation was analysed at the promoter by ChIP assays in order to link changes in the chromatin environment with any changes in gene expression. In control (untreated) hMSCs, hTR expression was detected but increased following treatment with TSA (Figure 5.4). However, no obvious changes in the levels of histones H3 and H4 acetylation at the hTR promoter were observed after treatment with TSA (Figure 5.5).

Although there was no obvious increase in histone acetylation, hMSCs treated with TSA did however show an increase in RNA polymerase II and TFIIB levels at the promoter sequence (Figure 5.6), although this was not statistically significant. Nominal levels of RNA polymerase II and TFIIB were detected before treatment with TSA and upon treatment elevated levels of RNA polymerase II and TFIIB at the promoter could indicate an increase in stability of the transcriptional complex and therefore an increase in transcription levels of hTR that would account for the increase in expression. As the hTR gene is relatively short, extended analysis of RNA polymerase II and TFIIB trafficking was not possible.

5.2.3 hTERT Expression, Chromatin Remodelling and Basal Transcriptional Machinery Dynamics at the hTERT Promoter in CD34+ hHSCs

All hHSCs were provided from the laboratory of Dr Tessa Holyoake (Section of Experimental Haematology and Haematopoietic Stem Cells, Division of Cancer Sciences

Figure 5.4 hTR Expression in hMSCs

hTR expression analysis of hMSC before and after treatment with TSA. hMSCs were treated with 350 μM TSA for 17hrs, while the control hMSC were treated with 100% Ethanol, after which RNA was extracted. RNA was extracted from each cell line and cDNA synthesised using 1ug RNA. hTR levels were detected using specific primers by Quantitative PCR (Q-PCR) using specific primers to the hTR coding sequence. hTR levels were normalised to the expression of GAPDH levels established using the same amount of cDNA as for hTR. Therefore, levels of hTR are expressed as being relative to GAPDH expression. Means and errors are established from triplication within the Q-PCR experiment from a single experiment. Asterisks denote a statistically significant difference between untreated and treated (ANOVA): $*p < 0.05$.

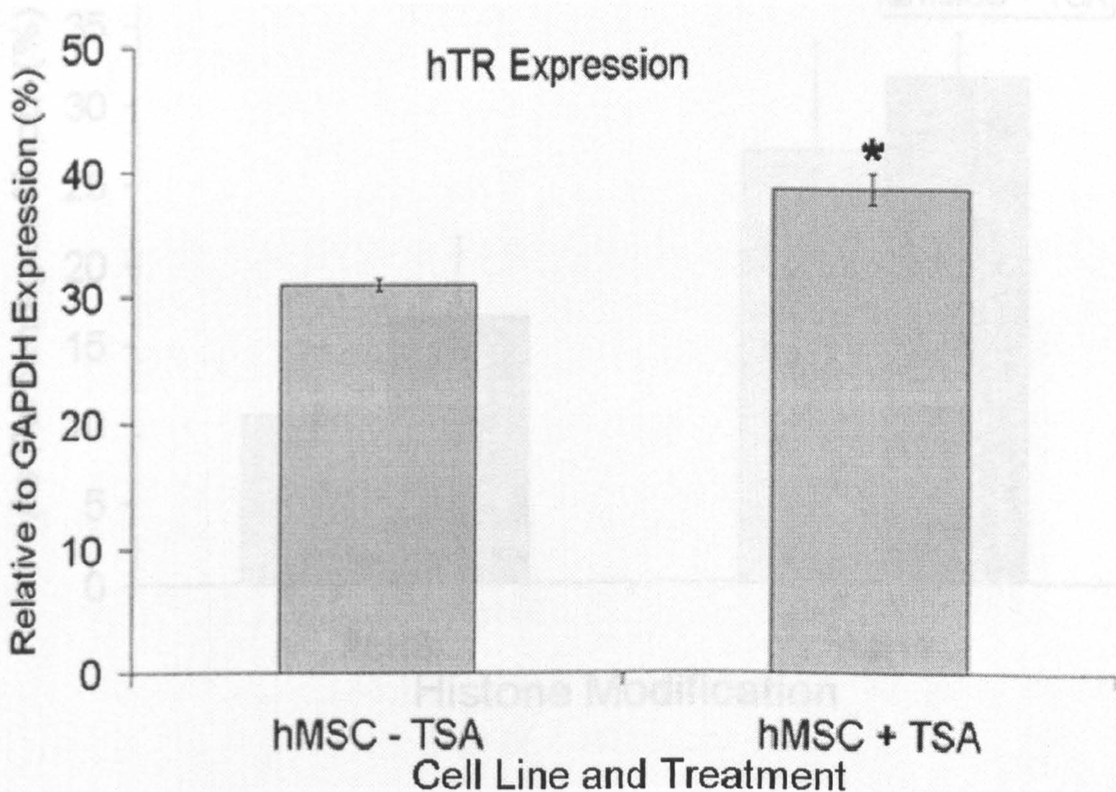


Figure 5.5 Acetylation Status of the hTR Promoter in hMSCs

Q-PCR results of the ChIP using antibodies against Acetylated H3 and H4, analysed by Q-PCR using primers which detect the hTR core promoter sequence before and after treatment with TSA (350 μM TSA for 17hrs, while the control hMSC were treated with 100% Ethanol). Results are expressed as a percentage of an Input sample, which is a 20% fraction of chromatin utilised for each IP experiment, and so normalises the data. Each IP is also corrected for a control IP experiment, done in parallel with other IP's, in which no antibody was added to control for any background (IP-). Means and standard errors are from triplication within a Q-PCR reaction from a single experiment.

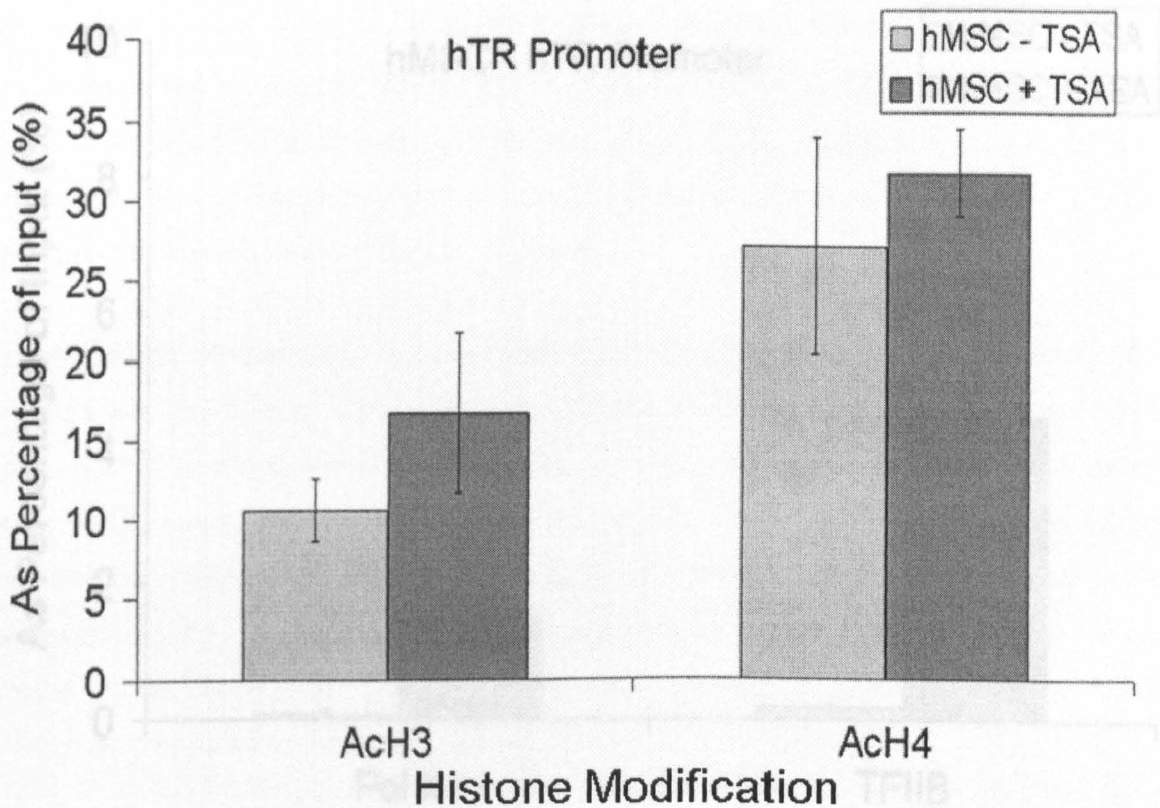
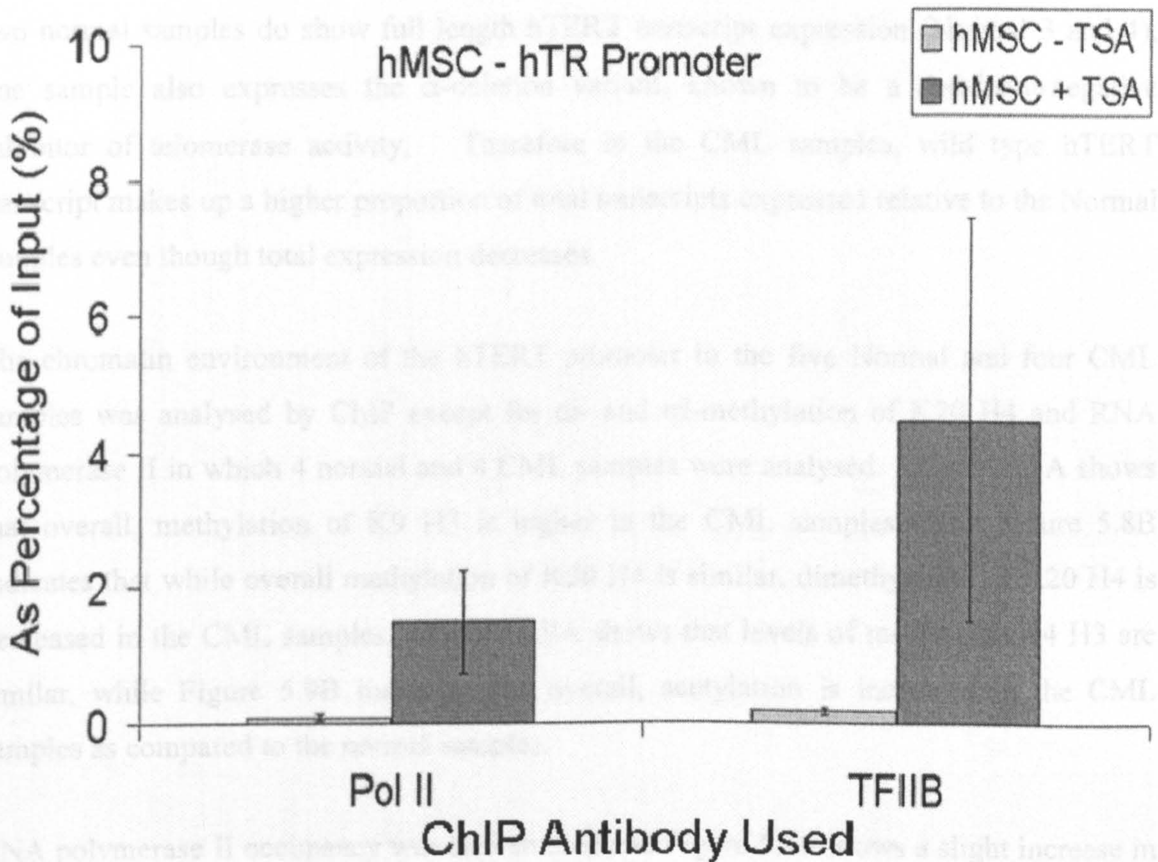


Figure 5.6 RNA polymerase II and TFIIB Occupancy of hTR Promoter

ChIP assays were performed for RNA Pol II occupancy and TFIIB occupancy of the promoter hTR before and after treatment of hMSCs with TSA (350 μM TSA for 17hrs, while the control hMSC were treated with 100% Ethanol). Results are expressed as a percentage of an Input sample, which is a 20% fraction of chromatin utilised for each IP experiment, and so normalises the data. Each IP is also corrected for a control IP experiment, done in parallel with other IP's, in which no antibody was added to control for any background (IP-). Means and standard errors are from triplication within a Q-PCR reaction from a single experiment.



and Molecular Pathology, University of Glasgow, Glasgow, Scotland, United Kingdom). hTERT expression was analysed in relation to the chromatin environment of the promoter in normal and CML CD34+ hHSCs. Figure 5.7A shows total hTERT expression, adjusted to levels of S15 expression, from five normal and four CML CD34+ hHSCs. hTERT expression is decreased in the CML samples as compared to the normal samples, but when splice variant expression is studied (non-normalised), it is observed that there tends to be more full-length active hTERT transcript in the CML samples as compared to the normal samples as a proportion of total expression. Full length hTERT transcript is expressed in each CML sample, but hTERT expression is totally absent in one Normal sample (Normal 5), two Normal samples show no full length hTERT transcript (Normal 1 and 2) and while two normal samples do show full length hTERT transcript expression (Normal 3 and 4), one sample also expresses the α-deletion variant, known to be a dominant-negative inhibitor of telomerase activity. Therefore in the CML samples, wild type hTERT transcript makes up a higher proportion of total transcripts expressed relative to the Normal samples even though total expression decreases.

The chromatin environment of the hTERT promoter in the five Normal and four CML samples was analysed by ChIP except for di- and tri-methylation of K20 H4 and RNA polymerase II in which 4 normal and 4 CML samples were analysed. Figure 5.8A shows that overall, methylation of K9 H3 is higher in the CML samples while Figure 5.8B indicates that while overall methylation of K20 H4 is similar, dimethylation of K20 H4 is decreased in the CML samples. Figure 5.9A shows that levels of methylated K4 H3 are similar, while Figure 5.9B indicates that overall, acetylation is increased in the CML samples as compared to the normal samples.

RNA polymerase II occupancy was also studied and Figure 5.10 shows a slight increase in RNA polymerase II tracking for hTERT in the CML samples signified by increases in levels of RNA polymerase II at the promoter at exon 12. However, this results may be distorted by CML sample four having large amounts of RNA polymerase II at the promoter, unlike other samples. Overall the data indicates an increase in K9 H3 methylation, a decrease in dimethylation of K20 H4 and an increase in acetylation of H3 and H4, coupled to increased RNA polymerase II tracking.

Figure 5.7 Expression of hTERT in Normal and CML CD34+ Haematopoietic Stem Cells

RNA was extracted from each sample and cDNA was synthesised. Q-PCR using specific primers for S15 expression was used to normalise the amount of cDNA used for hTERT splice variant PCR which was then quantified on the Agilent Bioanalyser. **A**, Total amounts of splice variant expression were totalled and plotted and show expression levels of hTERT in 5 normal and 4 CML CD34+ hHSCs samples. Means and errors are established from triplication of the Q-PCR reaction within Q-PCR for each sample. **B**, Un-normalised splice variant expression of hTERT showing the main splice variants. For each sample, each splice variant (WT, α,β and αβ) is expressed as a percentage of the samples total hTERT splice variant expression level.

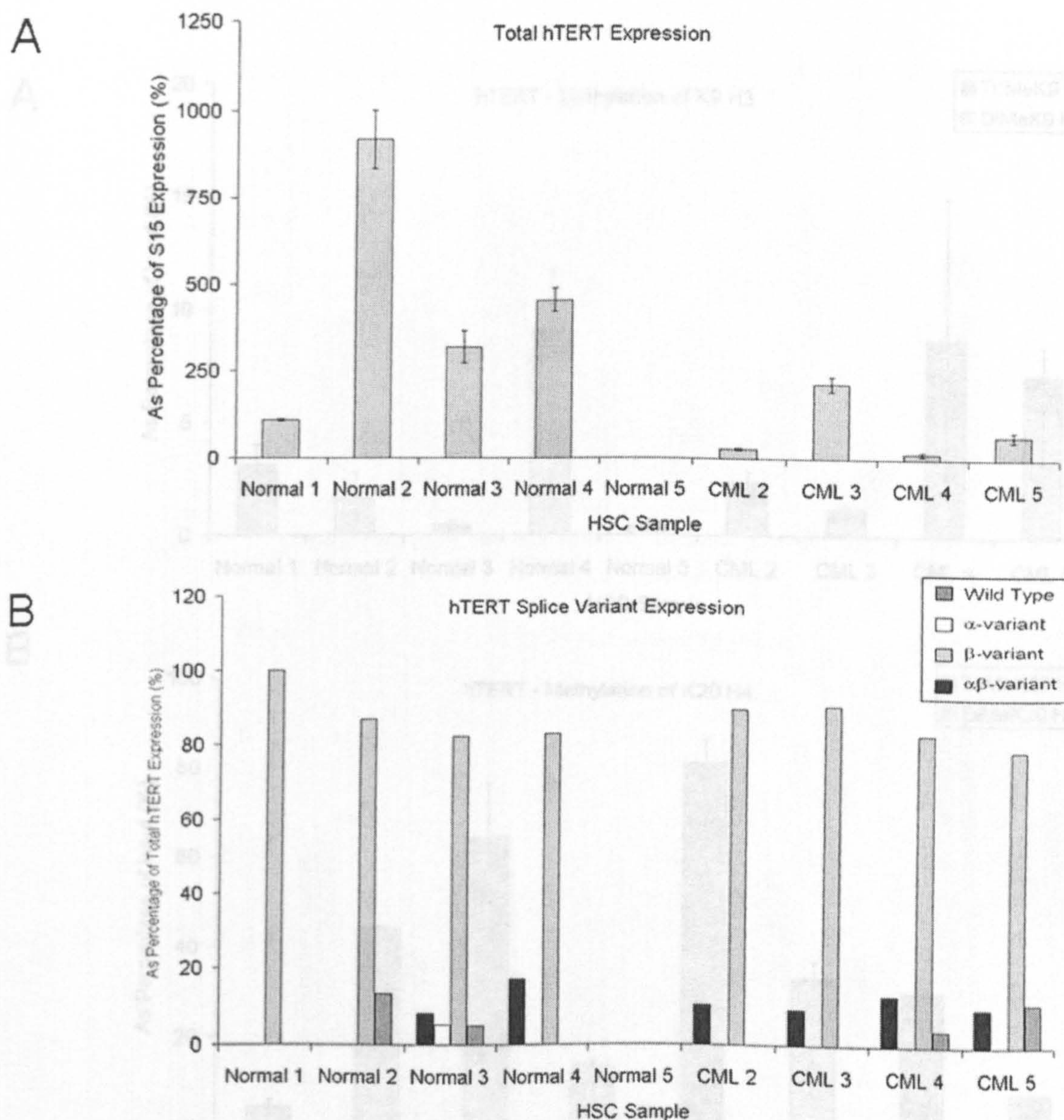


Figure 5.8 Repressive Modifications at the hTERT Promoter in Normal and CML CD34+ hHSCs

Levels of **A** Di- and tri-methylated K9 H3 from 5 normal and 4 CML CD34+ hHSCs and **B** di- and tri-methylated K20 H4 from 4 normal and 4 CML CD34+ hHSCs present at the hTERT promoter detected by Q-PCR using primers which detect the hTERT core promoter sequence, from products of chromatin immunoprecipitation assays. Results are expressed as the total IP amount for both the antibodies and are expressed as a percentage of an Input sample, which is a 20% fraction of chromatin utilised for each IP experiment, and so normalises the data. Each IP is also corrected for a control IP experiment, done in parallel with other IP's, in which no antibody was added to control for any background. Means and standard errors are from triplication within the Q-PCR reactions for each sample.

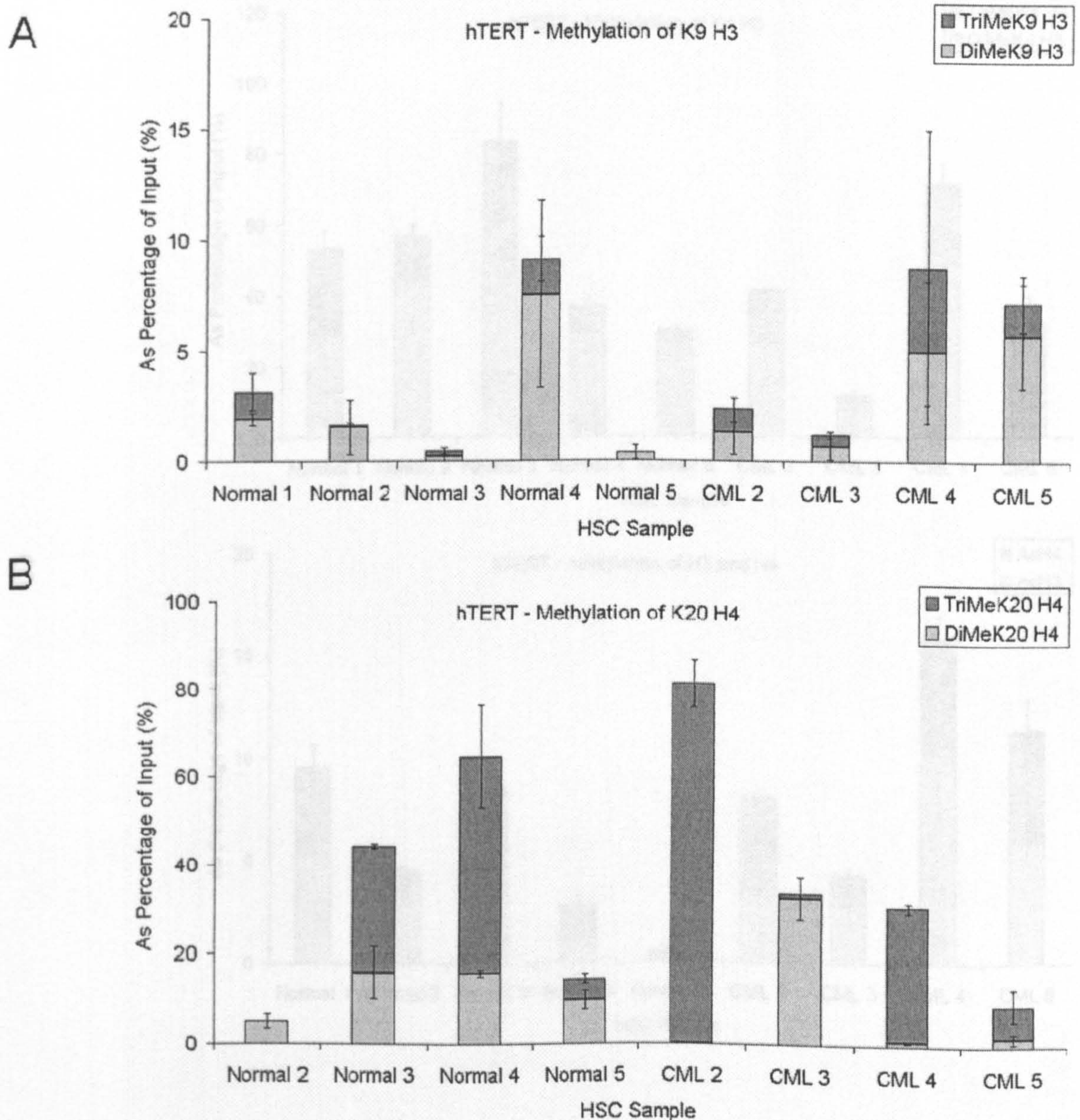


Figure 5.9 Permissive Modifications at the hTERT Promoter in Normal and CML CD34+ hHSCs

Levels of **A** Di- and Tri-methylated K4 H3 and **B** Acetylated H3 and H4 for 5 normal and 4 CML CD34+ hHSCs present at the hTERT promoter detected by Q-PCR using primers which detect the hTERT core promoter sequence, from products of chromatin immunoprecipitation assays. Results are expressed as the total IP amount for both the antibodies and are expressed as a percentage of an Input sample, which is a 20% fraction of chromatin utilised for each IP experiment, and so normalises the data. Each IP is also corrected for a control IP experiment, done in parallel with other IP's, in which no antibody was added to control for any background. Means and standard errors are from triplication within the Q-PCR reactions for each sample.

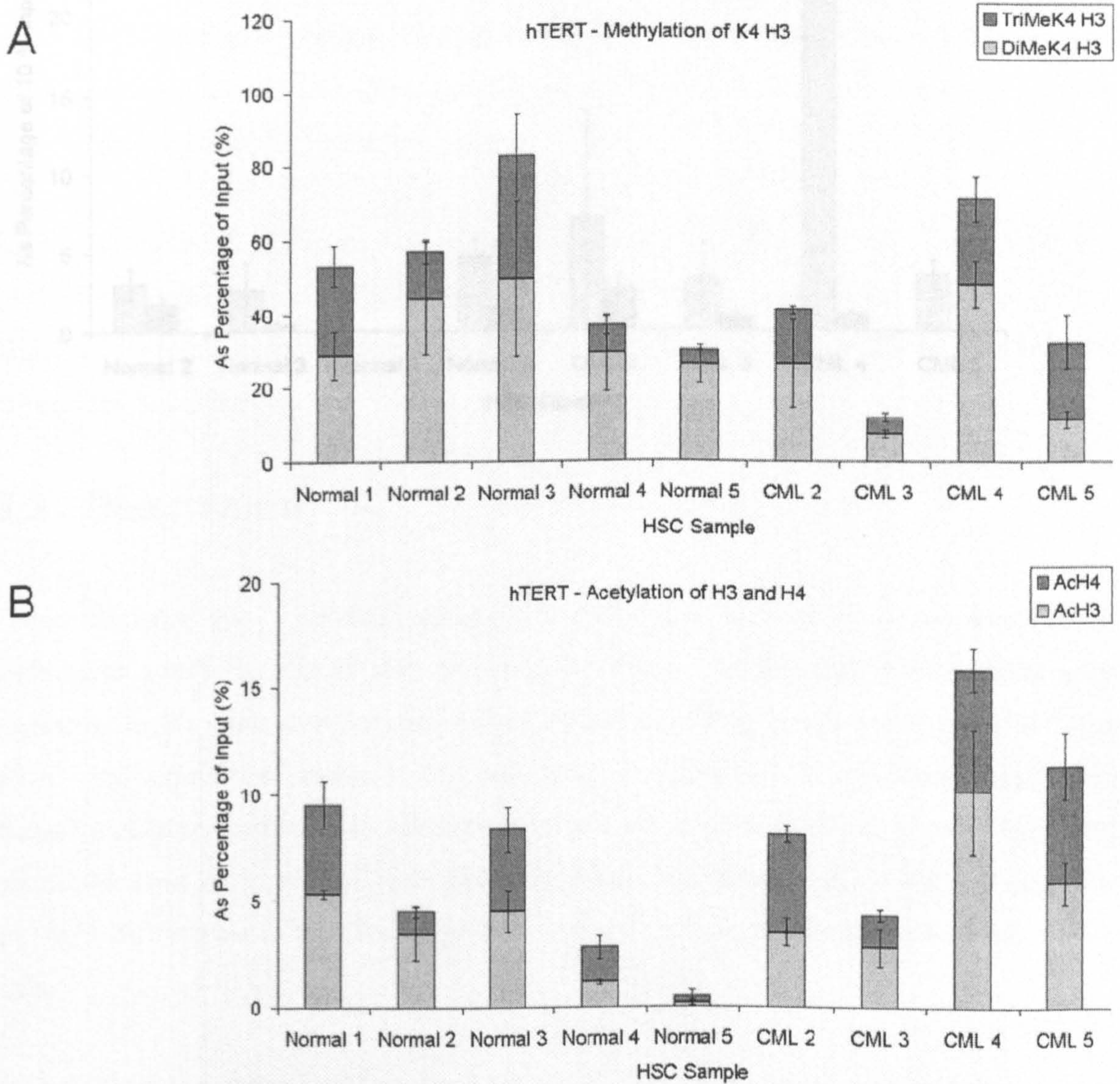
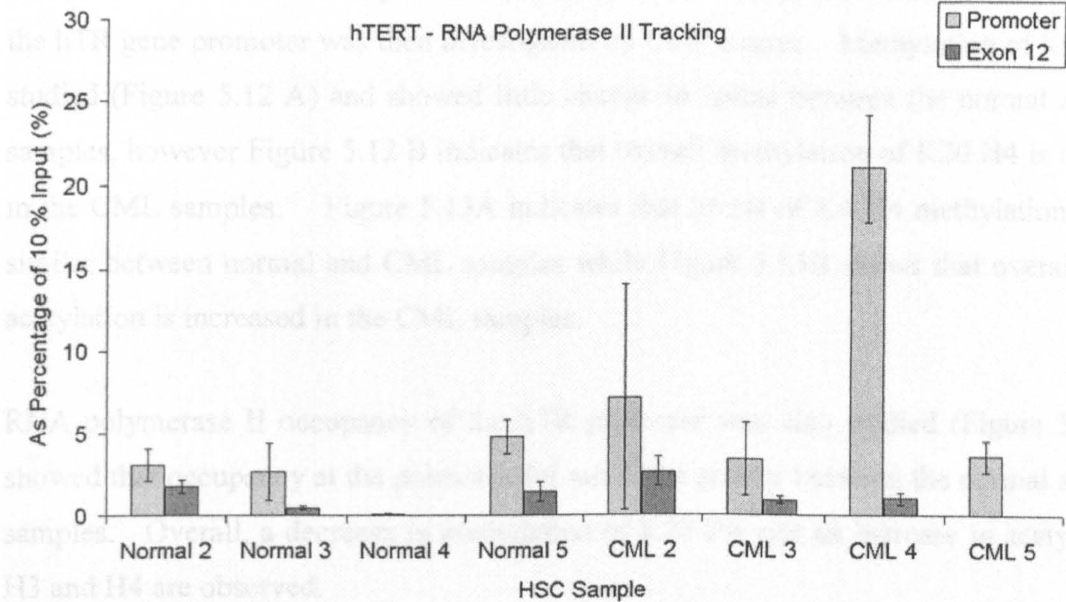


Figure 5.10 RNA Pol II Tracking at hTERT in Normal and CML CD34+ hHSCs

Q-PCR results of the ChIP for RNA Pol II detected by Q-PCR using primers which detect the hTERT core promoter sequence and Exon 12 of hTERT in 4 normal and 4 CML CD34+ hHSCs. Results are expressed as a percentage of a 10% Input sample, with the Input fraction being a 20% fraction of chromatin utilised for each IP experiment, and so normalises the data. Each IP is also corrected for a control IP experiment, done in parallel with other IP's, in which no antibody was added to control for any background. Means and standard errors are from triplication within the Q-PCR reactions for each sample.



5.2.4 hTR Expression, Chromatin Remodelling and Basal Transcriptional Machinery Dynamics at the hTR Promoter in CD34+ hHSCs

As for hTERT, hTR expression, as normalised to S15 expression, was studied in relation to the chromatin environment of the promoter. Figure 5.11 shows the hTR expression values for five normal and four CML CD34+ hHSC samples are decreased in the CML samples as compared to the normal samples. As for hTERT, the chromatin environment surrounding the hTR gene promoter was then investigated by ChIP assays. Methylation of K9 H3 was studied (Figure 5.12 A) and showed little change in levels between the normal and CML samples, however Figure 5.12 B indicates that overall methylation of K20 H4 is decreased in the CML samples. Figure 5.13A indicates that levels of K4 H4 methylation are very similar between normal and CML samples while Figure 5.13B shows that overall histone acetylation is increased in the CML samples.

RNA polymerase II occupancy of the hTR promoter was also studied (Figure 5.14) and showed that occupancy at the promoter did not differ greatly between the normal and CML samples. Overall, a decrease in methylation of K20 H4 and an increase in acetylation of H3 and H4 are observed.

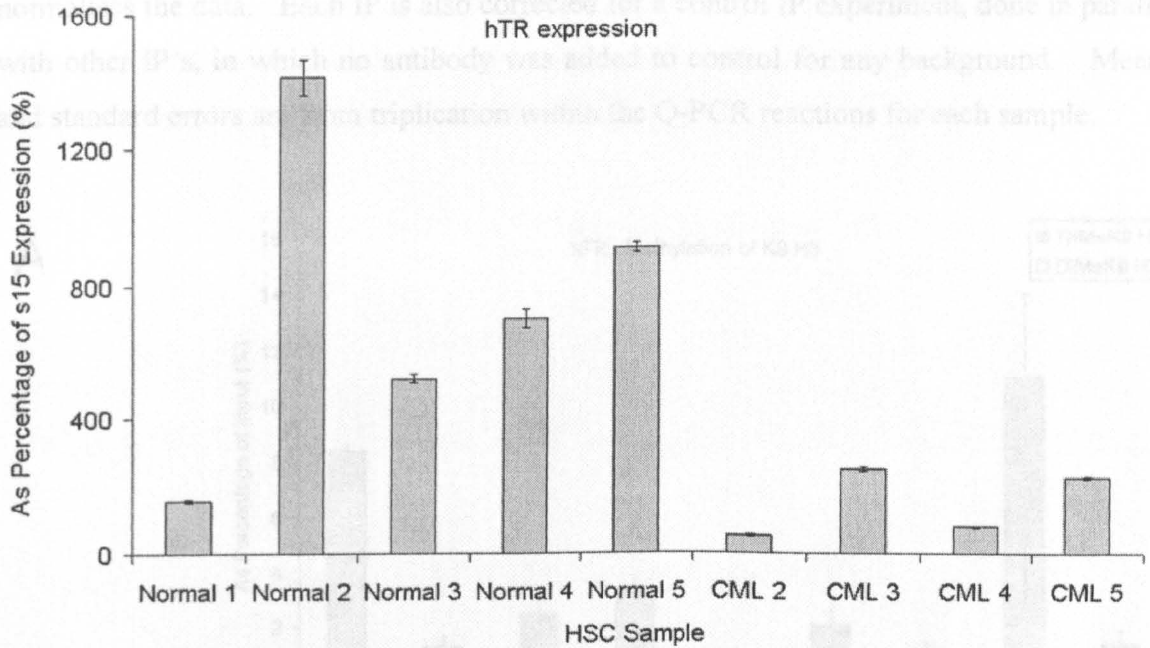
5.3 Discussion

These findings give essential information about the regulation of the endogenous telomerase genes in normal and cancer stem cells. Understanding telomerase gene regulation in the context of the chromatin environment of the promoters in the hMSC and hHSC, and similar cell types, is of importance as differences in regulation may reflect distinct regulatory pathways for telomerase gene expression in these cell types, which need not be the same as in normal and cancer cell lines (Anderson *et al.*, 2006) and may also highlight differences in telomerase gene regulation between different populations of stem cells.

This study shows that hTERT expression is repressed in the hMSC and that the mode of repression is chromatin based. Upon TSA treatment of hMSCs, hTERT becomes re-expressed concomitant with an increase in promoter histone acetylation. Beside hTERT expression, hTR expression is also increased upon TSA treatment although large changes

Figure 5.11 Expression of hTR in Normal and CML CD34+ hHSCs

RNA was extracted from each sample and cDNA was synthesised from 5 normal and 4 CML CD34+ hHSCs. Q-PCR using specific primers for S15 expression and used to normalise data established for hTR expression, using hTR specific primers in Q-PCR, established using the same amount of cDNA. Therefore, levels of hTR are expressed as being relative to S15 expression. Means and standard errors are from triplication within the Q-PCR reactions for each sample.



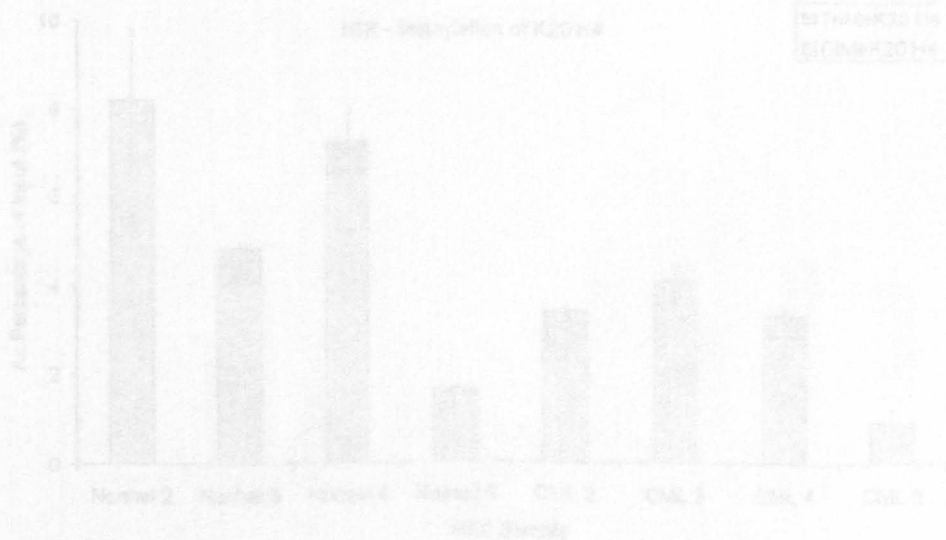
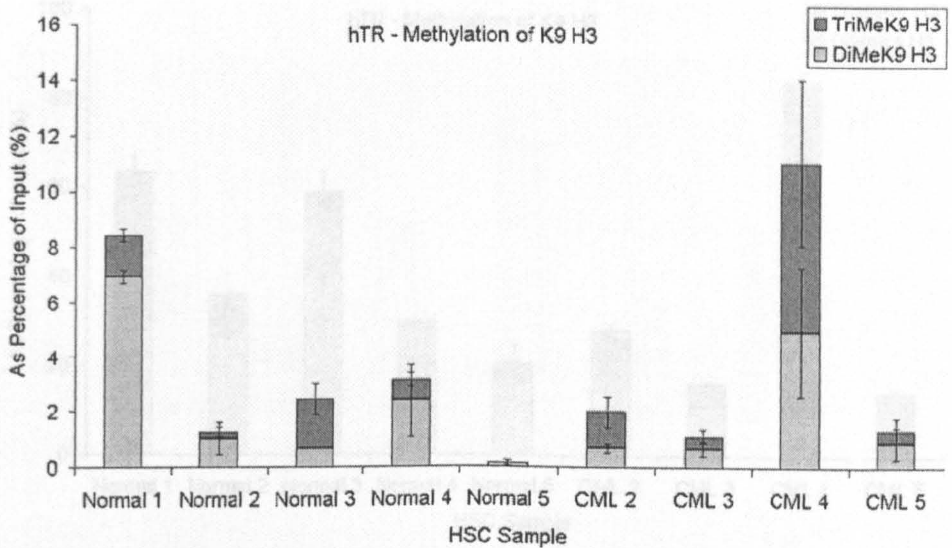


Figure 5.12 Repressive Modifications at the hTR Promoter in Normal and CML CD34+ hHSCs

Levels of **A** Di- and tri-methylated K9 H3 from 5 normal and 4 CML CD34+ hHSCs and **B** di- and tri-methylated K20 H4 from 4 normal and 4 CML CD34+ hHSCs present at the hTR promoter detected by Q-PCR using primers which detect the hTR core promoter sequence, from products of chromatin immunoprecipitation assays. Results are expressed as the total IP amount for both the antibodies and are expressed as a percentage of an Input sample, which is a 20% fraction of chromatin utilised for each IP experiment, and so normalises the data. Each IP is also corrected for a control IP experiment, done in parallel with other IP's, in which no antibody was added to control for any background. Means and standard errors are from triplication within the Q-PCR reactions for each sample.

A



B

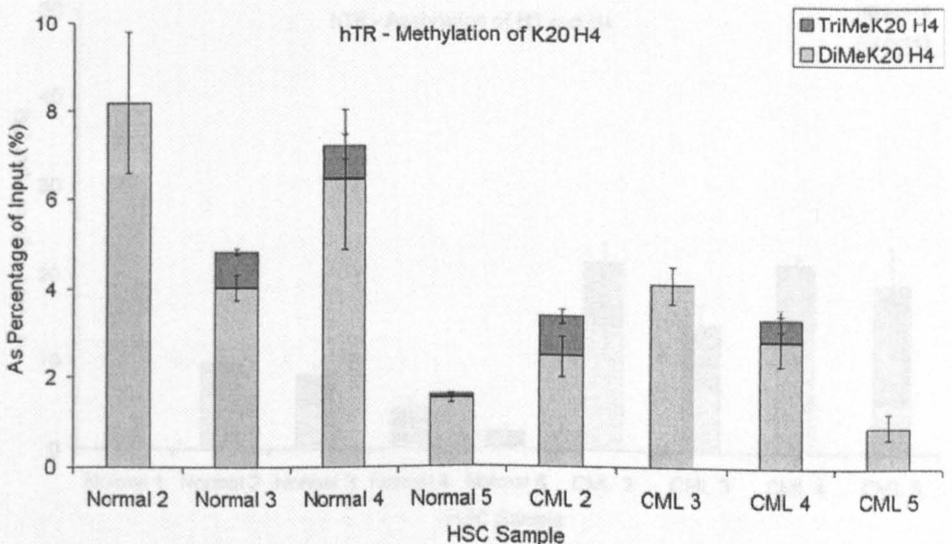


Figure 5.13 Permissive Modifications at the hTR Promoter in Normal and CML CD34+ hHSCs

Levels of **A** Di- and Tri-methylated K4 H3 and **B** Acetylated H3 and H4 for 5 normal and 4 CML CD34+ hHSCs present at the hTR promoter detected by Q-PCR using primers which detect the hTR core promoter sequence, from products of chromatin immunoprecipitation assays. Results are expressed as the total IP amount for both the antibodies and are expressed as a percentage of an Input sample, which is a 20% fraction of chromatin utilised for each IP experiment, and so normalises the data. Each IP is also corrected for a control IP experiment, done in parallel with other IP's, in which no antibody was added to control for any background. Means and standard errors are from triplication within the Q-PCR reactions for each sample.

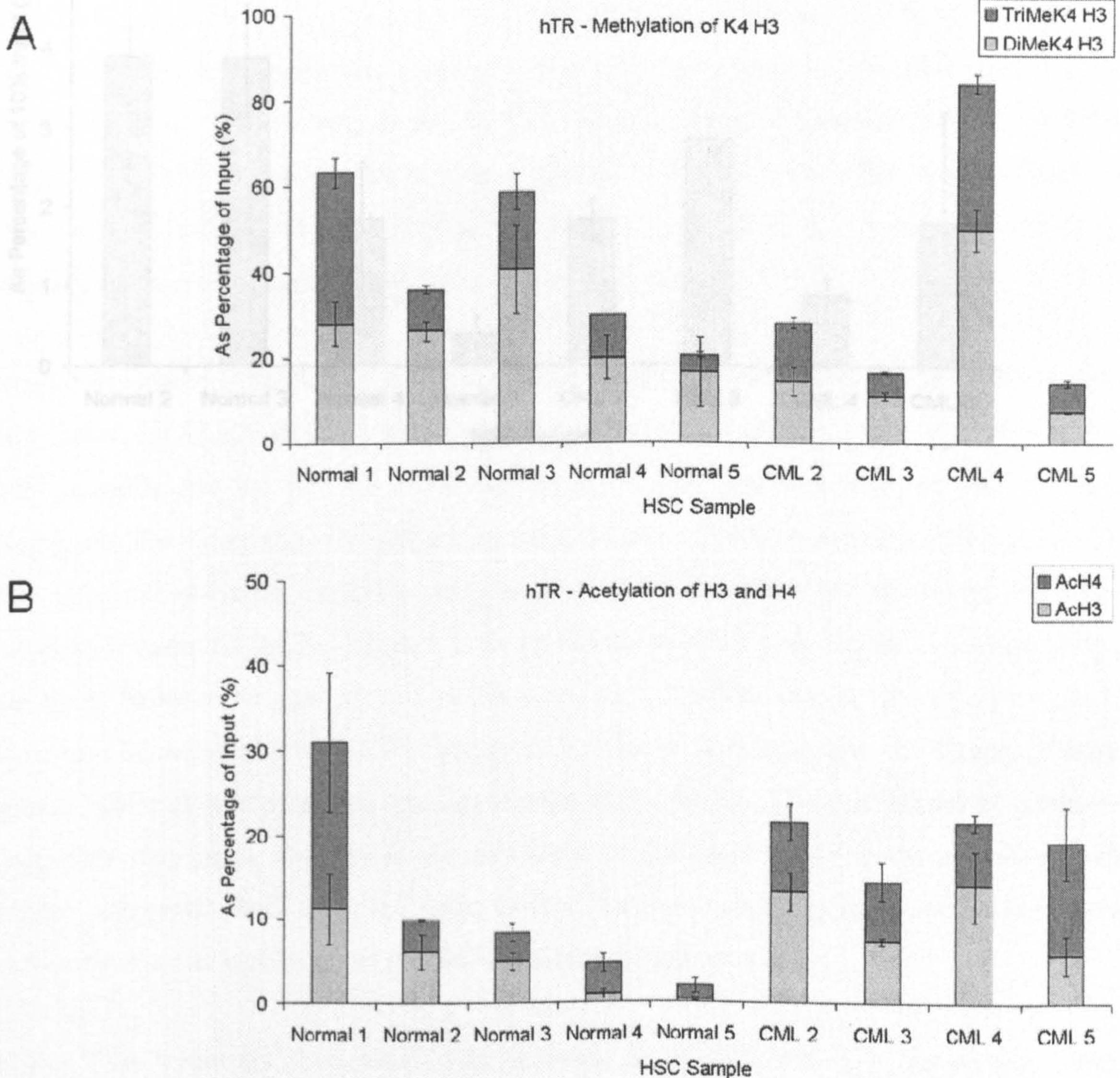
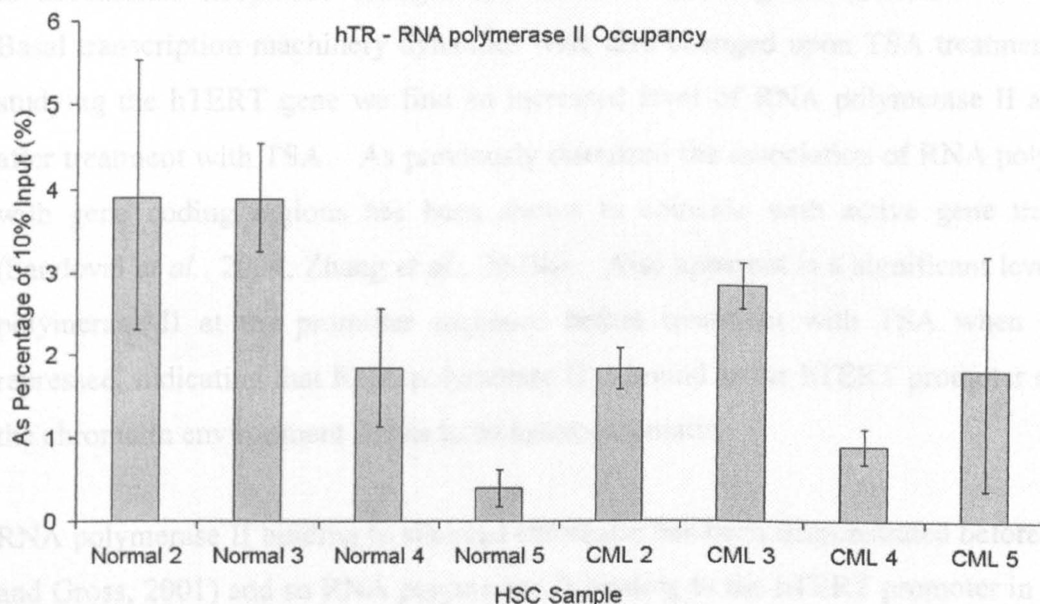


Figure 5.14 RNA Pol II occupancy of the hTR Promoter Normal and CML CD34+ hHSCs

Q-PCR results of the ChIP for RNA Pol II detected by Q-PCR using primers which detect the hTR core promoter sequence in 4 normal and 4 CML CD34+ hHSCs. Results are expressed as a percentage of a 10% Input sample, with the Input fraction being a 20% fraction of chromatin utilised for each IP experiment, and so normalises the data. Each IP is also corrected for a control IP experiment, done in parallel with other IP's, in which no antibody was added to control for any background. Means and standard errors are from triplication within the Q-PCR reactions for each sample.



in histone acetylation are not apparent. This suggests that chromatin and epigenetic mechanisms are involved in telomerase gene regulation in the hMSC and it has been previously suggested that dysregulation of epigenetic mechanisms in this stem cell type could alter the cells neoplastic potential (Serakinci *et al.*, 2004). Therefore, dysregulation of chromatin modifications in the hMSC could lead to the accumulation of neoplastic changes leading to neoplastic transformation. The chromatin based repression of hTERT transcription and hence repression of telomerase activity may act as a tumour suppressor

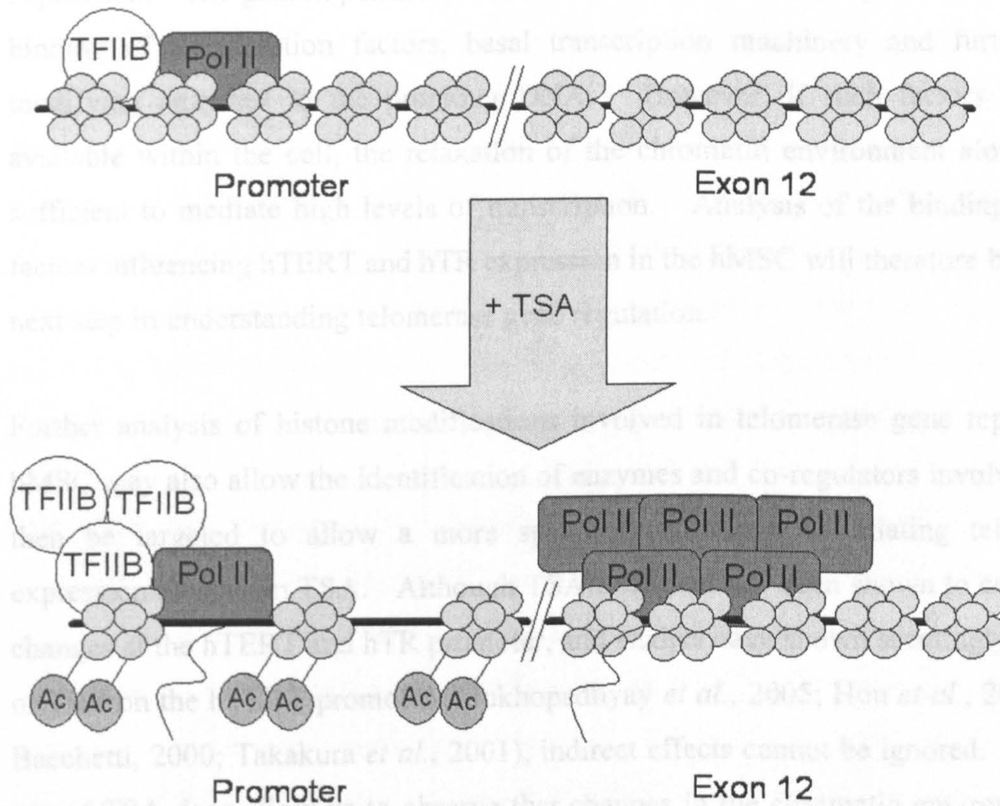
mechanism, whereby hMSCs would have a limited lifespan and cannot divide long enough to accumulate neoplastic changes and become tumourigenic (Serakinci *et al.*, 2004). Basal transcription machinery dynamics were also changed upon TSA treatment as when studying the hTERT gene we find an increased level of RNA polymerase II at Exon 12 after treatment with TSA. As previously discussed the association of RNA polymerase II with gene coding regions has been shown to coincide with active gene transcription (Sandoval *et al.*, 2004; Zhang *et al.*, 2003b). Also apparent is a significant level of RNA polymerase II at the promoter sequence before treatment with TSA when hTERT is repressed, indicating that RNA polymerase II is bound at the hTERT promoter even when the chromatin environment seems to be heterochromatic.

RNA polymerase II binding to silenced chromatin has been demonstrated before (Sekinger and Gross, 2001) and so RNA polymerase II binding to the hTERT promoter in the hMSC may indicate that the hTERT gene may be permissive for transcription, only needing changes in the chromatin environment of the promoter to allow transcription (Figure 5.15). The apparent decrease in RNA polymerase II binding at the promoter and the large increase at exon 12 can be accounted for by increased RNA polymerase II tracking along the gene, being no longer paused at the promoter. Differences in RNA polymerase II dynamics between hTR and hTERT may reflect the different regulation of each of these genes. Overall it is proposed that modulation of the chromatin environment of promoter sequences may drive changes in the dynamics of the basal transcription machinery and further suggests that complex interactions between transcriptional complexes and chromatin may be important in mediating transcriptional activity.

While TSA treatment does lead to an increase in hTERT and hTR expression, when compared to the telomerase positive MCF7 breast carcinoma cell line (Figure 5.1), hTERT expression in the hMSC treated with TSA is very low and it has been observed that these cells display no detectable telomerase activity as measured by TRAP (Data not included).

Figure 5.15 Chromatin and Basal Transcriptional Machinery Dynamics in hMSCs

As shown in this diagram, changes in the chromatin environment of promoter sequences may lead to changes in gene transcription through the relaxation of the chromatin environment.



This observation indicates that while modulation of the chromatin environment by TSA is important in the control of hTERT and hTR expression, this action alone is not enough to increase levels of hTERT and hTR expression to a level high enough to reconstitute telomerase activity, perhaps due to the lack of other factors which can influence gene expression. The gain in permissive modifications at the hTERT promoter could allow the binding of transcription factors, basal transcription machinery and further chromatin modifying enzymes to the promoter DNA. However, if such factors are not freely available within the cell, the relaxation of the chromatin environment alone will not be sufficient to mediate high levels of transcription. Analysis of the binding of regulatory factors influencing hTERT and hTR expression in the hMSC will therefore be an important next step in understanding telomerase gene regulation.

Further analysis of histone modifications involved in telomerase gene repression in the hMSC may also allow the identification of enzymes and co-regulators involved, which can then be targeted to allow a more specific method of modulating telomerase gene expression other than TSA. Although TSA treatment has been shown to cause chromatin changes at the hTERT and hTR promoter, and studies have shown seemingly direct actions of TSA on the hTERT promoter (Mukhopadhyay *et al.*, 2005; Hou *et al.*, 2002; Cong and Bacchetti, 2000; Takakura *et al.*, 2001), indirect effects cannot be ignored. However, the use of TSA does allow us to observe that changes in the chromatin environment, be they direct or indirect, can lead to changes in telomerase gene expression.

Overall, these data provides an impetus for further, more detailed analysis of the interplay between the chromatin environment, general transcription machinery and other important regulatory factors which may affect telomerase gene expression in the hMSC and has implications for tissue regeneration and studies in cancer stem cells.

Understanding how gene expression changes in the hHSC upon acquisition of the BCR-ABL oncogene will lead to greater understanding of the disease and how it can possibly be treated. Several studies have been undertaken to understand gene expression changes involved in CML (Ohmine *et al.*, 2001; Nowicki *et al.*, 2003; Salesse and Verfaillie, 2003; Kronenwett *et al.*, 2005; Kaneta *et al.*, 2003; Hakansson *et al.*, 2004) and it has been shown that genes deregulated in CML are important in the regulation of the chromatin environment (Nowicki *et al.*, 2003). Therefore, dysregulation of the expression of proteins controlling epigenetic mechanisms have a role in controlling gene expression profiles in CML vs. normal CD34+ hHSCs. Also important is the expression of the telomerase genes in relation to disease progress in CML and how this expression is

regulated. Normal and CML CD34+ hHSCs give us an opportunity to study the epigenetic regulation of expression of hTR and hTERT and therefore how epigenetic dysregulation could impact on disease progression.

Telomerase gene expression has been shown to become dysregulated in CP CML and so gives us an excellent system in which to dissect modes of telomerase gene regulation in a stem cell population (Drummond *et al.*, 2005; Campbell *et al.*, 2006). This study demonstrated that total hTERT expression decreased in the CML samples compared to the Normal samples, contradictory to some previous data (Drummond *et al.*, 2005) but in agreement with the another (Campbell *et al.*, 2006) and so the reduction in telomere length could be due to the decrease in hTERT expression observed. However changes in splicing favouring the full length hTERT transcript are observed in the CML samples agreeing with both previous studies (Drummond *et al.*, 2005; Campbell *et al.*, 2006).

The ChIP data presented here initially suggests the formation of a more repressive chromatin environment at the hTERT promoter in the CML samples as there is an increase in methylation of K9 H3. However an increase in acetylated H3 and H4 and a decrease in dimethylation of K20 H4 were also observed in the CML samples and would generally correlate with a more permissive chromatin environment and would correlate with enhanced hTERT expression. Changes in hTERT splice variant expression are observed in the CML samples and contributions of the chromatin environment to alternate splicing have been shown before and therefore may be influencing hTERT splice variant expression. Use of TSA, which enhances histone hyperacetylation, has been shown to lead to the skipping of alternate exons, and this was suggested to be due to the facilitation of the passage of RNA polymerase II across the transcribed region of the gene (Nogues *et al.*, 2002). The human STAGA complex contains the histone acetyltransferase GCN5 and studies have suggested cellular roles of STAGA in transcription-coupled processes through direct interactions transcriptional activators and with components of the splicing and DNA repair machineries (Martinez *et al.*, 2001).

A recent study has also shown that the ATP-dependent chromatin remodeller Brm also has a role in regulating splice variant transcription, although its catalytic activity was shown to be dispensable for this role (Batsche *et al.*, 2006). The hTERT promoter has also been shown to be relatively weak in terms of transcriptional activity (Bilsland *et al.*, 2003; Plumb *et al.*, 2001) and therefore the formation of a more permissive chromatin environment may allow more efficient splicing perhaps by increased association of the transcriptional machinery to the hTERT promoter. RNA polymerase II analysis shows an

increase in tracking in the CML samples, suggesting that changes in the chromatin environment may be sufficient to alter RNA polymerase II dynamics, which can in turn alter splicing events and may also allow the promoter to be more permissive to binding of factors which may influence hTERT gene splicing. Therefore, changes in the chromatin environment of the coding sequence of the genes may be able to influence splice variant expression and therefore further detailed analysis of chromatin modifications covering the hTERT coding sequence may uncover any roles that the chromatin environment has to play in the regulation of hTERT splice variant expression.

hTR expression was observed to decrease in the CML samples in agreement with a previous study (Drummond *et al.*, 2005). ChIP analysis showed a decrease in methylated K20 H4, but also showed an increase in acetylated H3 and H4 in the CML CD34+ hHSCs. With a decrease in expression one would perhaps expect a more repressive chromatin environment to be present at the hTR promoter, not as it seems a more permissive environment, as observed with the increase of histone acetylation and decrease in repressive histone methylation at the promoter. In this case, hTR expression may be regulated by different means or other important modifications of the chromatin environment not encompassed by this study may be linked to transcription. DNA methylation has been shown to be important in the regulation of hTR expression in some cell types and may have some influence here (Hoare *et al.*, 2001). However, the data does suggest that further studies may be fruitful in further uncovering the role of the chromatin environment in hTR gene regulation in CML. Therefore the reduction in hTR expression observed could lead to the telomere loss observed in CML.

Interestingly, acetylation of H3 and H4 was upregulated for both hTR and hTERT in the CML CD34+ hHSCs. This is consistent with gene expression analysis of BCR-ABL-dependent leukaemogenesis in which it was observed that the histone acetyltransferases HAT1 and TRRAP were overexpressed in CML cells (Nowicki *et al.*, 2003). It was noted that the profiling studies in which samples from CML patients and normal donors were compared, a novel phenotype was uncovered. Many genes upregulated in CML were observed to play a role in modification of chromosomes/chromatin/DNA dynamics. Transcriptional regulators such as MYC, which are known to be able to interact with histone modifying enzymes, are also upregulated.

Of note, is a study which has shown that inhibition of BCR-ABL signal transduction by the use of the tyrosine kinase inhibitor Imatinib mesylate (IM) (Uziel *et al.*, 2005) can cause inhibition of telomerase activity and cell proliferation in various malignant cell lines.

Inhibition of telomerase activity was observed to be mainly by post-translational modifications by the dephosphorylation of AKT but the early downregulation of hTERT transcription was also implicated. Changes in histone modification were not studied, but investigating the chromatin environment of the hTERT promoter and globally before and after treatment of CML BCR-ABL CD34+ hHSCs with IM would be of interest. Also, antisense inhibition of BCR-ABL/c-ABL has been shown to lead to the enhancement of hTERT levels in human leukaemic cells (Bakalova *et al.*, 2004).

Interestingly, a recent study has shown an important role for BCR-ABL in H4 acetylation (Brusa *et al.*, 2006). BCR/ABL was shown to be associated with H4 hyperacetylation which was decreased upon treatment with IM and conversely, trimethylation of K20 H4 was decreased in the presence of BCR-ABL and increased upon treatment with IM. This agrees with data shown here, as for hTR and hTERT, H4 acetylation was increased while a decrease in levels of dimethylation of K20 H4, rather than tri-methylation of K20 H4 was observed. Loss of methylation of K20 H4 has been observed in cancer (Fraga *et al.*, 2005) alongside the loss of acetylated K16 H4 and although we do see increases in H4 acetylation, it is conceivable that we do lose K16 H4 acetylation, and overall acetylation may be increased by increased acetylation at other residues within the histone tail. The interaction of pRB with K20 H4 histone methyltransferases has also been noted (Gonzalo *et al.*, 2005) which can lead to the formation of heterochromatin observed in senescing cells. pRB has been shown to be phosphorylated by BCR-ABL which could lead to the loss of interactions with K20 H4 methyltransferases (Nagano *et al.*, 2006) and therefore may be a mechanism by which we see the reduction of K20 H4 methylation noted in the CML samples.

Overall, this study of normal and CML CD34+ hHSCs show that a reduction in hTERT and hTR expression could lead to reductions in telomerase expression in CP CML in CD34+ hHSCs leading to telomere shortening and that the chromatin environment of the hTR and hTERT genes may have a role in the differential regulation of the telomerase genes in CML.

Overall, it has been shown that the chromatin environment has an important role on the regulation of the telomerase gene promoters in these two stem cell populations. Further more detailed studies would be of interest and but these studies do provide an excellent platform from which more detailed studies can now be started.

6 Summary and Conclusions

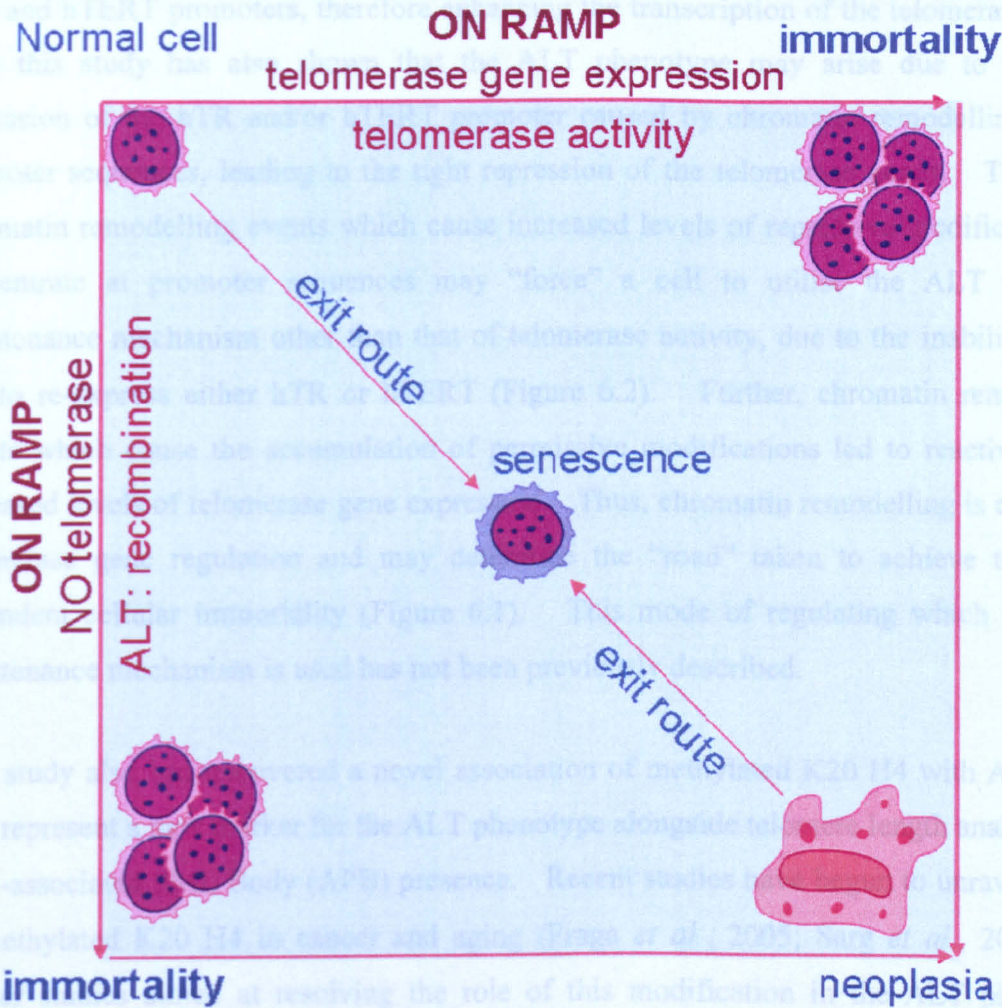
A common set of features distinguish cancer cells from their normal counterparts and includes the loss of cell cycle regulation, loss of control over invasion and metastasis, failure of apoptotic mechanisms and bypass of senescence (Keith *et al.*, 2002; Hahn and Weinberg, 2002). Cellular models designed to recapitulate the features of multistage neoplastic progression have been developed by a number of groups and epitomised by the work from the Weinberg lab (Hahn and Weinberg, 2002). A common element of these models is to use ectopic expression of the hTERT gene to induce telomerase activity and to overcome senescence and thus provide the immortalisation step of cancer progression. However, whilst this model for neoplastic transformation may be of considerable use in defining a number of the key events in progression, by ectopically expressing hTERT, the molecular events required to upregulate hTERT in cancer development are inevitably missed. Further, the role of hTR in telomerase activity and senescence and tumourigenesis is often overlooked. Therefore, given the critical role that telomerase has in tumour progression and with the cancer cell reliant on telomerase for its survival, understanding that the mechanisms regulating telomerase gene expression could be of great interest (Keith *et al.*, 2002). Further, it has also been proposed that epigenetic changes have a role to play in the transition of a cell through senescence and towards neoplasia through altering the expression of genes important in overcoming senescence and promoting neoplasia.

For the first time, the chromatin environment of the hTR and hTERT gene promoters in multiple cell lines; telomerase positive tumour, ALT, normal, and in stem cell populations, both normal and cancer stem cells, was extensively evaluated. The use of multiple models allowed commonalities in telomerase gene regulation to be apparent. The wide range of histone modifications studied also makes this study unique in its scope, by trying to understand fully the contributions that multiple modifications, both acetylation and methylation, can have on transcriptional regulation.

The first part of the study, utilising normal, telomerase positive cancer and telomerase negative ALT cells provided an excellent cellular system in which to study the epigenetic regulation of telomerase gene expression and also allowed us to understand how epigenetics may impact on the mechanisms utilised to maintain/elongate telomeres. A cell has two routes it can utilise to gain immortality and move towards neoplasia, reactivation

Figure 6.1 Telomere Maintenance Mechanisms and the Routes to Neoplasia

Cells can utilise two "on-ramps" towards immortality; either telomerase activity or the ALT mechanism. These routes lead to telomere elongation/maintenance and lead to the bypass of the "exit route" of senescence, and can eventually lead to neoplasia.

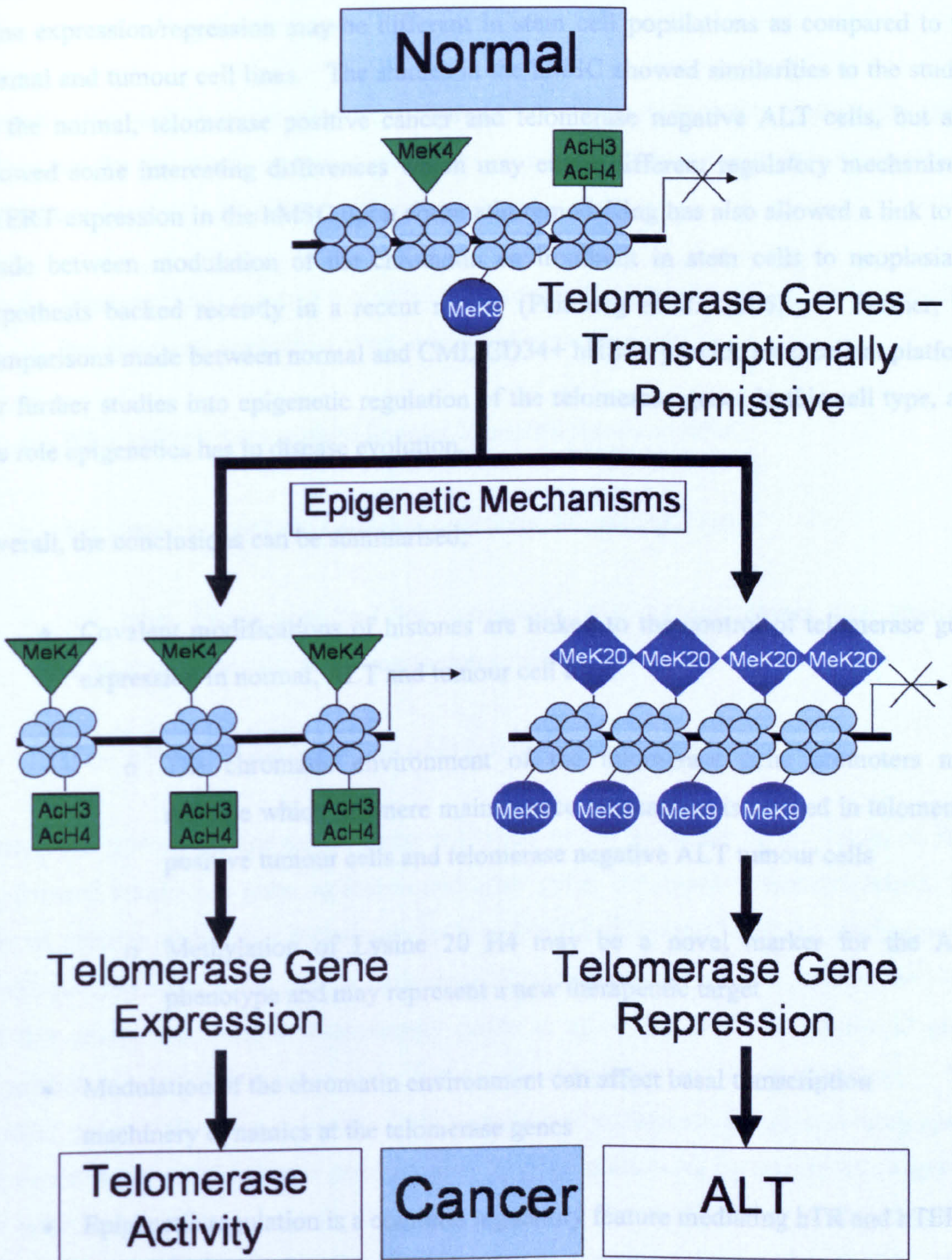


or upregulation of telomerase activity or ALT (Figure 6.1). However, why some cells utilise the ALT pathway while others reactivate telomerase is not fully understood, but this study illustrates that the chromatin environment of the telomerase gene promoters may influence this choice. This study has shown that telomerase reactivation may occur by chromatin remodelling allowing a more permissive state of transcription to occur at the hTR and hTERT promoters, therefore enhancing the transcription of the telomerase genes. Also this study has also shown that the ALT phenotype may arise due to the tight repression of the hTR and/or hTERT promoter caused by chromatin remodelling of the promoter sequences, leading to the tight repression of the telomerase genes. Therefore, chromatin remodelling events which cause increased levels of repressive modifications to concentrate at promoter sequences may "force" a cell to utilise the ALT telomere maintenance mechanism other than that of telomerase activity, due to the inability of the cell to re-express either hTR or hTERT (Figure 6.2). Further, chromatin remodelling events which cause the accumulation of permissive modifications led to reactivation or increased levels of telomerase gene expression. Thus, chromatin remodelling is central to telomerase gene regulation and may determine the "road" taken to achieve telomere-dependent cellular immortality (Figure 6.1). This mode of regulating which telomere maintenance mechanism is used has not been previously described.

This study also has uncovered a novel association of methylated K20 H4 with ALT, and may represent a new marker for the ALT phenotype alongside telomere length analysis and ALT-associated PML Body (APB) presence. Recent studies have begun to unravel a role of methylated K20 H4 in cancer and aging (Fraga *et al.*, 2005; Sarg *et al.*, 2002) and further studies aimed at resolving the role of this modification in the ALT phenotype would be of great interest. The clarification of the role of methylated K20 H4 in ALT may also provide a novel therapeutic target, which could be used along side telomerase-targeted therapeutics in cancer treatment. ALT occurs in a small but clinically relevant percentage of all tumours overall and can be the prevalent telomere maintenance mechanism in some mesenchymal tumours so the ability to somehow target or even to better understand the molecular mechanism inherent in these ALT cells would be an attractive proposition.

The next part of the study utilised hMSC and hHSC stem cell populations, allowing different models to study epigenetic regulation of the telomerase gene promoters and further allowing the study of normal and cancer stem cells. Moving these studies from normal and tumour cell lines into stem cells was an important step in order to fully

Figure 6.2 How Chromatin Modifications May Influence Telomere Maintenance Mechanisms
Telomerase gene expression is linked to the accumulation of permissive histone modifications, which could lead to telomerase expression and the elongation/maintenance of telomere length. However, heavy repression of the telomerase gene promoters by the accumulation of repressive histone modifications could lead to the utilisation of the ALT phenotype for telomere elongation/maintenance due to the repression of telomerase activity.


Normal
MeK4
AcH3
AcH4
MeK9
Telomerase Genes –
Transcriptionally
Permissive
Epigenetic Mechanisms
MeK4
MeK4
MeK4
AcH3
AcH4
AcH3
AcH4
AcH3
AcH4
MeK20
MeK20
MeK20
MeK20
MeK9
MeK9
MeK9
MeK9
Telomerase Gene
Expression
Telomerase Gene
Repression
Telomerase
Activity
Cancer
ALT

understanding telomerase regulation. Epigenetic regulatory mechanisms of telomerase gene expression/repression may be different in stem cell populations as compared to the normal and tumour cell lines. The studies in the hMSC showed similarities to the studies in the normal, telomerase positive cancer and telomerase negative ALT cells, but also showed some interesting differences which may entail different regulatory mechanisms. hTERT expression in the hMSC upon chromatin remodelling has also allowed a link to be made between modulation of the chromatin environment in stem cells to neoplasia, a hypothesis backed recently in a recent review (Feinberg *et al.*, 2006). Further, the comparisons made between normal and CML CD34+ hHSCs provide an excellent platform for further studies into epigenetic regulation of the telomerase genes in this cell type, and the role epigenetics has in disease evolution.

Overall, the conclusions can be summarised;

- Covalent modifications of histones are linked to the control of telomerase gene expression in normal, ALT and tumour cell lines
    - The chromatin environment of the telomerase gene promoters may mediate which telomere maintenance mechanism is utilised in telomerase positive tumour cells and telomerase negative ALT tumour cells
    - Methylation of Lysine 20 H4 may be a novel marker for the ALT phenotype and may represent a new therapeutic target
- Modulation of the chromatin environment can affect basal transcription machinery dynamics at the telomerase genes
- Epigenetic regulation is a common regulatory feature mediating hTR and hTERT expression/repression
- Covalent modifications of histones are linked to the control of telomerase gene expression in stem cells and cancer stem cells
    - Epigenetic dysregulation of normal stem cells may lead to the emergence of cancer stem cells

  - Chromatin mediated telomerase gene regulation may be a common regulatory mechanism in multiple different cell types

Overall, this study has provided an excellent platform towards understanding the global effect that the chromatin environment has on gene expression in the bypass of senescence and tumourigenesis. This study also raises several questions;

- What enzymes catalyse the addition/removal of histone modifications important in the epigenetic regulation of telomerase gene regulation?

- What pathways are utilised in order to control these enzymes?

- Are these enzymes and pathways conserved between cell types?

- Are other genes important for senescence and tumourigenesis also similarly regulated by the chromatin environment of their promoters?

- If so, what are these genes and what cellular roles do they play?

These are interesting questions which will need to be answered in the future to fully understand telomerase gene regulation and other genes important in tumourigenesis. The use of genome wide ChIP techniques such as GMAT (Genome Mapping Technique) or ChIP-on-Chip could allow us to answer some of these questions by incorporating the ideals of this study into a more wide-ranging mode to allow the study of groups of genes regulated in a similar manner upon bypass of senescence and immortalisation. The GMAT process allows us to use the ChIP technique to understand the genome wide spread of a chromatin modification or patterns of modifications allowing for an unbiased approach to understanding how gene regulation is controlled by modulations of the chromatin environment. Linking data such as this to gene expression analysis such as SAGE (serial analysis of gene expression) would allow an excellent comparison between gene expression and gene promoter chromatin landscape on a genome wide scale

Although regulation of the chromatin environment of the telomerase gene promoters has been shown to be important in the control of expression, it is noted that this is but one layer of complexity. Further studies will be required in order to identify the proteins involved in lysine methylation (Figure 6.3) and histone acetylation/deacetylation (Figure 6.4), which impact on the epigenetic control of hTR and hTERT gene regulation. Understanding the

enzymes and complexes which orchestrate chromatin modifications, both locally and globally, will be the next target in such studies. Such studies may allow identification of new regulatory pathways, provide therapeutic drug targets and allow further understanding of the processes controlling telomerase gene expression in normal development and tumourigenesis. Studies intent on unravelling regulatory pathways have already started to uncover some interesting regulatory mechanisms (Bilsland *et al.*, 2006; Ge *et al.*, 2006) while specific targeting by chromatin remodelling enzymes to the hTR and hTERT promoters to allow a level of transcriptional control is also an exciting prospect.

Figure 6.3 Enzymes which Mediate Lysine Methylation and Demethylation

A Enzymes which mediate Lysine 20 H4 methylation and possible other interactions. Interaction of the yeast protein Crb2 with Lysine 20 H4 methylation is also noted. **B** Enzymes which mediate Lysine 9 H3 methylation and possible other interactions. **C** Enzymes which mediate Lysine 4 H3 methylation and possible other interactions.

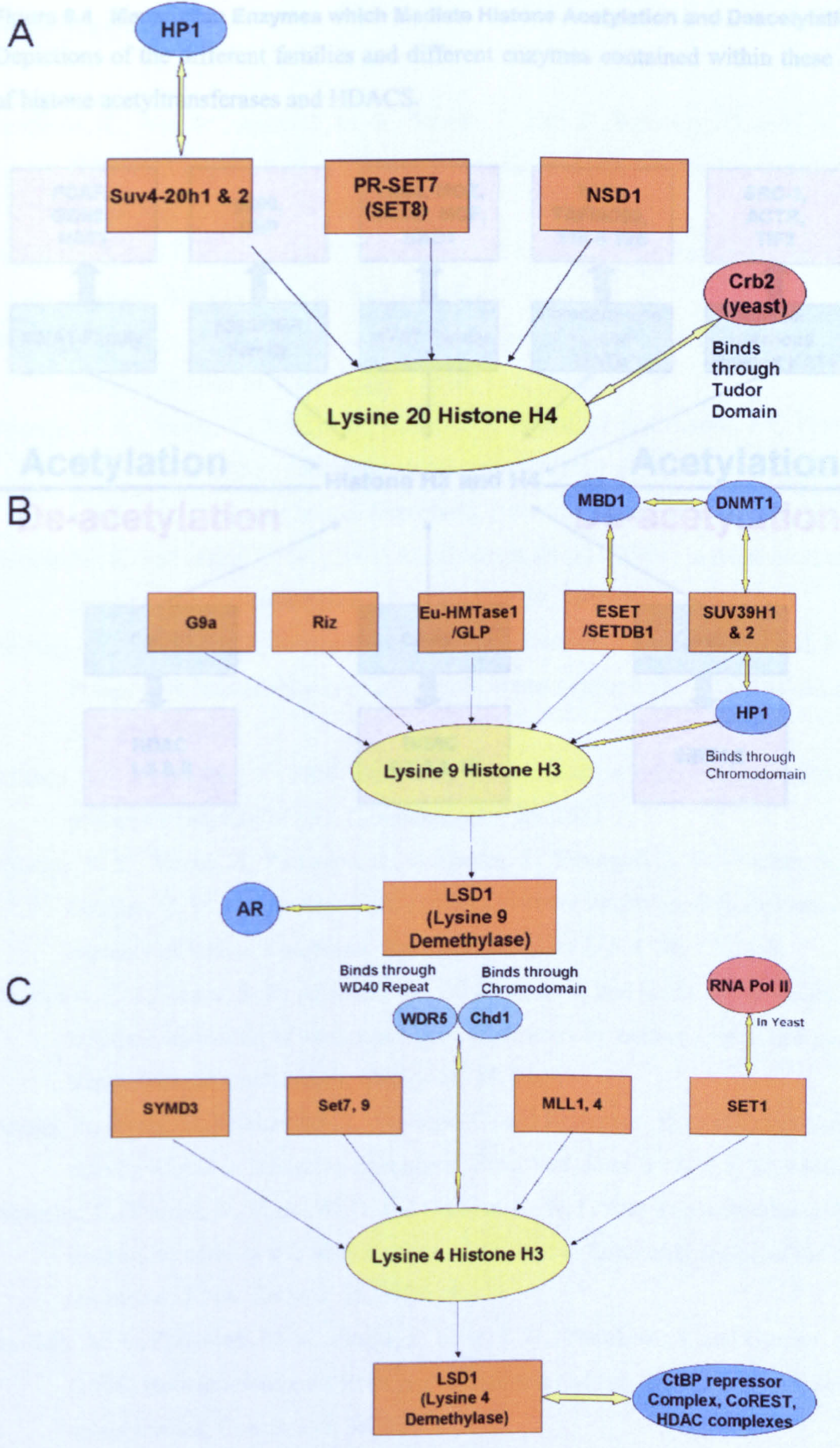
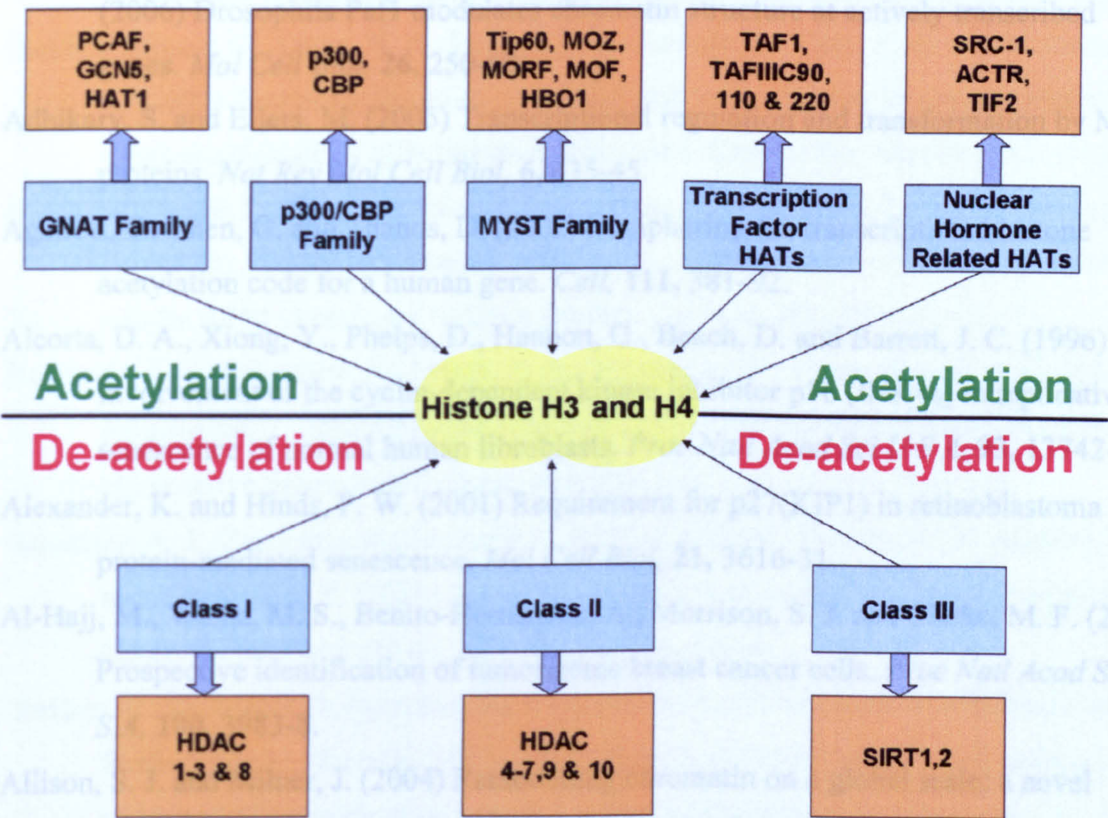

A
HP1
Suv4-20h1 & 2
PR-SET7 (SET8)
NSD1
Crb2 (yeast)
Binds through Tudor Domain
Lysine 20 Histone H4
B
MBD1
DNMT1
G9a
Riz
Eu-HMTase1 /GLP
ESET /SETDB1
SUV39H1 & 2
HP1
Binds through Chromodomain
Lysine 9 Histone H3
AR
LSD1 (Lysine 9 Demethylase)
C
Binds through WD40 Repeat
Binds through Chromodomain
WDR5
Chd1
RNA Pol II
In Yeast
SYMD3
Set7, 9
MLL1, 4
SET1
Lysine 4 Histone H3
LSD1 (Lysine 4 Demethylase)
CtBP repressor Complex, CoREST, HDAC complexes

Figure 6.4 Mammalian Enzymes which Mediate Histone Acetylation and Deacetylation
Depictions of the different families and different enzymes contained within these families of histone acetyltransferases and HDACS.



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Lack of Telomerase Gene Expression in Alternative Lengthening of Telomere Cells Is Associated with Chromatin Remodeling of the *hTR* and *hTERT* Gene Promoters

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Abstract

The presence of active telomere maintenance mechanisms in immortal cells allows the bypass of senescence by maintaining telomere length. In most immortal cell lines and tumors, telomere maintenance is attributable to telomerase reactivation. However, a number of immortal cell lines and tumors can achieve telomere maintenance in the absence of detectable telomerase activity by the alternative lengthening of telomere (ALT) mechanism. Epigenetic mechanisms have been implicated in the regulation of telomerase expression. We show that specific modifications within the chromatin environment of the *hTR* and *hTERT* promoters correlate with expression of *hTR* and *hTERT* in ALT, normal and telomerase-positive tumor cell lines. Lack of expression of *hTR* and *hTERT* in ALT cell lines is associated with histone H3 and H4 hypoacetylation and methylation of Lys^9 histone H3. Conversely, *hTR* and *hTERT* expression in telomerase-positive cell lines is associated with hyperacetylation of H3 and H4 and methylation of Lys^4 H3. Methylation of Lys^{20} H4 was not linked to gene expression but instead was specific to the *hTR* and *hTERT* promoters of ALT cells. This may provide an insight into the differences between ALT and telomerase-positive cells as well as a novel marker for the ALT phenotype. Treatment of normal and ALT cells with 5-azadeoxycytidine in combination with Trichostatin A caused chromatin remodeling of both promoters and reactivation of *hTR* and *hTERT* expression in ALT and normal cell lines. This data establishes a definite link between the chromatin environment of the telomerase gene promoters and transcriptional activity. (Cancer Res 2005; 65(17): 7585-90)

Introduction

In most cancer cell lines and tumors, telomere maintenance is achieved by the reactivation of telomerase (1). However, some cancer cell lines and tumors can maintain their telomeres in the absence of telomerase by the alternative lengthening of telomere (ALT) mechanism (2), but it is unclear why some cells use the ALT pathway whereas other cells reactivate telomerase. ALT lines represent an interesting model for telomerase gene regulation as some express hTR but not hTERT and others lack expression of both components. *hTR* and *hTERT* gene expression is regulated on many levels during development and tumorigenesis and evidence suggests that epigenetic mechanisms may also be involved. We have previously shown that CpG methylation at the promoter of *hTR* in ALT cell lines is associated with gene repression (3), whereas other groups have linked CpG methylation with transcriptional status of the *hTERT* promoter, although the correlation is not clear (4, 5). The influence of DNA methylation of gene expression suggests that histone modification may also regulate telomerase gene expression and previous studies have provided links between transcription factor binding and chromatin remodeling leading to changes in *hTERT* gene expression (6), although these studies have analyzed a limited number of modifications in the *hTERT* promoter alone. The histone code hypothesis (7) proposes that it is through the combination of several histone modifications that gene expression can be altered. Thus, to investigate how the chromatin environment might influence telomerase gene expression and how this may influence the mechanism of telomere maintenance in different cell types, we studied histone acetylation (histones H3 and H4 and Lys^9 histone H3) and histone methylation (Lys^4 and Lys^9 H3 and Lys^{20} H4) at the *hTR* and *hTERT* promoters in tumor, normal, and ALT cell lines.

Materials and Methods

Cell lines. Cell lines used were C33a (cervical carcinoma); A2780 (ovarian adenocarcinoma); 5637 (bladder carcinoma); WI38 (normal fetal lung fibroblast); and SUSM-1, SKLU, GM847, KMST-6, and WI38-SV40 (all ALT).

Chromatin immunoprecipitation assays. Cells were used when at 70% to 80% confluence and chromatin immunoprecipitation (ChIP) assays were done following the instructions recommended by the kit supplier (Upstate Biotechnology, Dundee, United Kingdom). Sonication was optimized to give chromatin fragments of around 500 bp to 1 kb in length (8 × 10-second pulses at 5 μm with 20-second rest between each pulse on ice using an MSE Soniprep150 sonifier). Resultant DNA from each immunoprecipitate was purified using the QIAquick PCR Purification Kit (Qiagen, West Sussex, United Kingdom). Also included in each experiment was a no antibody control immunoprecipitate to detect any background, which if present was subtracted from each immunoprecipitate within that experiment.

Antibodies. Antibodies used are the following: TriMeK4 H3, DiMeK9 H3, and TriMeK9 H3 (all Abcam, Cambridge, United Kingdom); DiMeK4 H3, DiMeK20 H4, TriMeK20 H4, AcH3, AcH4, and AcK9 H3 (all Upstate Biotechnology).

Quantitative PCR. Products from the ChIP assay were quantified by quantitative PCR on an Opticon2 DNA Engine (MJ Research, Inc., Waltham, MA) using primers to hTR and hTERT core promoter sequences and the SYBR Green Q-PCR Buffer (Finnzymes, Espoo, Finland). hTR primers, 29SF 5′-CCCGCCCGAGAGAGTGAC-3′ and 5ALTR 5′-AAGTCAGCGAGAAAAA-CAGC and hTERT primers, TERTSF 5′-TCCCCT TCACGTCCGGCATT-3′ and TERTSR 5′-AGCGGAGAGAGGTCGAATCG-3′.

Note: S.P. Atkinson and S.F. Hoare contributed equally to this work.

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Expression analysis. hTR expression was analyzed by quantitative PCR using the following primers: TRC3F, 5′-CTAACCCTAACTGAGAAGGGCGTA-3′ and TRC3R, 5′-GGCGAACGGGCCAGCAGCTGACATT-3′ and adjusted to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression (primers GAPDH0.45F 5′-ACCACAGTCCATGCCATCAC-3′ and GAPDH0.45R 5′-CCACCACCCTGTTGCTGTA-3′). hTERT expression was analyzed by methods outlined in Keith et al. (8) using primers which detect all splice variants (primers HT2026F 5′-GCCTGAGCTGTACTTTGTCAA-3′ and HT2482R 5′-GCCAAACAGCTTGTTCTCCATGTC-3′). hTERT expression is displayed as total amounts of all four main splice variants (wild type, α-deletion, β-deletion, and αβ-deletion).

Trichostatin A and 5-azadeoxycytidine treatment. Cells were treated twice for 24 hours with 5-azadeoxycytidine (5-azadC, Sigma, Dorset, United Kingdom) to a final concentration of 2.5 μmol/L and treated for 16 hours with Trichostatin A (TSA, Upstate Biotechnology) at a final concentration of 350 nmol/L.

Results

Profile of repressive histone lysine methylation states at the *hTR* promoter. To understand the relationship between expression levels of hTR and chromatin remodeling at the promoter, we first examined *hTR* gene expression levels from each cell line. In Fig. 1*A*, we see that *hTR* is expressed in GM847, 5637, A2780, SKLU, C33a, and WI38, whereas expression levels were negligible in SUSM-1, KMST-6, and WI38-SV40.

To examine the relationship between expression and histone modifications, ChIP assays using antibodies against specific histone modifications were used to generate a profile of the chromatin environment surrounding the *hTR* promoter in each cell line.

Methylation of Lys[9] histone H3 (MeK9 H3) facilitates the formation of heterochromatin (9, 10), and elevated levels of

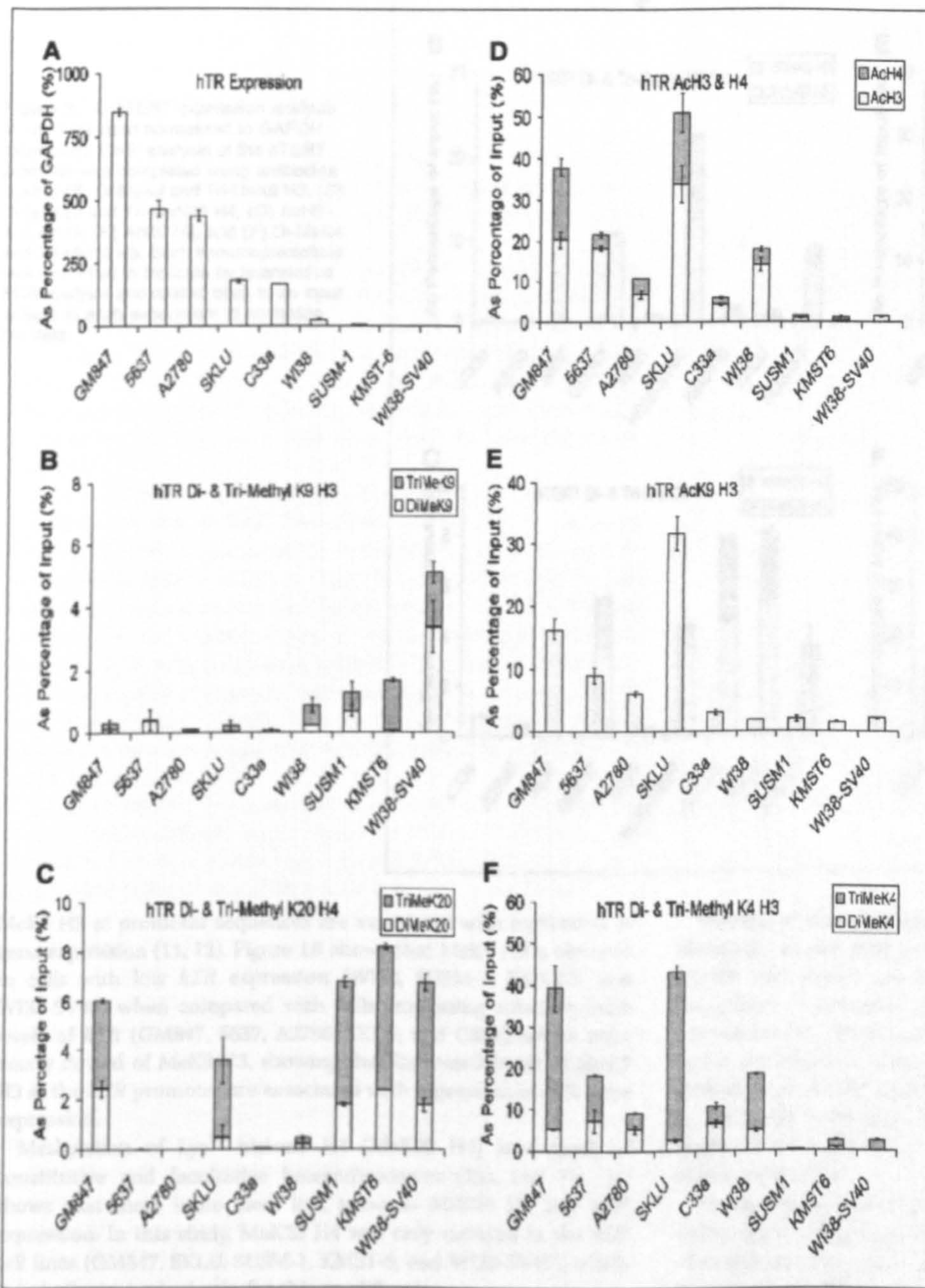

Figure 1. *A*, hTR expression analysis of cell lines used normalized to GAPDH expression. ChIP analysis of the hTR promoter was completed using antibodies against (*B*) Di-MeK9 and Tri-MeK9 H3, (*C*) Di-MeK20 and Tri-MeK20 H4, (*D*) AcH3 and AcH4, (*E*) AcK9 H3, and (*F*) Di-MeK4 and Tri-MeK4 H3. Each immunoprecipitate was quantified in triplicate by quantitative PCR analysis and related back to an input sample in each experiment to normalize the data.

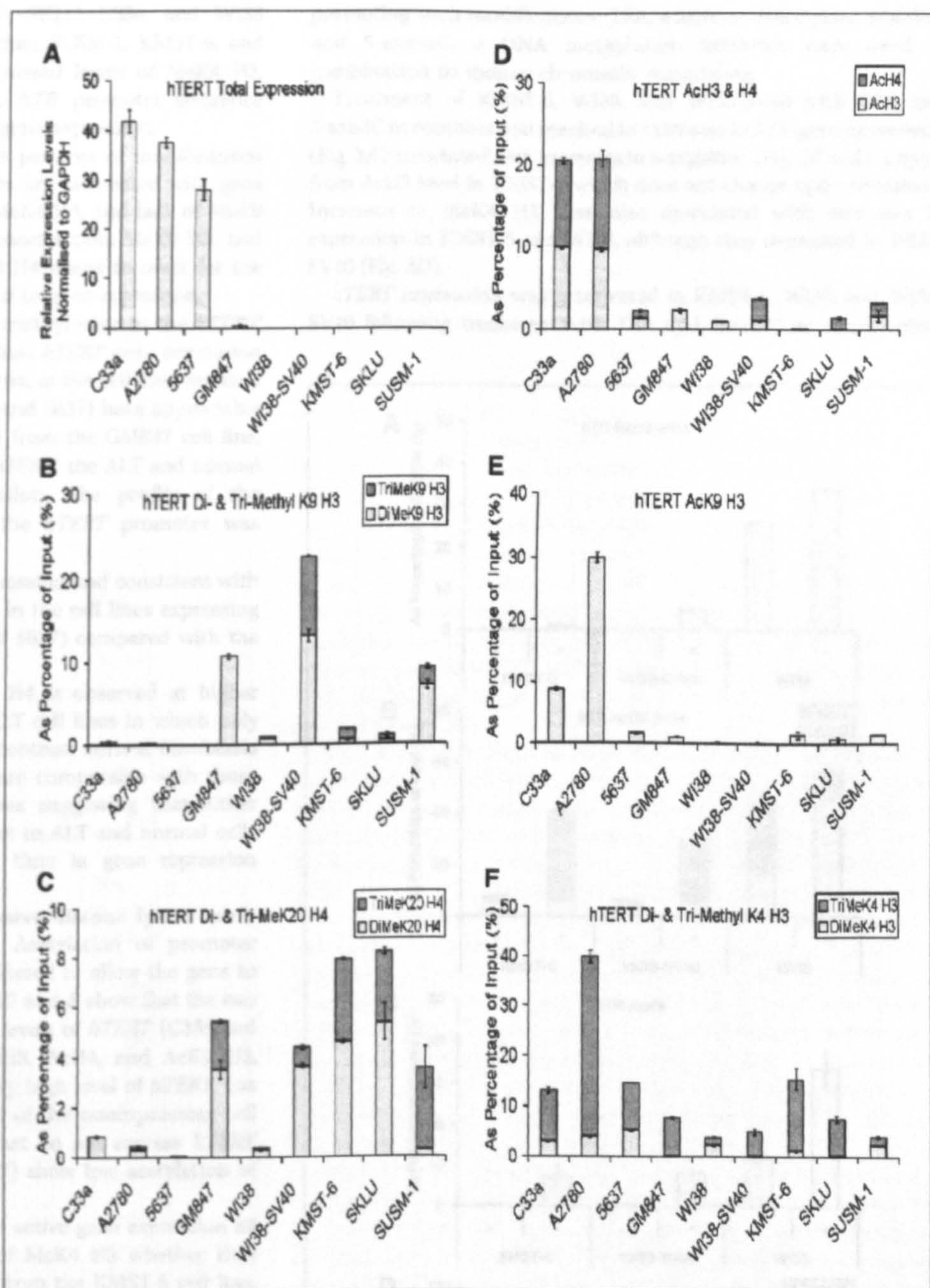

Figure 2. *A*, hTERT expression analysis of cell lines used normalized to GAPDH expression. ChIP analysis of the hTERT promoter was completed using antibodies against (*B*) Di-MeK9 and Tri-MeK9 H3, (*C*) Di-MeK20 and Tri-MeK20 H4, (*D*) AcH3 and AcH4, (*E*) AcK9 H3, and (*F*) Di-MeK4 and Tri-MeK4 H3. Each immunoprecipitate was quantified in triplicate by quantitative PCR analysis and related back to an input sample in each experiment to normalize the data.

MeK9 H3 at promoter sequences are associated with repression of gene expression (11, 12). Figure 1*B* shows that MeK9 H3 is elevated in cells with low *hTR* expression (WI38, SUSM-1, KMST-6, and WI38-SV40) when compared with cells expressing relatively high levels of *hTR* (GM847, 5637, A2780, SKLU, and C33a), which were nearly devoid of MeK9 H3, showing that increased levels of MeK9 H3 at the *hTR* promoter are associated with repression of *hTR* gene expression.

Methylation of Lys^{20} histone H4 (MeK20 H4) is a mark of constitutive and facultative heterochromatin (13), but Fig. 1*C* shows that there is no clear link between MeK20 H4 and *hTR* expression. In this study, MeK20 H4 was only elevated in the ALT cell lines (GM847, SKLU, SUSM-1, KMST-6, and WI38-SV40), which may indicate a novel role for this modification.

Profile of transcriptionally permissive histone lysine modifications at the *hTR* promoter. Acetylated histones H3 and H4 (AcH3 and AcH4) are associated with euchromatin (14), and acetylation of promoter proximal histones is associated with gene expression (15, 16). Figure 1*D* shows that high levels of AcH3 and AcH4 are linked to elevated levels of *hTR* gene expression, with GM847, 5637, A2780, SKLU, C33a, and WI38 exhibiting higher levels of AcH3 and AcH4 than SUSM-1, KMST-6, and WI38-SV40. Higher levels of AcK9 H3 (Fig. 1*E*) are also observed in cell lines with higher expression.

Methylation of Lys^4 histone H3 (MeK4 H3) is also associated with active gene transcription (17) and elevated levels have been observed at active gene promoters (18). Figure 1*F* indicates that high levels of MeK4 H3 are associated with elevated *hTR* gene

expression with GM847, 5637, A2780, SKLU, C33a, and WI38 exhibiting higher levels of MeK4 H3 than SUSM-1, KMST-6, and WI38-SV40. This data shows that increased levels of MeK4 H3, AcH3 and H4, and AcK9 H3 at the *hTR* promoter sequence correlate with increased levels of *hTR* gene expression.

Overall, the data shows that distinct patterns of modifications present at the *hTR* promoter sequences are associated with gene expression (histone hyperacetylation, MeK4 H3, and lack of MeK9 H3) and gene repression (histone hypoacetylation, MeK9 H3, and lack of MeK4 H3). Interestingly, MeK20 H4 seems to mark for the ALT phenotype rather than being linked to gene repression.

Profile of repressive histone lysine methylation at the *hTERT* promoter. As for *hTR*, we first established *hTERT* gene expression levels from each cell line. As Fig. 2*A* shows, of the cell lines studied, only the tumor cell lines (C33a, A2780, and 5637) have appreciable levels of *hTERT* gene expression. Apart from the GM847 cell line, which expresses very low amounts of *hTERT*, the ALT and normal cell lines showed no *hTERT* expression. The profile of the chromatin environment surrounding the *hTERT* promoter was then investigated.

MeK9 H3 is associated with gene repression and consistent with this the levels of MeK9 H3 were lowest in the cell lines expressing high levels of *hTERT* (C33a, A2780, and 5637) compared with the normal and ALT cell lines (Fig. 2*B*).

As with the hTR promoter, MeK20 H4 is observed at higher levels at the *hTERT* promoter in the ALT cell lines in which only GM847 has any *hTERT* expression. In contrast normal fibroblasts (WI38) have levels of MeK20 H4 that are comparable with those found in hTERT-expressing tumor lines suggesting that either different mechanisms of repression exist in ALT and normal cells or that MeK20 H4 has a role other than in gene repression (Fig. 2*C*).

Profile of transcriptionally permissive histone lysine modifications at the *hTERT* promoter. Acetylation of promoter histones H3 and H4 are generally considered to allow the gene to be permissive for transcription. Figure 2*D* and *E* show that the two tumor cell lines expressing the highest levels of *hTERT* (C33a and A2780) have the highest levels of AcH3, AcH4, and AcK9 H3. However, 5637, which express a relatively high level of *hTERT*, has acetylation levels comparable with that of the nonexpressing cell lines. The ALT and normal cell lines that do not express *hTERT* (except for very low amounts in GM847) show low acetylation of AcH3, AcH4, and AcK9 H3.

Although MeK4 H3 is associated with active gene expression all the cell lines exhibited some degree of MeK4 H3 whether they express hTERT or not. However, apart from the KMST-6 cell line, MeK4 H3 is higher in the *hTERT*-expressing tumor cell lines (C33a, A2780, and 5637) than in the *hTERT*-nonexpressing normal and ALT cell lines Fig. 2*F*.

Thus, as with *hTR*, the data shows that distinct patterns of modifications present at the *hTERT* promoter sequences are associated with gene expression (histone hyperacetylation, MeK4 H3, and lack of MeK9 H3) and gene repression (histone hypoacetylation and MeK9 H3). Interestingly, MeK20 H4 again seems to mark for the ALT phenotype rather than being linked to gene repression.

Reactivation of telomerase gene expression by chromatin remodeling. As active *hTR* and *hTERT* promoters show similar patterns of histone modifications (high levels of AcH3, AcH4, AcK9 H3, and MeK4 H3), we asked whether expression of repressed telomerase genes in telomerase-negative cells could be induced by promoting such modifications. TSA, a histone deacetylase inhibitor and 5-aza-dC, a DNA methylation inhibitor were used in combination to induce chromatin remodeling.

Treatment of KMST-6, WI38, and WI38-SV40 with TSA and 5-azadC in combination resulted in increases in *hTR* gene expression (Fig. 3*A*) associated with increases in acetylation (Fig. 3*B* and *C*), apart from AcH3 level in KMST-6 which does not change upon treatment. Increases in MeK4 H3 were also associated with increases in expression in KMST-6 and WI38, although they decreased in WI38-SV40 (Fig. 3*D*).

hTERT expression was reactivated in KMST-6, WI38, and WI38-SV40 following treatment with TSA and 5-azadC in combination

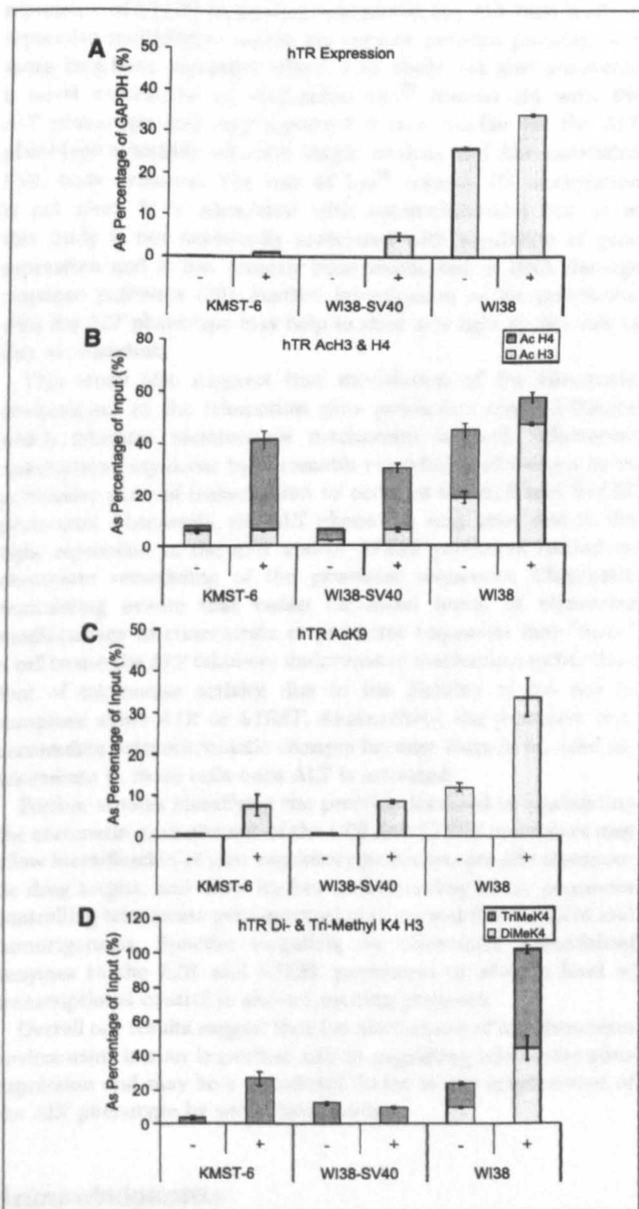

Figure 3. *A*, hTR expression analysis before and after treatment with TSA and 5-azadC. ChIP analysis of the hTR promoter was completed for (*B*) AcH3 and AcH4, (*C*) AcK9 H3, and (*D*), Di-MeK4 and Tri-MeK4 H3. Each immunoprecipitate was quantified in triplicate by quantitative PCR analysis and related back to an input sample in each experiment to normalize the data.

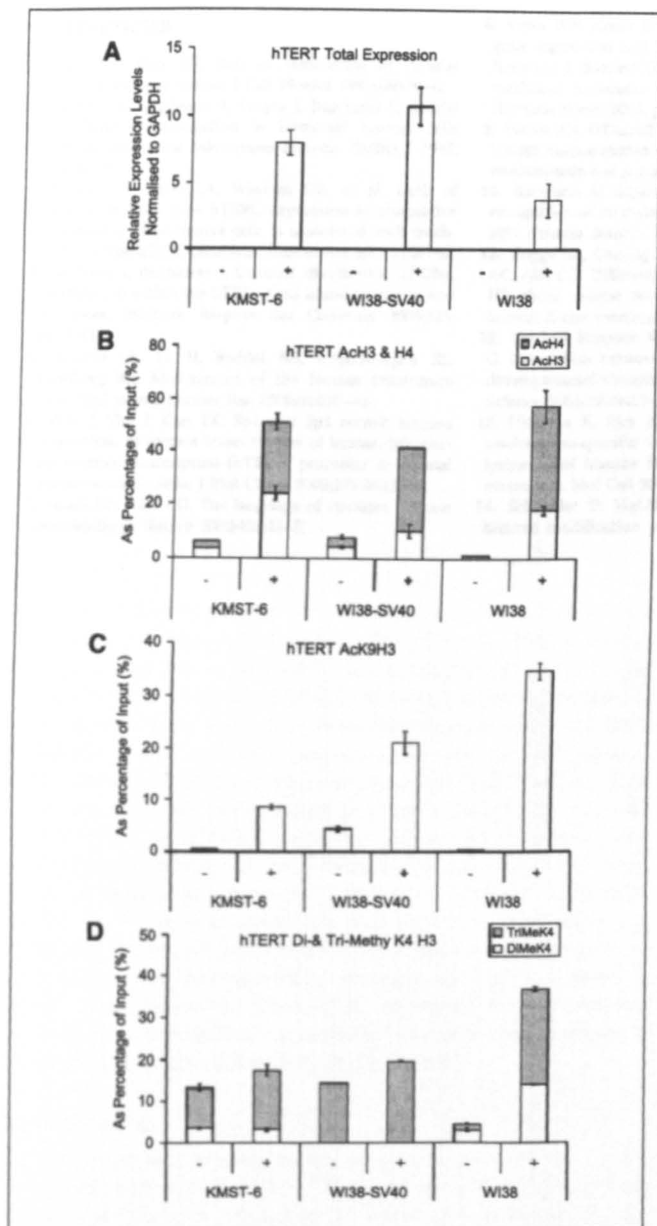

Figure 4. *A*, hTERT expression analysis before and after treatment with TSA and 5-azadC. ChIP analysis of the hTERT promoter was completed for (*B*) AcH3 and AcH4, (*C*) AcK9 H3, and (*D*) Di-MeK4 and Tri-MeK4 H3. Each immunoprecipitate was quantified in triplicate by quantitative PCR analysis and related back to an input sample in each experiment to normalize the data.

(Fig. 4*A*). This increase in expression was also associated with increases in AcH3, AcH4 (Fig. 4*B*), AcK9 (Fig. 4*C*), and MeK4 H3 (Fig. 4*D*).

This data shows that for *hTR* and *hTERT*, an increase in the levels of histone modification linked to gene expression at the promoter can cause reexpression or increases in expression.

Discussion

This study emphasizes the importance of the chromatin environment in the regulation of telomerase gene expression. Common chromatin patterns at promoter sequences are associated with repression and activation of both *hTR* and *hTERT* in normal, ALT, and cancer cell lines, and forced chromatin remodeling of the *hTR* and *hTERT* promoter sequences can induce the reexpression of *hTR* and *hTERT*. Our results are consistent with a recent study by Kumakaru et al. (19) on *hTERT*; furthermore, the proposal that the chromatin-mediated regulation of expression is of importance for both *hTR* and *hTERT*. We have also shown that the normal cell line WI38 has a promoter chromatin profile which shows neither euchromatic nor heterochromatic hallmarks compared with the normal and ALT cell lines. It may be in this cell line that the lack of permissive modifications may be enough to repress hTERT transcription without needing the added effect of the repressive modifications. This may also be the hallmark of a short-term repression of hTERT expression, whereas in the ALT high levels of repressive methylation marks are present perhaps pointing to a more long-term repressive effect. This study has also uncovered a novel association of methylated Lys[20] histone H4 with the ALT phenotype and may represent a new marker for the ALT phenotype alongside telomere length analysis and ALT-associated PML body presence. The role of Lys[20] histone H4 methylation is not clear. It is associated with heterochromatin but as in this study is not necessarily associated with regulation of gene expression and it has recently been implicated in DNA damage response pathways (20). Further investigation of its association with the ALT phenotype may help to shed new light on the role of this modification.

This study also suggests that modulation of the chromatin environment of the telomerase gene promoters could influence which telomere maintenance mechanism is used. Telomerase reactivation may occur by chromatin remodeling allowing a more permissive state of transcription to occur at the *hTR* and *hTERT* promoters. Conversely, the ALT phenotype may arise due to the tight repression of the *hTR* and/or *hTERT* promoter, caused by chromatin remodeling of the promoter sequences. Chromatin remodeling events that cause increased levels of repressive modifications to concentrate at promoter sequences may "force" a cell to use the ALT telomere maintenance mechanism rather than that of telomerase activity, due to the inability of the cell to reexpress either *hTR* or *hTERT*. Alternatively, the promoter may accumulate heterochromatic changes because there is no need for telomerase in these cells once ALT is activated.

Further studies identifying the proteins involved in modulating the chromatin environment of the *hTR* and *hTERT* promoters may allow identification of new regulatory pathways, provide therapeutic drug targets, and allow further understanding of the processes controlling telomerase gene expression in normal development and tumorigenesis. Specific targeting by chromatin remodeling enzymes to the *hTR* and *hTERT* promoters to allow a level of transcriptional control is also an exciting prospect.

Overall our results suggest that the modulation of the chromatin environment has an important role in regulating telomerase gene expression and may be a significant factor in the acquirement of the ALT phenotype by some tumor cells.

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Transcriptional Repression of Telomerase RNA Gene Expression by c-Jun-NH_2-Kinase and Sp1/Sp3

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Abstract

Telomerase is essential for immortalization of most human cancer cells. Expression of the core telomerase RNA (hTR) and reverse transcriptase (hTERT) subunits is mainly regulated by transcription. However, hTR transcriptional regulation remains poorly understood. We previously showed that the core hTR promoter is activated by Sp1 and is repressed by Sp3. Here, we show that the mitogen-activated protein kinase kinase kinase 1 (MEKK1)/c-Jun-NH_2-kinase (JNK) pathway represses hTR expression by a mechanism that involves Sp1 and Sp3. Promoter activity was induced by the JNK inhibitor SP600125 and was repressed by activated MEKK1. Repression by MEKK1 was blocked by SP600125 or enhanced by coexpression of wild-type but not phosphoacceptor mutated JNK. SP600125 treatment also increased levels of endogenous hTR. Mutations in the hTR promoter Sp1/Sp3 binding sites attenuated SP600125-mediated promoter induction, whereas coexpression of MEKK1 with Sp3 enhanced hTR promoter repression. Chromatin immunoprecipitation showed that levels of immunoreactive Sp1 associated with the hTR promoter were low in comparison with Sp3 in control cells but increased after JNK inhibition with a reciprocal decrease in Sp3 levels. No corresponding changes in Sp1/Sp3 protein levels were detected. Thus, JNK represses hTR promoter activity and expression, apparently by enhancing repression through Sp3. (Cancer Res 2006; 66(3): 1363-70)

Introduction

Telomerase is a ribonucleoprotein reverse transcriptase minimally composed of core RNA (hTR) and catalytic (hTERT) subunits, which stabilizes the telomeres of linear chromosomes (1, 2). Although telomerase activity is present during human embryonic development, its expression and activity are repressed in most adult tissues. In contrast, it is essential for long-term proliferation of most human cancer cell lines and most human tumors express high levels of telomerase (3-6).

Levels of telomerase in cancer cells are primarily determined by transcriptional activity of the *hTR* and *hTERT* genes. Both transcripts are either absent or at very low levels in most normal human cell types but are readily detectable in most cancer cell lines (7). Interestingly, ectopic delivery of hTR and hTERT promoter constructs results in selective activity in cancer cells as shown by recent preclinical models of transcriptionally targeted gene therapy (7-9). Because of the potential for exploitation of telomerase as a relatively specific therapeutic target in a broad range of tumor types, expanded understanding of the regulation of hTR and hTERT expression is of immediate interest.

Recently, key promoter elements contributing to hTR promoter regulation have begun to emerge. The core promoter is essential for hTR expression *in vivo* because its methylation in some cell lines is sufficient to silence hTR expression (10). In the 5637 bladder cancer cell line, basal activity of transfected core promoter constructs (nucleotides −107/+69) is dependent on NF-Y binding to a CCAAT box sequence. The core promoter also contains four Sp1/Sp3 binding sites, which serve as sites of positive regulation by Sp1 and negative regulation by Sp3 (11-13).

We have recently identified, in archived DNA from the buccal smear of a paroxysmal nocturnal hemoglobinuria patient, a mutation in one of these sites that disrupts Sp1/Sp3 binding and increases the activity of a minimal hTR promoter in transfection assays in 5637 cells (14). In the same cells, NF-Y, Sp1, and TFIIB interact directly with hTR promoter chromatin (12). hTR promoter activity can also be regulated by overexpression of pRb and MDM2, which act, respectively, as positive and negative regulators (15). Notably, upstream signaling events that might regulate transcription factors at the hTR promoter are not well characterized.

Accumulating evidence indicates that multiple kinases, including mitogen-activated protein kinases (MAPK), directly phosphorylate Sp1 and modulate its DNA binding and/or transactivation activity (16-25). MAPK pathways are evolutionarily conserved signaling cascades with a minimal core structure in which an upstream MAPK kinase kinase (MAP3K) activates a MAPK kinase, which in turn activates a terminal effector MAPK (26). Because the hTR promoter is regulated *in vitro* by Sp1, MAPK pathways might be involved in hTR regulation (Fig. 1). This hypothesis was tested in this study.

Indeed, Sp1 sites are required for stimulation of several promoters by the extracellular signal-regulated kinase (ERK) and c-Jun-NH_2-kinase (JNK) MAPK pathways, including the urokinase plasminogen activator and $p21^{waf1/cip1}$ promoters (22, 25). Epidermal growth factor stimulation of gastrin promoter activity in gastric adenocarcinoma cells also involves Sp1 and ERK although the direct Sp1 kinase activity in these cells seems to be an ERK effector rather than ERK itself (24). Nevertheless, Sp1 can be directly phosphorylated by ERK *in vitro*, a modification that stimulates Sp1 DNA binding activity in gel shift assays (19). Furthermore, endogenous Sp1 and ERK coimmunoprecipitate in fibroblasts and, in these cells, Raf induction leads to direct phosphorylation by ERK of Sp1 residues Thr^{453} and Thr^{739}. These modifications are required for efficient stimulation of vascular endothelial growth factor expression (20). In this study, we show

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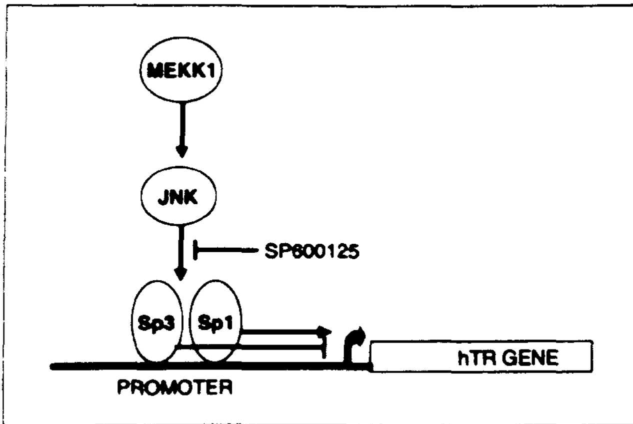

Figure 1. Rationale of the study. The hTR promoter is positively and negatively regulated *in vitro* by Sp1 and Sp3, respectively. MAPK pathways are known to phosphorylate and regulate DNA binding of Sp1. We tested whether the JNK pathway regulates hTR promoter activity and expression using overexpression of an activated mutant of MEKK1, wild-type JNK1, phosphoacceptor mutated JNK2, and the JNK-specific inhibitor SP600125.

that JNK signaling represses the hTR promoter and endogenous hTR levels and provide evidence for a JNK regulated transcriptional switch that enhances binding and/or activity of the transrepressor Sp3 at the hTR promoter and may inhibit Sp1.

Materials and Methods

Cell lines, plasmids, and inhibitors. A2780 (ovarian adenocarcinoma) cells were used throughout. The other cell lines used were 5637 bladder carcinoma cells, C33A cervical carcinoma cells, HT29 colon carcinoma cells, A549 lung adenocarcinoma cells, and HCT116 colon carcinoma cells. Construction of both the 176 bp hTR core promoter reporter (nucleotides 107/+69) and the derived Sp1 mutant construct from pGL3-Basic (Promega Ltd., Madison, WI) has previously been reported in detail (11-13). The Sp1 site-mutated reporter was constructed by PCR mutagenesis of the wild-type promoter and carries mutations in all Sp1 sites that disrupt binding and regulation by Sp1 and Sp3 but not regulation by other key transcription factors. Plasmid pCMV-Sp3 was provided by Dr. G. Suske (Institut fur Molekularbiologie und Tumorforschung, Marburg, Germany). SP600125 was obtained from Merck Biosciences Ltd. (Nottingham, United Kingdom). SP600125 was either titrated as indicated or used at a concentration of 25 μmol/L in medium containing 0.1% DMSO per 10 μmol/L SP600125.

Transfections and luciferase assay. All transfections were done using superfect transfection reagent according to the instructions of the manufacturer (Qiagen Ltd., West Sussex, United Kingdom). A 1:2.5 ratio of DNA/superfect was used and 250 ng hTR reporter plasmid per well was transfected in 96-well luminometer plates (Fisher Scientific UK, Leicestershire, United Kingdom) together with varying amounts of pCMV empty vector or expression vectors encoding constitutively active MEKK1, wild-type or dominant-negative JNK, and Sp3. 30 ng pSV40-Renilla luciferase expression plasmid (Promega) was also cotransfected in each well for normalization of hTR promoter activity. hTR activity was also normalized to protein equivalents using the Bio-Rad assay (Bio-Rad Laboratories Ltd., Hemel Hempstead, United Kingdom). Forty-eight hours posttransfection, cells were lysed and luciferase activities were determined using Dual Luciferase Assay reagents (Promega) according to the instructions of the manufacturer. All transfections were done in quadruplicate and all experiments were repeated a minimum of thrice.

Western blotting. Protein extracts were prepared in SDS lysis buffer (10% SDS, 500 mmol/L EDTA, and 1 mol/L Tris-HCl). Protein concentrations were estimated at A_{595} using the Bio-Rad protein assay (Bio-Rad Laboratories). Twenty-microgram protein equivalents were separated by SDS-PAGE then blotted onto polyvinylidene difluoride filter (Millipore, Watford, United Kingdom) and blocked overnight at 4°C in PBS-T containing 5% nonfat dried milk. Filters were probed for 2 hours with 1:500 to 1:2,000 dilutions of primary antibodies and then with a 1:3,000 dilution of horseradish peroxidase (HRP)–conjugated antirabbit or antimouse secondary antibody. HRP was detected using enhanced chemiluminescence HRP detection reagents (Amersham Pharmacia, Buckinghamshire, United Kingdom). Antibodies raised against RSK1, c-Jun, c-Jun phospho-Ser^{73}, JNK, phospho-JNK, and phospho-ERK were all obtained from Upstate Ltd. (Buckingham, United Kingdom). Antibody against RSK phospho-Ser^{363} was obtained from Abcam (Cambridge, United Kingdom) and antibodies against ERK, Sp1, and Sp3 were obtained from Autogen Bioclear UK Ltd. (Wiltshire, United Kingdom).

Chromatin immunoprecipitation assays. Formaldehyde cross-linking and chromatin immunoprecipitation were done as described previously (12, 15). A2780 cell cultures were treated with formaldehyde for 10 minutes followed by the addition of glycine to a final concentration of 0.125 mol/L. Cells were then washed twice with cold PBS and were resuspended in lysis buffer [1% SDS, 10 mmol/L EDTA, and 50 mmol/L Tris-HCl (pH 8.1)] containing proteinase inhibitor. DNA was sonicated to an average fragment size of 500 bp and cross-linked proteins were enriched by immunoprecipitation with Sp1 and Sp3 antibodies. A "no Ab" sample was included as a negative control for the immunoprecipitation step. After reversal of the cross-links and DNA purification, the extent of enrichment was monitored by PCR amplification of the hTR promoter using the primers detailed below. The PCR product was analyzed both by gel electrophoresis and by real-time PCR. For real-time PCR analysis, the ratio of specifically precipitated versus input chromatin was calculated, with the background (no Ab) subtracted. The input sample was processed with the rest of the samples from the point at which the cross-links were reversed.

Quantitative real-time reverse transcription-PCR. Quantitative PCR was done using GRI Opticon monitor equipment and software (Genetic Research Instrumentation, Essex, United Kingdom). Sybr green was used as the fluorophore for detection of amplified DNA. Reactions were done in triplicate. The primers 5′-CTAACCCTAACTGAGAAGGGCGTA-3′ and 5′-GGCGAACGGGCCAGCAGCTGACATT-3′ were used for detection of endogenous hTR and the primers 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′ were used for detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers 5′-CCCGCCCGA-GAGAGTGAC-3′ and 5′-AAGTCAGCGAGAAAAACAGC′3′ were used for detection of the hTR promoter following chromatin immunoprecipitation experiments. Primer dimers were excluded from the quantification by performing the optical read step at 81°C.

To analyze effects of JNK inhibition on endogenous hTR levels, exponentially growing cells were treated for 16 hours with 25 μmol/L SP600125 or with DMSO followed by RNA extraction, cDNA synthesis, and quantitative PCR. The mean value of the triplicate measurements of hTR levels was normalized to the mean value of the GAPDH triplicates.

To analyze the relative levels of Sp1 and Sp3 at the hTR promoter *in vivo*, cells were treated for 16 hours with DMSO or 25 μmol/L SP600125 followed by chromatin immunoprecipitation and quantitative PCR. Background (no Ab) was subtracted and immunoprecipitated samples were compared with the input sample. Additionally, Sp1 immunoprecipitate levels were compared with those of Sp3. Quantitative PCR was repeated twice for each experiment, and the experiments were repeated four times.

Statistical analysis. Statistical analysis of all experiments was done by ANOVA using the Microsoft Excel data analysis tool pack. Mean values from each independent experiment were included in the analyses. Differences were statistically significant with $P < 0.05$ or highly significant with $P < 0.01$.

Results

The JNK pathway represses the hTR promoter *in vitro*. To determine whether the JNK pathway regulates the hTR promoter *in vitro*, we transfected A2780 ovarian adenocarcinoma cells with a 176 bp hTR promoter-luciferase reporter construct. Our previous studies using this cell line have indicated that the basal hTR

promoter activity is very strong in these cells by comparison with other cell lines and they, therefore, provide a robust model for analysis of promoter regulation (8, 9). Thirty-two hours post-ransfection, cells were treated for 16 hours with a titration of the JNK inhibitor SP600125. Concentrations of SP600125 over 12.5 μmol/L increased hTR promoter activity (Fig. 2*A*). The activity of the hTR promoter was induced by 2-fold at 12.5 μmol/L ($P < 0.05$) and by 2.4-fold at 50 μmol/L ($P < 0.01$). Therefore, despite high-level basal hTR promoter activity in these cells, manipulation of signal transduction pathways can still lead to significant promoter up-regulation. These data also suggested that JNK signaling might repress the hTR promoter.

To verify these results, expression vector encoding the constitutively active kinase domain of MEKK1, a major MAP3K for the JNK pathway, was used to test whether activation of the JNK pathway would repress hTR promoter activity. Figure 2*B* shows that MEKK1 led to strong repression of the hTR promoter. Using a 1:1 ratio of hTR/MEKK1 vectors, hTR promoter activity was reduced to 1.8% of basal levels in A2780 cells. We also did this experiment using five other cancer cell lines (5637 bladder carcinoma cells, C33A cervical carcinoma cells, HT29 colon carcinoma cells, HCT116 colon carcinoma cells, and A549 lung adenocarcinoma cells). MEKK1 transfection led to strong repression of the hTR promoter in all cells tested ($P < 0.01$). Together, these data suggested that the canonical JNK pathway might repress the hTR promoter.

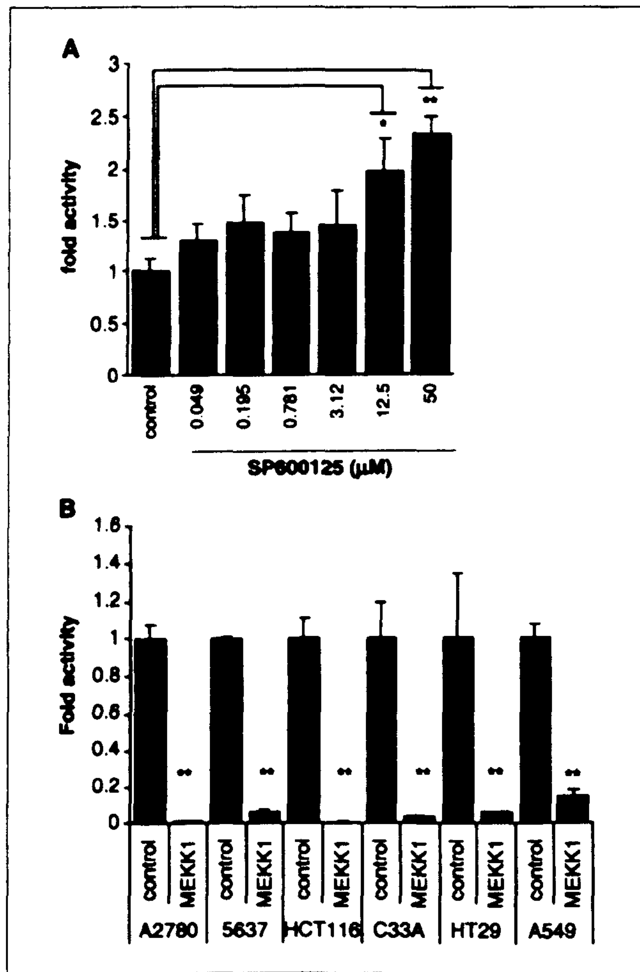

Figure 2. The JNK pathway represses the hTR promoter *in vitro*. *A*, a luciferase reporter driven by a 176 bp fragment of the wild-type hTR core promoter (nucleotides -107/+69) was transiently transfected into A2780 ovarian cancer cells. Thirty-two hours after transfection, the cells were exposed to a titration of the JNK inhibitor SP600125 for a further 16 hours before luciferase assay. *B*, the hTR core promoter was cotransfected in the cells indicated at a 1:1 ratio with either empty vector or pCMV-MEKK1, encoding a fusion protein comprising the constitutively active kinase domain of MEKK1. Luciferase activities were quantified 48 hours later. Relative luciferase activities were normalized using both an internal control vector and by measurement of luciferase activity in protein equivalents. All experiments were done in quadruplicate and repeated thrice. *Columns*, means derived from three independent experiments and represented as fold reporter activity relative to control; *bars*, SE. Results were analyzed by ANOVA (*, $P < 0.05$, **, $P < 0.01$).

To confirm that JNK is the effector of MEKK1-mediated hTR promoter repression, we examined the effect of blocking or augmenting activated MEKK1 signaling in A2780 and 5637 cells. As shown in Fig. 3*A*, a 10:1 ratio of hTR reporter/MEKK1 repressed hTR promoter activity to 42% of basal levels in A2780 cells. MEKK1-mediated repression was completely blocked by 25 μmol/L SP600125 ($P < 0.05$) and hTR promoter activity was even activated relative to basal levels in the presence of both MEKK1 and SP600125. In 5637 bladder cancer cells, a 2.5:1 ratio of hTR reporter/MEKK1 repressed hTR promoter activity to 55% of basal levels. In cells treated with both MEKK1 and 25 μmol/L SP600125, this effect was completely blocked ($P < 0.05$) and hTR promoter activity was strongly elevated relative to control. Thus, chemical inhibition of JNK blocks MEKK1-mediated repression of the hTR promoter.

We also hypothesized that repression of hTR promoter activity by MEKK1 could be enhanced by additional overexpression of JNK (Fig. 3*B*). A2780 or 5637 cells were transfected with a 10:1:4 ratio of hTR reporter/MEKK1/wild-type JNK1 or $JNK2^{APF}$, which harbors mutated phosopho-acceptor residues and, therefore, cannot be activated by upstream MAPK kinase. In A2780 cells, MEKK1 alone reduced hTR promoter activity to 22% of basal levels. As predicted, repression of the hTR promoter by MEKK1 was further enhanced in the presence of wild-type JNK1 (10% of basal levels, $P < 0.05$), whereas $JNK2^{APF}$ was not able to enhance MEKK1-mediated repression. In 5637 cells, this dilution of MEKK1 only had a mild effect on hTR promoter activity, which was not significant (83% of basal levels). However, the addition of wild-type JNK1 enhanced repression to 62% of basal levels ($P < 0.05$ compared with control). Additionally, the phosphoacceptor mutant $JNK2^{APF}$ blocked mild repression by MEKK1 in these cells. These results confirm that JNK activation can mediate repression of the hTR promoter by MEKK1.

The JNK pathway represses endogenous hTR expression. To determine whether the JNK pathway affects the endogenous expression of hTR, exponentially growing A2780 cells, 5637 cells, or C33A cervical cancer cells were incubated with 25 μmol/L SP600125 or DMSO control. After 16-hour treatment, RNA was extracted from the cells and the levels of hTR were analyzed by quantitative PCR and compared with those of GAPDH. The results from three independent paired treatments were analyzed twice by quantitative PCR. The pooled data, shown in Fig. 4*A*, show that SP600125 treatment led to a 63% increase in endogenous hTR levels in A2780 cells and increases of 88% and 94% in 5637 and C33A cells, respectively. GAPDH levels were not affected by the treatment (data not shown), whereas induction of hTR in the presence of SP600125 was statistically significant as determined by ANOVA ($P < 0.05$ in A2780 cells, $P < 0.01$ in C33A and 5637 cells). These data indicate that JNK inhibition can substantially increase the steady-state hTR level in diverse cell lines even where hTR expression levels are already high (10).

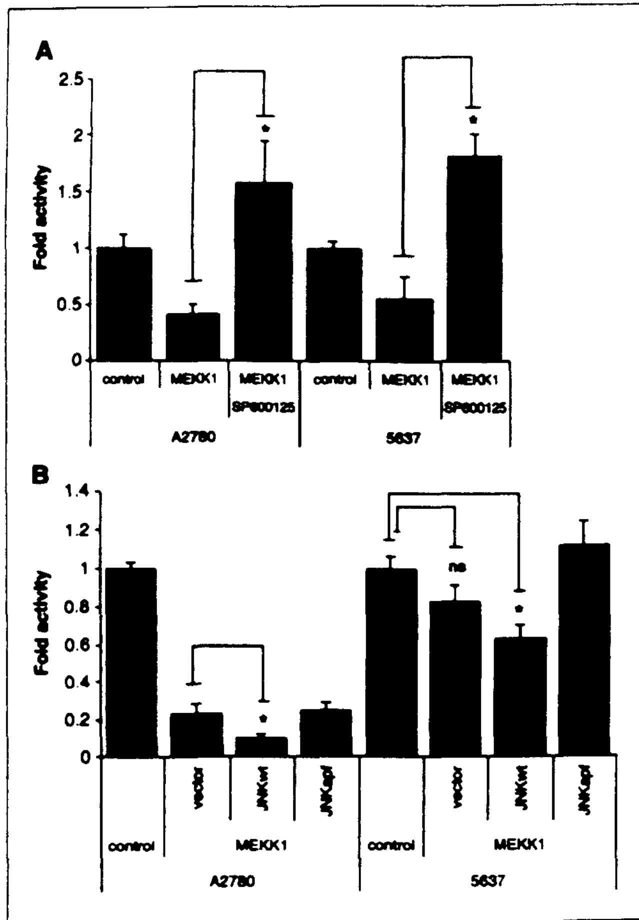

Figure 3. JNK activation mediates repression of the hTR promoter by MEKK1. *A*, a luciferase reporter driven by the wild-type hTR core promoter (nucleotides 107/+69) was transiently cotransfected into A2780 ovarian cancer cells or 5637 bladder cancer cells with empty vector or with pCMV-MEKK1 at a 10:1 ratio (reporter/MEKK1) in A2780 cells or a 2.5:1 ratio on 5637 cells. Thirty-two hours after transfection, MEKK1-transfected cells were treated with 25 μmol/L SP600125 or DMSO for a further 16 hours before luciferase assay. *B*, a luciferase reporter driven by the wild-type hTR core promoter was transiently cotransfected into A2780 ovarian cancer cells or 5637 bladder cancer cells with empty vector or with pCMV-MEKK1 alone, or in combination with wild-type pCMV-JNK1 or pCMV-JNK2^{APF} at a 10:4:1 ratio (reporter/JNK/MEKK1). JNK2^{APF} encodes an inactive JNK2 mutant in which the activating Thr-Pro-Tyr phosphoacceptor residues are mutated to Ala-Pro-Phe. Forty-eight hours posttransfection, luciferase activities were determined. Relative luciferase activities were normalized using both an internal control vector and by measurement of luciferase activity in protein equivalents. All experiments were done in quadruplicate and repeated thrice. *Columns*, means derived from three independent experiments and represented as fold reporter activity relative to control; bars, SE. Results were analyzed by ANOVA (*, $P < 0.05$).

To confirm that JNK activity was specifically inhibited by SP600125, exponentially growing A2780 cells were treated for 16 hours with 25 μmol/L SP600125. After treatment, protein was extracted and Western blot analysis was done for total expression and phosphorylation of ERK and the ERK-specific substrate p90-RSK1, and for JNK and the major JNK substrate c-Jun. As shown in Fig. 4*B*, SP600125 did not significantly affect the levels or phosphorylation of ERK, p90-RSK1, JNK, or the level of c-Jun protein; however, c-Jun Ser^{73} phosphorylation was thoroughly inhibited, demonstrating that SP600125 does not interfere with the activating phosphorylation of JNK but with its downstream signaling as expected. In contrast, SP600125 neither inhibited the phosphorylation of ERK or its downstream substrate RSK. Thus, under conditions of JNK inhibition, hTR expression is increased. Taken together, Figs. 2 to 4 suggest that the JNK pathway is a genuine repressor of hTR levels and that elements in the core promoter are likely to participate in JNK-mediated repression.

Repression of the hTR promoter involves Sp1 and Sp3. Several studies have indicated that MAPK pathways may regulate gene expression partly through Sp1/Sp3 binding elements (19, 20, 24, 25). To determine whether the hTR core promoter Sp1/Sp3 sites are involved in JNK-mediated repression of hTR promoter activity, we used an hTR reporter construct carrying functional mutations in all Sp1/Sp3 sites (construct no Sp1). Previous

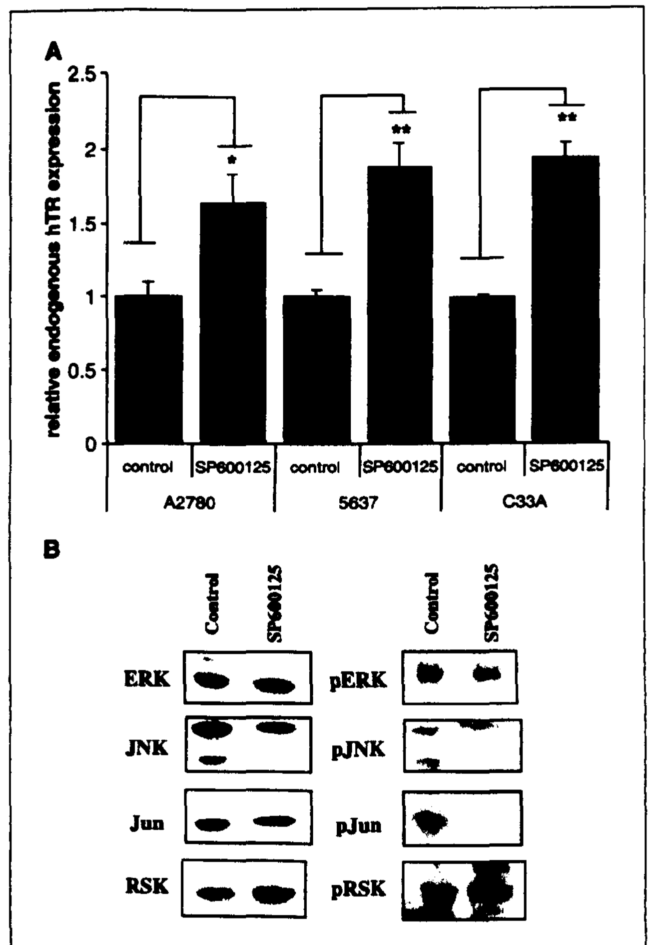

Figure 4. JNK inhibition increases endogenous levels of hTR. *A*, up-regulation of endogenous hTR levels. Exponentially growing A2780 cells, 5637 cells, or C33A cervical cancer cells were treated for 16 hours with 25 μmol/L SP600125 or with DMSO control. After treatment, cDNA was synthesized from RNA extracted from treated and untreated cells. Levels of endogenous hTR in control and treated samples were analyzed by quantitative PCR using hTR-specific primers and normalized to levels of GAPDH. The experiment was repeated thrice and quantitative PCR was done twice on each independent experiment. *Columns*, means of pooled data from all independent experiments; *bars*, SE. Results were analyzed by ANOVA (*, $P < 0.05$, **, $P < 0.01$). *B*, inhibition of JNK activity. Exponentially growing A2780 cells were treated for 16 hours with 25 μmol/L SP600125 or DMSO control. After treatment, protein extracts were made and probed for inhibition of the JNK pathway by Western blotting 20 μg extract for total and phosphorylated levels of ERK and its substrate RSK1 and for JNK and its substrate c-Jun. Experiments were repeated at least twice. Representative blots are shown.

characterization determined that binding and regulation by Sp1 and Sp3 but not by NF-Y or MDM2 are specifically ablated in this construct (11, 12).

A2780 and 5637 cells were transfected in parallel with the wild-type or no-Sp1 reporters and were treated with DMSO or 25 μmol/L SP600125 (Fig. 5A). In these experiments, 25 μmol/L SP600125 resulted in 2.2-fold induction of wild-type promoter activity in A2780 cells but induction of the reporter lacking Sp1/Sp3 sites was attenuated, reaching only 1.5-fold induction ($P < 0.05$). In 5637 cells, SP600125 induced the wild-type hTR promoter by 1.61-fold but the mutant promoter was induced by only 1.26-fold ($P < 0.05$). These results suggested that part of the mechanism for induction of hTR promoter activity by JNK inhibition involves Sp1 and/or Sp3 proteins, although the incomplete attenuation indicates that other factors may also be involved. Our previous studies have shown that Sp1 activates the hTR promoter, whereas Sp3 is a repressor. We, therefore, sought to investigate whether Sp3 is involved in repression of hTR by JNK.

A2780 or 5637 cells were cotransfected the wild-type hTR promoter along with vector or MEKK1 and vector or Sp3 in a 5:1:2 ratio (reporter:MEKK1/vector:Sp3/vector). For each experiment, we also calculated the product of the repression mediated by MEKK1 and Sp3 individually as a representation of the expected promoter activity assuming additive effects between MEKK1 and Sp3. As shown in Fig. 5B, MEKK1 alone repressed the hTR to 30% of basal levels in A2780 cells, whereas Sp3 alone repressed the hTR to 68% of basal levels. The calculated expected promoter activity (*exp column*), assuming only additive effects between Sp3 and MEKK1, was 18% of basal levels. However, cotransfection of both MEKK1 and Sp3 repressed hTR promoter activity to 9% of basal levels ($P < 0.05$).

In 5637 cells, MEKK1 transfection alone resulted in 50% reduction of hTR promoter activity, whereas Sp3 alone decreased promoter activity to 55% of basal levels. Across all experiments, the expected promoter activity assuming perfect additive effects between MEKK1 and Sp3 (Fig. 5B, *exp column*), would have been 27% of basal levels. Instead, in the presence of both MEKK1 and Sp3, hTR was reduced to only 11.5% of basal levels ($P < 0.05$). Therefore, in both 5637 and A2780 cells, Sp3 and MEKK1 seem to act cooperatively to repress the hTR promoter.

JNK regulates Sp1/Sp3 binding at the hTR promoter *in vivo*. Several studies have suggested that regulated Sp1/Sp3-dependent promoter activation may be mediated by a change in the ratio of Sp1 and Sp3 expression (27-29). To determine whether our results reflect altered expression of Sp1 or Sp3 (e.g., repression of Sp1 expression by JNK), we did Western blots for total cellular levels of Sp1 and Sp3 in control cells or cells treated for 16 hours with SP600125. As shown in Fig. 6A, 16-hour treatment with 25 μmol/L SP600125 had no significant effect on the levels of Sp1 or Sp3 protein. Therefore, any JNK-dependent regulation of Sp1 or Sp3 is likely to be mediated through a posttranslational mechanism.

To investigate the hypothesis that the JNK pathway enhances repression of the hTR promoter by Sp3 and/or constrains Sp1-mediated activation in these cells, we did chromatin immunoprecipitation experiments, pulling down either Sp1 or Sp3 from control cells or from cells treated for 16 hours with 25 μmol/L SP600125. The presence of the hTR promoter in chromatin immunoprecipitates was assessed using both conventional and quantitative PCR.

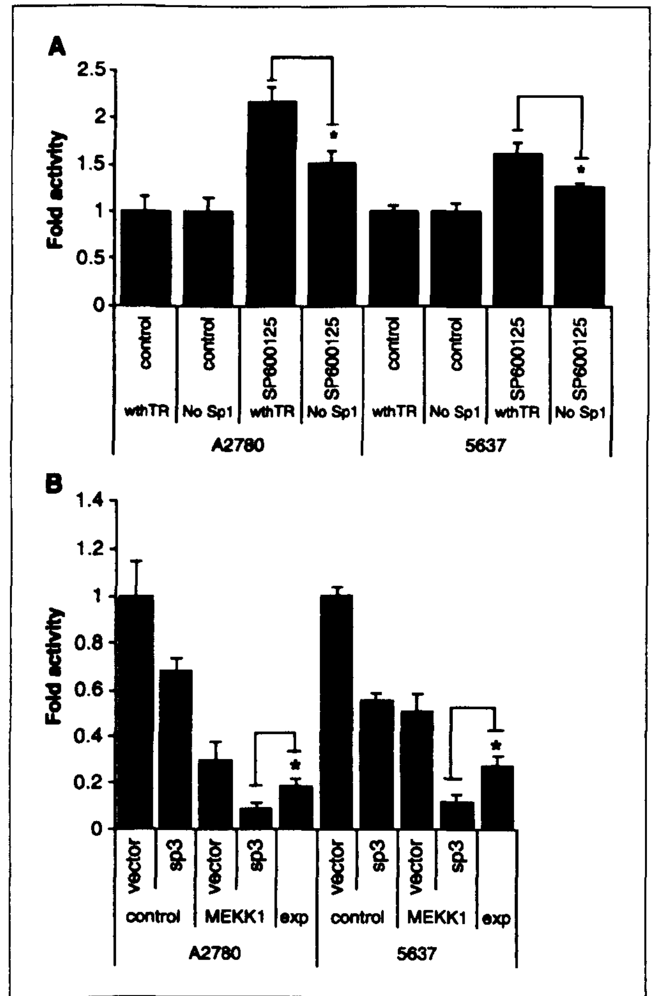

Figure 5. Repression of the hTR promoter JNK involves Sp1 and Sp3. *A,* induction of promoter activity by SP600125 requires Sp1 binding sites. Luciferase reporters driven by either the wild-type hTR core promoter (nucleotides −107/+69) or a mutation construct in which all the Sp1 binding sites are ablated (*construct no Sp1*) were transiently transfected into A2780 ovarian cancer cells or 5637 bladder cancer cells. Thirty-two hours after transfection, cells were treated with 25 μmol/L SP600125 or DMSO for a further 16 hours before luciferase assay. *B,* MEKK1 enhances Sp3-dependent repression of the hTR promoter. Wild-type hTR core promoter reporter was transiently cotransfected into A2780 ovarian cancer cells or 5637 bladder cancer cells with vector or MEKK1 and vector or Sp3. Forty-eight hours posttransfection, luciferase activities were determined. The expected repression by MEKK1 + Sp3 assuming ideal additive effects was calculated in each experiment from the product of the fold repressions by Sp3 and MEKK1 alone. All experiments were done in quadruplicate and repeated thrice. *Columns,* means derived from three independent experiments and are represented as fold reporter activity relative to control; *bars,* SE. Results were analyzed by ANOVA (*, $P < 0.05$).

Sp1-specific products seemed weak compared with the Sp3-specific products in control A2780 cells (Fig. 6B and C). This may simply reflect the different affinities of the two antibodies for their targets or it may indicate that Sp3 occupancy of the promoter is greater than Sp1. More importantly, after 16-hour treatment with SP600125, the intensity of the Sp1-specific product had increased, whereas that of Sp3 was reduced. As shown in Fig. 6D, pooled data from all experiments showed that Sp1 represented 33% of the total Sp1/Sp3 associated with the hTR promoter in control cells but increased to 55% in JNK-inhibited cells ($P < 0.05$). In contrast, Sp3 constituted 67% of the total Sp1/Sp3 protein pulled down in

control cells but the proportion fell to 45% in JNK-inhibited cells ($P < 0.05$). Thus, JNK inhibition results in a shift in the ratio of immunoreactive Sp1 and Sp3 at the hTR promoter *in vivo*, favoring an increase in the levels of the Sp1 epitope.

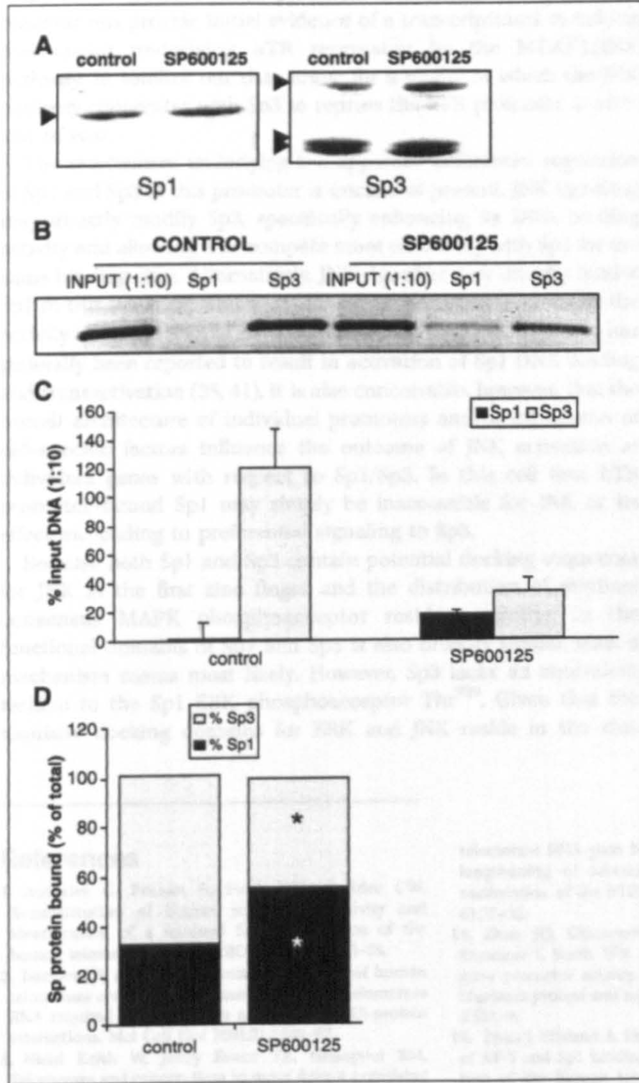

Figure 6. The JNK pathway modulates Sp1 and Sp3 binding to the hTR promoter *in vivo*. *A*, cellular levels of Sp1 and Sp3 are not affected by SP600125. Exponentially growing A2780 cells were treated for 16 hours with 25 μmol/L SP600125 or DMSO control. After treatment, protein extracts were made and probed for total cellular levels of Sp1 and Sp3 protein. Sp1 p95 was the main product; Sp3 p100 (full-length) and p60 (alternative translation initiation site) isoforms were detected. The experiment was repeated twice and representative blots are shown. *B* and *C*, SP600125 increases detection of Sp1 in chromatin immunoprecipitates. Exponentially growing A2780 cells were treated either with DMSO or with 25 μmol/L SP600125 for 16 hours. After the treatment periods, proteins were cross-linked to DNA using formaldehyde and chromatin was immunoprecipitated using antibodies specific for Sp1 or Sp3. The presence of the hTR promoter in precipitated chromatin was monitored by conventional PCR (*B*) and quantitative PCR (*C*) using primers specific for the hTR promoter. One representative experiment. Input DNA samples were diluted 1:10. *D*, increased ratio of immunoreactive Sp1 relative to Sp3 detected at the hTR promoter *in vivo* after SP600125 treatment. After subtraction of background, Sp1 and Sp3 levels were compared with the total amount of Sp1 + Sp3 detected in chromatin immunoprecipitation. Chromatin immunoprecipitation experiments were repeated four times and each experiment was analyzed twice by quantitative PCR. Pooled data for all experiments. The relative shifts in Sp1 or Sp3 levels were analyzed by ANOVA (*, $P < 0.05$).

Discussion

Significant interest in recent years has focused on *hTERT* gene regulation as a mechanism controlling telomerase in cancer cells. However, recent findings in mice and humans have indicated that control of hTR levels may also contribute to overall regulation of telomerase. In the mTR knockout mouse, telomerase activity is abrogated resulting in telomere shortening and late-generation phenotypic abnormalities in multiple organs (30, 31). Interestingly, and, more recently, it has been shown that haploinsufficiency of mTR in mTR+/− heterozygotes results in defects of telomere elongation independent of telomerase activity detected by telomere repeat amplification protocol assay (32). In humans, hTR haploinsufficiency has been documented in autosomal dominant dyskeratosis patients (33). Indeed, several mutations that have the effect of reducing the stability of hTR are documented in autosomal dominant dyskeratosis (34).

Together, these data indicate that hTR levels are essential for telomerase holoenzyme function *in vivo*, although sparse data exist regarding signal transduction pathways affecting hTR expression. *In situ* hybridization analysis of 800 tumor biopsy samples show clearly that hTR levels are up-regulated specifically in cancer cells (35–40). It is, therefore, critical to begin to define pathways affecting hTR.

In this report, we provide evidence that JNK represses the hTR core promoter in transfection assays and also represses endogenous hTR levels partly via regulation of Sp1 and Sp3 at the hTR promoter. We found that the JNK inhibitor SP600125 up-regulated activity of a transiently transfected hTR core promoter reporter in a dose-dependent manner and also increased endogenous levels of hTR at 25 μmol/L. At this concentration, SP600125 also abrogated phosphorylation of c-Jun but not of RSK1, a major ERK specific effector, suggesting that the JNK pathway was indeed specifically inhibited at SP600125 concentrations leading to elevation of endogenous hTR.

Conversely, overexpression of active MEKK1, a major MAP3K for the JNK pathway, strongly repressed promoter activity. Further, repression by MEKK1 could be completely blocked by 25 μmol/L SP600125 or enhanced by coexpression of wild-type JNK1 but not the phosphoacceptor mutant $JNK2^{APF}$. Together, these data suggest strongly that JNK is a major effector of MEKK1-mediated repression of the hTR promoter and that elements in the core promoter may be involved in induction of hTR following JNK inhibition.

We next assessed whether repression by the JNK pathway could be attributed to Sp1/Sp3-dependent mechanisms. Induction of the core promoter by SP600125 was partly dependent on intact, functional Sp1 binding sites, indicating that Sp1/Sp3 could indeed have a role in integrating JNK signaling at the hTR core promoter. Overexpression of MEKK1 enhanced repression by Sp3, suggesting that MEKK1 might selectively favor binding or activity of Sp3 at the hTR promoter.

Finally, we showed by chromatin immunoprecipitation that SP600125 treatment increases detection of Sp1 at the hTR promoter *in vivo* with a concomitant relative decrease in detectable Sp3. These observations are consistent with a model in which the JNK pathway enhances binding and/or trans-repression by Sp3 and suppresses Sp1-dependent transcription of hTR. It is important to note that altered Sp1/Sp3 immunoreactivity does not necessarily reflect overall levels of Sp1/Sp3 binding. It may instead reflect other changes within promoter bound complexes

that unmask the Sp1 epitope. However, in light of our other findings reported here, there remains a strong possibility that Sp1 epitope unmasking in this context might reflect relief from inhibition. In context of our finding that MEKK1 cooperates with Sp3 to repress the hTR promoter, and based on our previous analyses of positive hTR promoter regulation by Sp1, these observations provide initial evidence of a transcriptional switching mechanism underlying hTR repression by the MEKK1/JNK pathway. In totality, our data argue for a model in which the JNK pathway cooperates with Sp3 to repress the hTR promoter *in vitro* and *in vivo*.

The mechanism underlying the apparent differential regulation of Sp1 and Sp3 at this promoter is unclear at present. JNK signaling may directly modify Sp3, specifically enhancing its DNA binding activity and allowing it to compete more effectively with Sp1 for the same binding sites. Alternatively, JNK signaling may directly inhibit Sp1 in this instance, which would also be expected to enhance the activity of Sp3. It should be noted, however, that JNK signaling has generally been reported to result in activation of Sp1 DNA binding and transactivation (25, 41). It is also conceivable, however, that the overall architecture of individual promoters and/or expression of cell-specific factors influence the outcome of JNK activation at individual genes with respect to Sp1/Sp3. In this cell line, hTR promoter-bound Sp1 may simply be inaccessible for JNK or its effectors, leading to preferential signaling to Sp3.

Because both Sp1 and Sp3 contain potential docking sequences for JNK in the first zinc finger and the distribution of minimal consensus MAPK phosphoacceptor residues relative to the functional domains of Sp1 and Sp3 is also broadly similar, such a mechanism seems most likely. However, Sp3 lacks an equivalent residue to the Sp1 ERK phosphoacceptor Thr^{739}. Given that the requisite docking domains for ERK and JNK reside in the zinc fingers and are, therefore, likely to be proximal in the tertiary structure of Sp1, it is possible that JNK binding to its putative domain might mask the ERK docking site, thereby inhibiting phosphorylation on Thr^{739} and, thus, DNA binding. Such a mechanism would not be expected to affect Sp3 due to the absence of an equivalent residue, although it is uncertain whether a JNK active site inhibitor could unmask such an effect.

In conclusion, this report provides the first evidence that Sp1 and Sp3 may act to integrate stress and/or growth signals at the hTR promoter. It will be of particular interest to investigate whether JNK also represses hTR in cells with low hTR levels, such as normal cell strains, and, if so, what its relative contribution to hTR repression is. From a therapeutic perspective, improved understanding of the signal transduction pathways that regulate the hTR gene promoter should allow for the development of lead drug candidates targeting pathways involved in hTR transcription. Several clinically relevant cytotoxic drugs in widespread use, including cisplatin, are known to activate the JNK pathway as part of their mechanism of action (42). Interestingly, some ovarian cancer cells acquire drug resistance through down-regulation of DNA damage pathways while retaining competent but compromised JNK activation (43, 44). It will also be of interest to determine whether long-term subtoxic schedules of such drugs might have a beneficial effect resulting from inhibition of telomerase even in cells that have acquired resistance to apoptosis.

Acknowledgments

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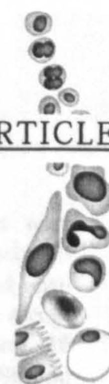

Telomerase promoter reprogramming and interaction with general transcription factors in the human mesenchymal stem cell

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The human adult mesenchymal stem cell (hMSC) does not express telomerase and has been shown to be the target for neoplastic transformation after transduction with hTERT. These findings lend support to the stem cell hypothesis of cancer development but by supplying hTERT, the molecular events required to upregulate hTERT expression in cancer development are missed. Therefore, the hMSC is ideal for the identification of molecular mechanisms regulating telomerase gene expression in stem cells. This study shows that the repression of hTERT expression in hMSC is chromatin based and that modifications of the chromatin environment lead to reactivation of telomerase gene expression. It is shown that repression of hTERT expression in hMSCs is due to promoter-specific histone hypoacetylation coupled with low Pol II and TFIIB trafficking. This repression is overcome by treatment with Trichostatin A (TSA), an HDAC inhibitor, concomitant with increases in promoter-specific histone acetylation and increases in Pol II and TFIIB tracking. hTR expression is also increased in TSA-treated hMSCs, concomitant with changes in Pol II and TFIIB dynamics.

Stem cells are unique in their ability to both self renew and to give rise to differentiated tissues. Mesenchymal stem cells (hMSC) are multipotential cells. Thus, hMSC have the ability both *in vivo* and *in vitro*, to differentiate into a variety of adult mesenchymal tissues, such as bone, cartilage, adipose and muscle [1,2] and therefore may be of use in the treatment of a diverse variety of clinical conditions. In addition, there are now strong links between normal stem cells and cancer stem cells [3], suggesting that stem cells are targets for neoplastic transformation. Interestingly, despite having stem cell properties, when passaged in culture the hMSC gradually reduce their proliferative rate and enter replicative senescence [1,2]. hMSCs also lack activity for the immortalizing enzyme telomerase which can counteract senescence by maintaining telomere sequence [1,2]. Further, when hTERT is ectopically expressed in the hMSC, neoplastic changes are accumulated suggesting that the adult hMSCs can be targets for neoplastic transformation [4].

The expression of telomerase is regulated in normal human cells. During development, telomerase expression is extinguished after embryonic differentiation in most cells. Notable exceptions are male germ cells, lymphocytes and some stem cell populations, which maintain their proliferative capacity in adult life, are telomerase competent and can express low levels of telomerase. By contrast, telomerase is highly active in the vast majority of human tumors [5–7]. Thus, a key issue is how this complex pattern of expression is maintained in adult tissues and in particular the adult stem cell and how this is deregulated during neoplastic progression [8].

Telomerase activity is achieved through the expression of two genes, the *hTR* gene, which codes for the essential RNA component of telomerase, and the *hTERT* gene, which codes for the telomerase protein component that has reverse transcriptase activity. A major mechanism to differentially regulate telomerase is transcriptional control of the *hTERT* and *hTR* genes. Although much remains to be clarified, the cloning of the *hTR* and *hTERT* promoter region has enabled the identification of several positive and negative regulators of transcription [9].

However, gene expression is not only determined by the availability of combinations of transcription factors but also by chromatin context and in particular by covalent modifications of the core histones including acetylation, phosphorylation and methylation [10]. Distinct modification patterns at promoter sequences have been linked to expression and repression, and the dynamics of histone modification in transcriptional control is ideally suited to the task of organizing complex programs of gene activity necessary for many cellular processes including cell lineage commitment, cellular senescence and tumorigenesis [11–13]. Formation

Keywords: chromatin, epigenetics, hTERT, hTR, mesenchymal, telomerase, stem cell

future medicine

of the basal transcriptional machinery into transcriptional complexes on promoter sequences is also a major step in gene transcription. It is known that telomerase has a critical role in tumor progression and cancer cell survival; therefore it is crucial that the mechanisms regulating telomerase gene expression are well understood. Studies have shown that distinct patterns of histone modifications are linked to *hTR* and *hTERT* gene regulation [14] and alterations in expression levels are concomitant with dynamic changes in basal transcription machinery binding [15].

Thus, the lack of *hTERT* expression in the hMSC provides an ideal model to study telomerase gene regulation in the normal adult stem cell. This allows us to study all stages of promoter function including the key steps of transcription preinitiation complex formation, promoter clearance and elongation in the context of chromatin remodeling. The histone deacetylase inhibitor Trichostatin A (TSA) has been shownpreviously to cause an increase in histone acetylation at the hTERT promoter and cause re-expression of the *hTERT* gene [16–18]. To this end, we have studied *hTERT* and *hTR* expression, the acetylation status of the hTR and hTERT promoters and RNA Pol II and TFIIB dynamics in the hMSC by chromatin immunoprecipitation (ChIP), before and after treatment with TSA.

Material & methods

Isolation of hMSC & cell culture

Primary hMSC isolated from bone marrow aspirates by centrifugation (700 g for 15 min at 4°C) over a Ficoll–Hypaque gradient (Sigma) as previously described by Pittenger and colleagues [19]. The resulting cells were cultivated in high-glucose (4.5 g/l) Dulbecco's modified Eagles medium (DMEM, Gibco, Life Technology, USA) supplemented with 10% fetal bovine serum (Gibco, Life technology), 100 U/ml of penicillin and streptomycin (Gibco, Life technology) and 2 mM of L-glutamine. Cells were plated out with a confluence of 75% and the next day TSA was added at a final concentration of 350 µM for 17 h. This was followed by fixing in neutral buffered formalin (at a final concentration of 3.7%) for ChIP assay.

Chromatin immunoprecipitation assays

ChIP assays for histone acetylation were performed following the instructions recommended by the kit supplier (Upstate Biotechnology, UK). Antibodies used were anti-acetylated histone H3 (AcH3), which recognizes acetylated lysines 9 and 14 and anti-acetylated histone H4 (AcH4), which recognizes acetylated lysines 5, 8, 12 and 16 (Upstate Biotechnology, Cat. No. 06-599 and 06-866). Sonication was optimized to give chromatin fragments of around 500 bp in length (8 × 10 second pulses at 5 µ with 20 s rest between each pulse on ice using an MSE Soniprep150 sonifier) and the resultant DNA from each immunoprecipitation (IP) was purified using the QIAquick PCR Purification Kit™ (Qiagen, UK). ChIP assays using anti-Pol II and anti-TFIIB (both Autogen Bioclear, UK) were undertaken as in Gomez-Ramon and colleagues [20]. In both ChIP techniques and in each individual experiment, a no-antibody control is used to detect any background, which if present is subtracted from the IPs to normalize.

Quantitative polymerase chain reaction

Products from the ChIP assay were quantified by quantitative polymerase chain reaction (Q-PCR) Opticon2 DNA Engine (MJ Research, Massachusetts) using primers to hTR and hTERT core promoter sequences and exon 12 of hTERT using SYBR Green Q-PCR Buffer (Finnzymes, Espoo, Finland) [14,15]. hTR Primers, 29SF 5'CCC GCC CGA GAG AGT GAC 3' and 5ALTR 5' AAG TCA GCG AGA AAA ACA GC 3', hTERT primers, TERTSF 5' TCC CCT TCA CGT CCG GCA TT 3' and TERTSR 5' AGC GGA GAG AGG TCG AAT CG 3' and Exon 12 primers, Ex12Fa 5'CAG GAC AAG GAA GCG GGA GGA 3' Ex 12Rab 5'CAG CCG CAA GAC CCC AAA GA 3' were used.

Expression analysis

hTR expression was analyzed by Q-PCR using the following primers (TRC3F – 5' CTAACCCTAACTGAGAAGGGCGTA 3' and TRC3R – 5' GGCGAACGGGCCAGCAGCTGACATT 3' and adjusted to GAPDH expression (primers – GAPDH0.45F – 5' ACCACAGTCCATGCCATCAC 3' and GAPDH0.45R – 5' CCA CCA CCC TGT TGC TGT A 3') [14,15]. hTERT expression was analyzed by methods outlined in Keith and colleagues [21] using primers which detect all splice variants (primers HT2026F 5' GCCTGAGCTGTACTTTGTCAA 3' AND HT2482R 5' GCCAAACAGCTTGTTCTCCATGTC 3'). hTERT expression is displayed as total amounts of all four main splice variants.

Results

hTERT *gene expression in hMSC & chromatin remodeling at the hTERT promoter*

hTERT expression was studied before and after treatment with TSA and levels of histone acetylation at the promoter were also studied by ChIP assays in order to link changes in the chromatin environment with any changes in gene expression. In control (untreated) hMSCs, there was a lack of expression of any hTERT transcripts (Figure 1A). Acetylation of histone H3 was not detected and acetylation of histone H4 was low (Figure 1B), linking the lack of expression of *hTERT* with low levels of promoter proximal histone acetylation. Upon treatment with TSA, hMSCs showed re-expression of full-length hTERT transcripts (Figure 1A) and this was associated with increased levels of AcH3 and AcH4, (Figure 1B) linking remodeling of the chromatin environment of the hTERT promoter with re-expression of *hTERT*.

Basal transcription machinery dynamics at the hTERT *gene in hMSCs.*

As well as monitoring acetylation, Pol II and TFIIB occupancy at the hTERT promoter and exon 12 of hTERT were also studied, to understand the trafficking of the basal transcription machinery along the *hTERT* gene. Exon 12 is located some 31 kb downstream from the promoter and therefore can give excellent data on the extent of basal transcription machinery tracking along the gene-coding sequence. Pol II levels were low at the hTERT promoter before treatment with TSA and decreased slightly after treatment (Figure 1C) perhaps signifying that any Pol II that was present at the promoter but was not engaged in transcription. Levels of Pol II at exon 12 were increased after treatment with TSA (Figure 1C), indicating that increasing amounts of Pol II was involved in the process of transcription of the *hTERT* gene.

TFIIB levels in hMSCs were increased at the promoter upon TSA treatment, perhaps due to the relaxing of the chromatin environment allowing the stabilization of the preinitiation complex and increased transcription of hTERT by Pol II. Low levels of TFIIB at exon 12 before and after TSA treatment suggests that the role of TFIIB is primarily at the promoter in stabilizing the preinitiation complex (Figure 1D).

hTR *gene expression in hMSC & chromatin remodeling at the hTR promoter*

hTR gene expression and promoter chromatin environment was also studied in hMSC before and after treatment with TSA. Figure 2A shows that hMSCs express *hTR* and levels increase upon treatment with TSA. However, ChIP analysis did not detect any change in acetylation of histones H3 and H4 before and after treatment with TSA (Figure 2B).

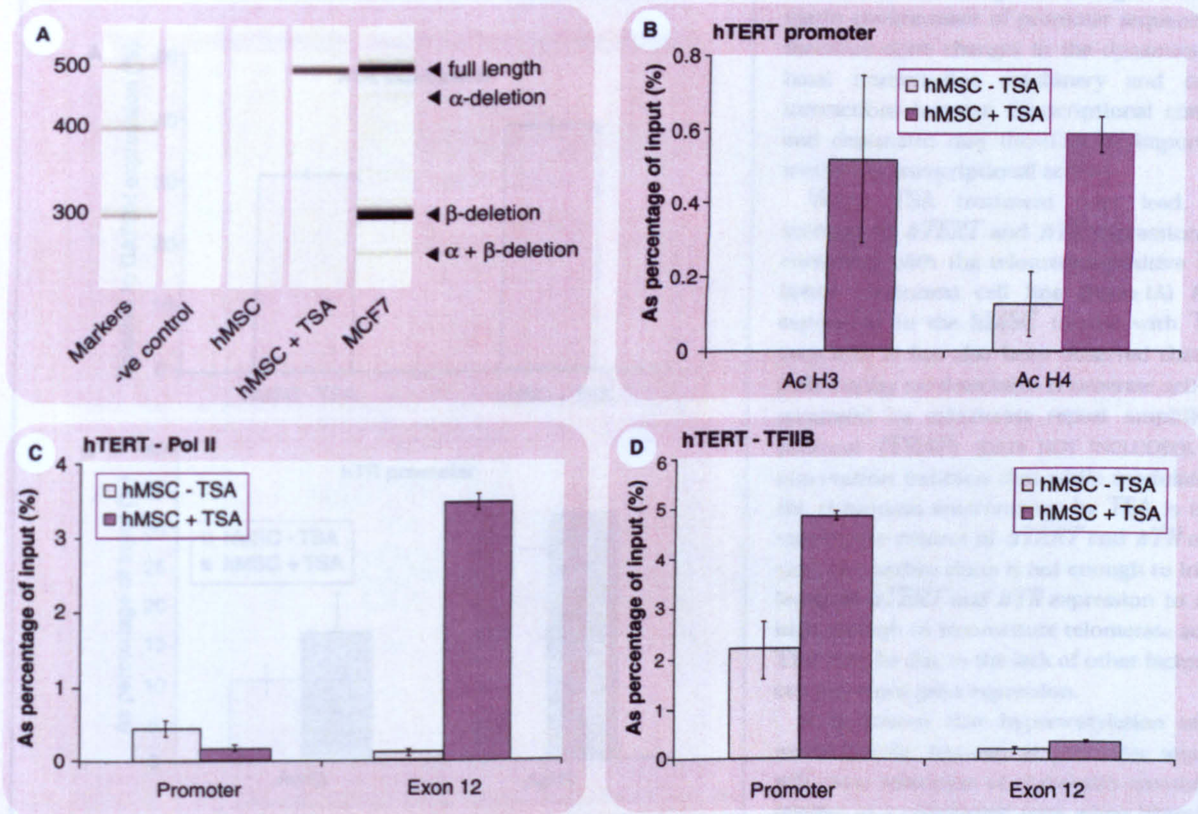
Basal transcription machinery dynamics at the hTR *gene in hMSCs*

Although there did not seem to be an increase in histone acetylation, hMSCs treated with TSA showed an increase in Pol II and TFIIB levels at the promoter sequence (Figure 2C). An increase in Pol II and TFIIB at the promoter could indicate an increase in stability of the transcriptional complex and therefore an increase in transcription levels of hTR that would account for the increase in expression. As the hTR gene is relatively short, extended analysis of Pol II and TFIIB trafficking was not possible.

Discussion

A common set of features distinguish cancer cells from their normal counterparts including loss of cell cycle regulation, loss of control over invasion and metastasis, failure of apoptotic mechanisms and bypass of senescence [7,22]. Cellular models designed to recapitulate these features of multistage neoplastic progression have recently been developed by a number of groups and epitomized by the work from the Weinberg lab [21]. A common element of these models is to use ectopic expression of the *hTERT* gene to overcome senescence and thus provide the immortalization step of cancer progression. However, whilst this model for neoplastic transformation may be of considerable use in defining a number of the key events in progression, by supplying telomerase, the molecular events required to upregulate *hTERT* in cancer development are inevitably missed. Given the critical role that telomerase has in tumor progression and with the cancer cell reliant on telomerase for its survival, it is essential that the mechanisms regulating telomerase gene expression are investigated [8]. Our findings give essential information about the regulation of endogenous telomerase gene expression in normal hMSCs. Understanding telomerase gene regulation in the hMSC, and similar cell types, is of importance. Any differences in regulation may reflect distinct regulatory pathways for telomerase gene expression in these cell types, which need not be the same as in normal and cancer cell lines, which has been published previously [14,15].

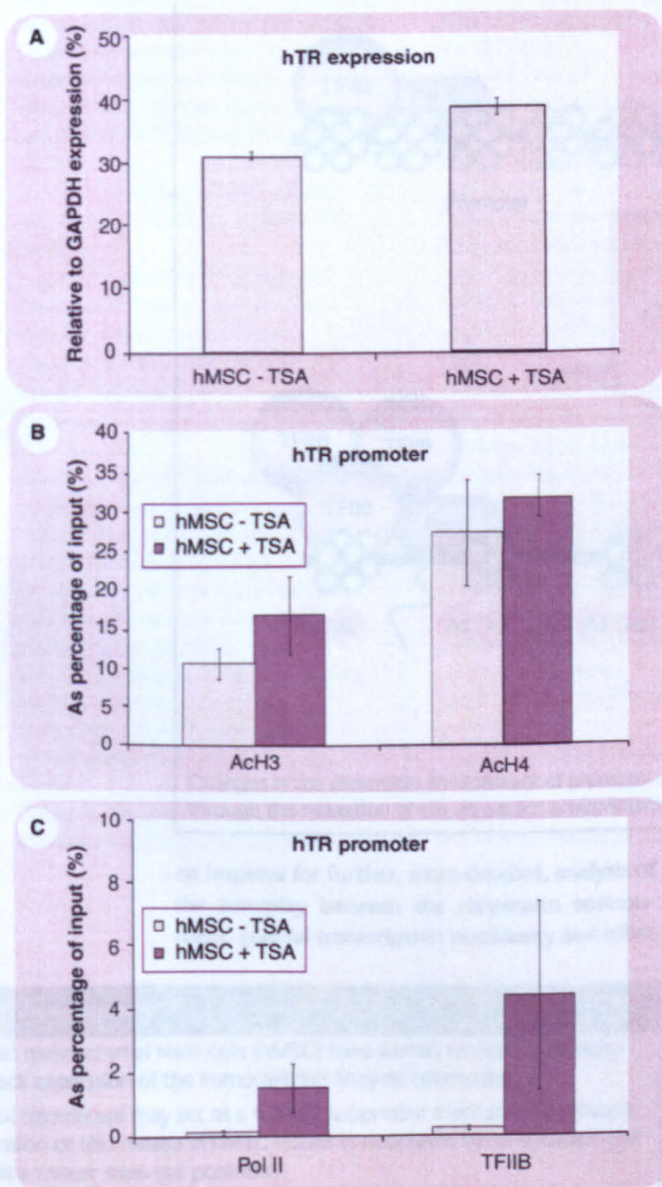
Figure 1. hTERT expression, chromatin environment and basal transcriptional machinery dynamics.



(A) hTERT spice variant expression analysis of hMSC before and after treatment with TSA, corrected to GAPDH expression and visualized using the Agilent Bioanalyser (Agilent Technologies). Lane 1 shows size markers, Lane 2, the negative control for the PCR reaction, Lane 3, hTERT expression for the hMSC, Lane 4, hTERT expression for the hMSC treated with TSA, and Lane 5, hTERT expression in the MCF-7 cell line as comparison. ChIP assays were performed for **(B)** histone acetylation (histones H3 and H4) status of the hTERT promoter **(C)** Pol II and **(D)** TFIIB occupancy of the promoter and exon 12 before and after treatment of hMSCs with TSA. Each IP is expressed as a percentage of an input sample to normalize and detected by Q-PCR in triplicate to generate error bars. No antibody controls were analyzed within each experiment and Q-PCR allowed the subtraction of any background from each IP.
hMSC: Human mesenchymal stem cell; IP: Immunoprecipitation; Q-PCR: Quantitative polymerase chain reaction; TSA: Trichostatin A.

The data presented shows that *hTERT* expression is repressed in the hMSC and that the mode of repression is chromatin based. Upon TSA treatment of hMSCs, *hTERT* becomes re-expressed concomitant with an increase in promoter histone acetylation. Beside *hTERT* expression, *hTR* expression is also increased upon TSA treatment although large changes in histone acetylation are not apparent. This suggests that chromatin and epigenetic mechanisms are involved in telomerase gene regulation in the hMSC. The chromatin-based repression of *hTERT* transcription and hence repression of telomerase activity may act as a tumor suppressor mechanism, whereby hMSCs would have a limited lifespan and cannot divide long enough to accumulate neoplastic changes and become tumorigenic. Basal transcription machinery dynamics were also changed upon TSA treatment. Association of Pol II with gene-coding regions has been previously shown to coincide with active gene transcription [23,24], and this agrees well with the data for *hTERT* for which we see an increased level of Pol II at exon 12 after treatment with TSA. Also apparent is a significant level of Pol II at the promoter sequence before treatment with TSA when hTERT is repressed. This may be due to a paused Pol II molecule being present at the promoter, perhaps indicating that the *hTERT* gene may be permissive for transcription, only needing changes in the

Figure 2. hTR expression, chromatin environment and basal transcriptional machinery dynamics.



(A) hTR expression analysis for hMSCs before and after treatment with TSA, detected by Q-PCR and corrected to GAPDH expression. ChIP assays were performed for (B) histone acetylation status (histones H3 and H4) of the hTR promoter and (C) Pol II and TFIIB occupancy of the hTR promoter.
Q-PCR: Quantitative polymerase chain reaction; TSA: Trichostatin A.

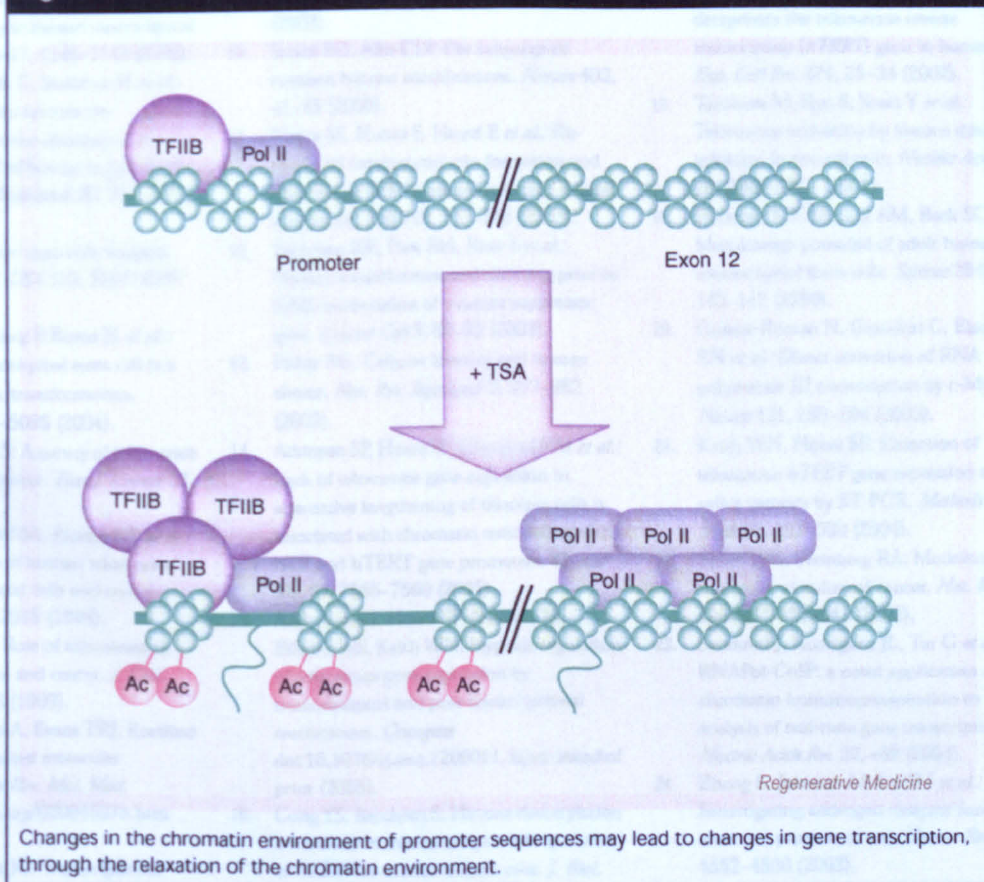
chromatin environment of the promoter to allow transcription (Figure 3). The apparent decrease in Pol II binding at the promoter and the large increase at exon 12 can be accounted for by increased Pol II tracking along the gene, being no longer paused at the promoter. Apparent differences in Pol II dynamics between *hTR* and *hTERT* may reflect the different regulation of each of these genes. Changes in the chromatin environment of promoter sequences may therefore drive changes in the dynamics of the basal transcription machinery and complex interactions between transcriptional complexes and chromatin may therefore be important in mediating transcriptional activity.

While TSA treatment does lead to an increase in *hTERT* and *hTR* expression when compared with the telomerase-positive MCF7 breast carcinoma cell line (Figure 1A) *hTERT* expression in the hMSC treated with TSA is very low. It has also been observed that these cells display no detectable telomerase activity as measured by telomerase repeat amplification protocol (TRAP) [DATA NOT INCLUDED]. This observation indicates that while modulation of the chromatin environment by TSA is important in the control of *hTERT* and *hTR* expression, this action alone is not enough to increase levels of *hTERT* and *hTR* expression to a level high enough to reconstitute telomerase activity. This may be due to the lack of other factors that can influence gene expression.

It is known that hyperacetylation of promoter-specific histones at promoter sequences will cause relaxation of chromatin environment relative to a compacted state when histones are hypoacetylated. This relaxation of the chromatin environment will allow the binding of transcription factors, basal transcription machinery and other chromatin modifying enzymes to the promoter DNA. However, if such factors are not freely available within the cell, the relaxation of the chromatin environment alone will not be sufficient to mediate high levels of transcription. Analysis of the binding of regulatory factors influencing *hTERT* and *hTR* expression in the hMSC will therefore be an important next step. Further analysis of histone modifications involved in telomerase gene repression in the hMSC may allow the identification of enzymes and co-regulators involved, which can then be targeted to allow a more specific method of modulating telomerase gene expression other than TSA.

Although TSA treatment has been shown to cause chromatin changes at the *hTERT* and *hTR* promoter, it is not known if this is a direct action. However, the use of TSA does allow us to see that changes in the chromatin environment, be they direct or indirect, can lead to changes in telomerase gene expression. These data provide

Figure 3. Chromatin and basal transcriptional machinery dynamics in hMSCs.



Changes in the chromatin environment of promoter sequences may lead to changes in gene transcription, through the relaxation of the chromatin environment.

an impetus for further, more detailed, analysis of the interplay between the chromatin environment, general transcription machinery and other important regulatory factors which may affect telomerase gene expression.

Overall, this study shows that the lack of *hTERT* expression in hMSCs is due to a repressive chromatin environment surrounding the *hTERT* gene promoter and that modulation of the chromatin environment has an important role in the re-expression of *hTERT* in these cells. This provides an excellent platform for the further investigation of the control of telomerase gene expression in the hMSC and its implications for tissue regeneration and cancer stem cells.

Executive summary

- Human mesenchymal stem cells (hMSC) have limited replicative capacity and lack expression of the immortalizing enzyme telomerase.
- Lack of telomerase may act as a tumour suppressor mechanism as ectopic expression of telomerase in hMSC results in neoplastic transformation and reveals a cancer stem cell potential.
- Lack of telomerase gene expression is due to the repressive chromatin environment surrounding the telomerase gene promoters.
- Activation of telomerase gene expression is accompanied by chromatin remodelling and dynamic shifts in the basal transcriptional complex.
- hMSC represent an excellent model for studying telomerase gene expression with direct implications for cell replacement therapies and tissue engineering.

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