UNIL | Université de Lausanne
Faculté de biologie
et de médecine

Implication of DNA methylation and PAX5 factor in the transcriptional regulation of hTERT, the human telomerase reverse transcriptase gene

Thèse de doctorat ès sciences de la vie (PhD)

effectuée à l'Institut de Pathologie,
Département de Pathologie et de Médecine de Laboratoire du CHUV

présentée à la Faculté de Biologie et de Médecine
de l'Université de Lausanne

par

Stéphanie BOUGEL

Diplômée en Microbiologie, Enzymologie, Nutrition
Université de Nancy

Jury
Dr. Luc Pellerin, Président
Dr. Jean Benhattar, Directeur de thèse
Prof. Fred T. Bosman, Co-directeur de thèse
Dr. Evelyne Ségal-Bendirdjian, Expert
Prof. Nicolas Mermod, Expert

Lausanne
2009

UNIL | Université de Lausanne
Faculté de biologie
et de médecine

Ecole Doctorale

Doctorat ès sciences de la vie

Imprimatur

Vu le rapport présenté par le jury d'examen, composé de

Président	Monsieur	Prof.	Luc **Pellerin**
Directeur de thèse	Monsieur	Dr	Jean **Benhattar**
Co-directeur de thèse	Monsieur	Prof.	Frederik **Bosman**
Experts	Madame	Dr	Evelyne **Ségal-Bendirdjian**
	Monsieur	Prof.	Nicolas **Mermod**

le Conseil de Faculté autorise l'impression de la thèse de

Madame Stéphanie Bougel

Titulaire d'un DEA microbiologie de l'Université H. Poincaré, Nancy, France

intitulée

Implication of DNA metylation and PAX5 factor in the transcriptional regulation of hTERT, the human telomerase reverse transcriptase gene

Lausanne, le 5 juin 2009

pour Le Doyen
de la Faculté de Biologie et de Médecine

Prof. Luc Pellerin

Remerciements

Avant tout, je veux adresser mes remerciements aux personnes qui ont été présentes pour moi au cours de ma thèse et grâce à qui ce travail a pu être possible.

Je tiens tout d'abord à remercier Monsieur Bosman, directeur de l'Institut de Pathologie, pour avoir accepté d'être mon co-directeur de thèse et pour avoir consacré beaucoup de temps à corriger et améliorer ce manuscrit, malgré les nombreuses charges qui lui incombent.

Mes remerciements les plus grands et les plus chaleureux sont pour mon directeur de thèse, Jean Benhattar, et toute son équipe. Je remercie Jean de m'avoir accueillie au sein de son laboratoire et de m'avoir donné sa confiance. J'ai eu un grand plaisir à travailler à ses cotés durant ces quatre années. J'ai particulièrement apprécié ses qualités scientifiques, ses convictions, mais aussi son grand cœur et sa patience. Merci de m'avoir conseillée, soutenue et motivée tout au long de ce travail de thèse, et de m'avoir transmis son amour pour les nouvelles technologies !

Geneviève, Stéphanie et Pu ont été mes modèles, voire même mes mentors !! Elles m'ont appris les bases pour devenir une bonne doctorante, d'un point de vue technique bien sûr, mais aussi humain, en me transmettant l'esprit d'équipe. Merci pour leur amitié et pour tous les moments privilégiés que nous avons partagés ensemble.

J'adresse aussi tous mes remerciements aux techniciennes, qui sont les piliers du laboratoire. Tout d'abord, merci à Charlotte de m'avoir initiée au fonctionnement du labo, de m'avoir prodigué ses nombreux conseils techniques, et surtout merci pour notre complicité et nos discussions passionnées ! Merci à Gaby pour son aide dans mes différents projets, sa gentillesse et son soutien à toute épreuve ! Merci à Patricia et Astrid pour avoir partagé avec moi leur savoir-faire et m'avoir prêté une oreille toujours attentive !

Je remercie également mes stagiaires de l'Ecole laborantine : Christel, Sabrina, Cora, Sébastien et Elena. J'espère vous avoir appris autant que vous m'avez appris.

Plus généralement, merci à tous ceux qui ont croisé ma route au labo, comme Adeline, Isa et bien d'autres, avec qui j'ai pu échanger un peu de savoirs et surtout d'agréables moments culinaires !

Je remercie vivement Lucienne Juillerat et toute son équipe pour m'avoir accueillie dans son laboratoire en culture cellulaire et lors de mes western blots. Les lab meetings partagés avec eux et les membres du laboratoire de Hans-Anton Lehr resteront d'agréables souvenirs, tout comme nos discussions non scientifiques autour de petits-dej' ou de soirées !

Un grand merci aux autres laboratoires « voisins » de Phil Shaw et Ivan Stamenkovic pour m'avoir permis d'emprunter du matériel et m'avoir aidée dans l'utilisation d'appareils tel que le laser de microdissection ou le microscope à fluorescence. Merci aussi au grand labo pour les coupes histologiques et à la cytologie pour m'avoir ouvert leurs portes lors de mes cytospins !

Merci à toute l'équipe des médecins de l'Institut, en particulier Richard et Pu, pour avoir pris le temps de commenter mes coupes histologiques et m'avoir transmis les bases de l'histologie. Je les remercie pour tout ce que j'ai appris grâce à eux.

Je remercie tout le personnel de l'Institut, avec un clin d'oeil particulier à Audisio, Yannick et Sam pour leur bonne humeur !

Je veux enfin témoigner toute ma reconnaissance aux personnes avec qui j'ai pu collaborer : Robert Dante et les filles de son laboratoire à Lyon et les groupes de Herbert C Morse et Victor Lobanenkov à Washington, sans qui ce travail n'aurait pas été si loin. Et surtout, merci de m'avoir accueillie pour d'agréables meetings à l'étranger !

En parallèle, je tiens à remercier tout particulièrement mes parents. Ce sont des parents formidables. Ils m'ont permis de réaliser de longues études, même si ce n'était pas dans mes plans de départ ! Je les remercie du fond du cœur pour tout ce qu'ils font pour moi au quotidien. Ils sont toujours présents et je sais que je peux toujours compter sur eux. A mes yeux, cela est rassurant et tellement important.

Je remercie aussi ma sœur, Frédérique, et ma sœur de cœur, Julie, avec lesquelles j'ai une relation particulière très complice et à qui je peux me confier en toute occasion. Grâce à elles, j'ai confiance en moi, et cela me permet de m'affirmer un peu plus chaque jour. Merci pour tout ce qu'elles m'apportent, nos fous rires, nos coups de gueules et tous les moments forts que nous avons déjà partagés et que nous partagerons encore !

Merci à toute ma famille, qui m'entoure et me soutient même à distance. En particulier, merci à ma marraine Danièle et à mes grands-parents pour leurs encouragements et leur confiance.

Pour terminer, j'adresse un chaleureux remerciement à tous mes amis, présents durant ces quatre années, et tout particulièrement, Alvaro, Nico et Fab, qui m'ont soutenue dans les moments difficiles et ont toujours cru en moi, même quand je n'étais plus sûre d'y arriver.

Aujourd'hui, je ressens une grande satisfaction pour avoir fourni le travail qu'une thèse représente et l'investissement qu'elle demande. Je suis surtout ravie de toutes les expériences enrichissantes que j'ai pu vivre pendant ce temps. Je suis fière d'avoir réussi, et c'est grâce à vous tous. C'est une revanche pour moi.

C'est avec un grand regret que je quitte le labo, dans lequel j'ai eu tant de plaisir à travailler au quotidien, et qui m'a fait et vu évoluer. Les petits-dej', « tea time » et diverses sorties me manqueront tout spécialement (surtout les sorties « fondue ») ! La bonne humeur qui y règne et la générosité de chacun resteront dans mon cœur. Je sais que nous garderons des relations fortes, et j'espère que nous aurons l'occasion de travailler ensemble dans le futur.

Je suis confiante en l'avenir et prête à embrasser la vie avec toute l'énergie et la joie de vivre que vous m'avez tous données.

A tous ceux qui partagent, ou qui ont partagé ma vie,

et grâce à qui je suis devenue celle que vous connaissez.

SUMMARY

Human telomerase is an "immortalizing" enzyme that enables cells to maintain telomere length, allowing unlimited replicative capacity to reproductive and cancer cells. Conversely, normal somatic cells that do not express telomerase have a finite replicative capacity. The catalytic subunit of telomerase, hTERT, is defined as the limiting factor for telomerase activity. Between activators and repressors, and the role of DNA methylation and histone acetylation, an abundance of hTERT regulatory models have been suggested. The discovery of the implication of CTCF in the transcriptional regulation of hTERT in part explained the mechanism of silencing of telomerase in most somatic cells and its reactivation in neoplastic cells. In telomerase-positive cells, the inhibitory activity of CTCF is blocked by methylation-dependent and -independent mechanisms.

In most carcinoma cells, hypermethylation of the hTERT 5’ region has been shown to block the inhibitory effect of CTCF, while a short hypomethylated region allows a low transcription level of the gene. We have demonstrated that MBD2 protein specifically binds the methylated 5’ region of hTERT in different cell lines and is therefore involved in the partial repression of hTERT transcription in methylated tumor cells. In contrast, we have shown that in normal and neoplastic B cells, hTERT regulation is methylation-independent. The PAX5 factor has been shown to bind to the hTERT 5’region downstream of the ATG translational start site. Ectopic expression of PAX5 in telomerase-negative cells or repression of PAX5 expression in B lymphoma cells respectively activated and repressed hTERT transcription. Thus, PAX5 is strongly implicated in hTERT expression activation in telomerase-positive B cells. These results reveal differences between the hTERT methylation patterns in telomerase-positive carcinoma cells and telomerase-positive normal B cells. The potential of hTERT methylation as a cancer biomarker was evaluated and applied to the detection of metastasis. We have shown that hTERT methylation correlates with the cytological diagnosis in cerebrospinal fluids.

Our results suggest a model of hTERT gene regulation, which helps us to better understand how hTERT transcription is regulated by CTCF in methylation-dependant and independent mechanisms. Our data also indicate that hTERT methylation is a promising new cancer biomarker.

RESUME

La télomérase est une enzyme dite "d'immortalité" qui permet aux cellules de maintenir la longueur de leurs télomères, ce qui confère une capacité de réplication illimitée aux cellules reproductrices et cancéreuses. A l'inverse, les cellules somatiques normales, qui n'expriment pas la télomérase, ont une capacité de réplication limitée. La sous-unité catalytique de la télomérase, hTERT, est définie comme le facteur limitant l'activité télomérasique. Entre activateurs et répresseurs, le rôle de la méthylation de l'ADN et de l'acétylation des histones, de nombreux modèles ont été suggérés. La découverte de l'implication de CTCF dans la régulation transcriptionnelle de hTERT explique en partie le mécanisme de répression de la télomérase dans la plupart des cellules somatiques et sa réactivation dans les cellules tumorales. Dans les cellules télomérase-positives, l'activité inhibitrice de CTCF est bloquée par un mécanisme dépendent ou non de la méthylation.

Dans la plupart des carcinomes, une hyperméthylation de la région 5' de hTERT bloque l'effet inhibiteur de CTCF, alors qu'une petite région hypométhylée permet un faible niveau de transcription du gène. Nous avons démontré que la protéine MBD2 se lie spécifiquement sur la région 5' méthylée de hTERT dans différentes lignées cellulaires et qu'elle est impliquée dans la répression partielle de la transcription de hTERT dans les cellules tumorales méthylées. Par contre, nous avons montré que dans les lymphocytes B normaux et néoplasiques, la régulation de hTERT est indépendante de la méthylation. Dans ces cellules, le facteur PAX5 se lie sur la région 5' de hTERT en aval du site d'initiation de la traduction (ATG). L'expression exogène de PAX5 dans les cellules télomérase-négatives active la transcription de hTERT, alors que la répression de PAX5 dans les cellules lymphomateuses inhibe la transcription du gène. PAX5 est donc directement impliqué dans l'activation de l'expression de hTERT dans les lymphocytes B exprimant la télomérase. Ces résultats révèlent des différences entre les niveaux de méthylation de hTERT dans les cellules de carcinomes et les lymphocytes B exprimant la télomérase. La méthylation de hTERT en tant que biomarqueur de cancer a été évaluée, puis appliquée à la détection de métastases. Nous avons ainsi montré que la méthylation de hTERT est positivement corrélée au diagnostic cytologique dans les liquides céphalorachidiens.

Nos résultats conduisent à un modèle de régulation de hTERT, qui aide à comprendre comment la transcription de ce gène est régulée par CTCF, avec un mécanisme lié ou non à la méthylation du gène hTERT. La méthylation de hTERT s'est aussi révélée être un nouveau et prometteur biomarqueur de cancer.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

Aicda or AID	Activation-induced cytidine deaminase
ALL	Acute lymphoblastic leukemia
ALT	Alternative lengthening of telomeres
AML	Acute myeloblastic leukemia
AP-1	Activator protein-1
APBs	ALT-associated promyelocytic leukemia bodies
APC	Adenomatous polyposis coli
BIO box	Biogenesis box
BORIS	Brother of the regulator of imprinting sites
Blimp1	B lymphocyte induced maturation protein
B-NHL	B-cell non-Hodgkin lymphomas
BSAP	B-cell-specific activator protein
CAB box	Cajal bodies box
CAF-1	Chromatin assembly factor
CBP	CREB-binding protein
CDKN	Cyclin dependent kinase inhibitor
ChIP	Chromatin immunoprecipitation
CIMP	CpG island methylator phenotype
cirDNA	circulating DNA or cell-free DNA
CLP	Common lymphoid progenitors
CMP	Common myeloid progenitors
COBRA	Combined bisulfite restriction analysis
CSF	Cerebrospinal fluids
CTCF	CCCTC-binding factor
DAPK	Death-associated protein kinase
DC	Dyskeratosis congenita
DC	Dendritic cell
DDR	DNA damage repair
DIG	Digoxigenin
DMH	Differential methylation hybridization
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DSB	Double stranded break
ECTR	Extrachromosomal telomeric repeat
EGF	Epidermal growth factor
EMSA	Electrophoretic mobility shift assay
ERE	Estrogen-responsive element
GC	Germinal centre

GSTP1	Glutathione S-transferase P1
hALP	human N-acetyltransferase-like protein
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HELP	HpaII tiny fragment enrichment by ligation-mediated PCR
HIF-1	Hypoxia-inducible factor 1
HL	Hodgkin lymphomas
HMT	Histone methyl transferases
hnRNP	Heterogeneous nuclear ribonucleoproteins
HP1	Heterochromatin protein 1
HPCE	High-performance capillary electrophoresis
HPLC	High-performance liquid chromatography
HPV	Human papillomavirus
HR	Homologous recombination
HRE	Hypoxia response element
HRM	high resolution melting analysis
HSC	Hematopoietic stem cells
Ig	Immunoglobulin
IHC	Immunohistochemistry
K	Lysine
KD	Knock down
LM	Leptomeningeal metastasis
LMP1	Latent membrane protein 1
LSD1	Lysine-specific demethylase 1
MAP	Mitogen-activated protein
MBD	Methyl-CpG binding domain proteins
MC	Methylcytosine
MCA	Methylated CpG-island amplification
M-CSF	Macrophage colony-stimulating factor
MECP	Methyl CpG binding protein
MGMT	O^6-methylguanine DNA methyltransferase
MLH1	MutL homolog 1 gene
MS-DBA	Methylation-sensitive dot blot assay
MS-DGGE	Methylation-specific denaturing gradient gel electrophoresis
MS-HRM	The methylation-sensitive resolution melting analysis
MSP	Methylation specific polymerase chain reaction
MS-SSCA	Methylation-sensitive single-strand conformation analysis
MZF-2	Myeloid-specific zinc finger protein 2
NHEJ	non-homologous DNA end-joining
NK	Natural killer
PARP	Poly(ADP-ribose) polymerase
PAX	Paired box
PCR	Polymerase chain reaction
PKC	Protein kinase C

PML	Promyelocytic leukemia
POT1	Protection of Telomere
PP2A	Protein phosphatase 2A
PRC	Polycomb repressive complex
pre-BCR	Pre-B-cell receptor
QAMA	Quantitative analysis of methylated alleles
RASSF1A	Ras association domain family 1 gene
RB	Retinoblastoma protein
RLGS	Restriction landmark genomic scanning
RNA	Ribonucleic acid
RNP	Ribonucleoproteins
RT-PCR	Reverse transcription polymerase chain reaction
SAGA	Spt–Ada–Gcn5 acetyltransferase
SCC	Small cell carcinoma
siRNA	small interfering RNA
SMART-MSP	Sensitive Melting Analysis after Real Time MSP
SMYD3	SET- and MYND-domain-containing protein-3
sno	small nucleolar
TBP	TATA-binding protein
TCR	T cell antigen receptor
TEIF	Transcriptional elements-interacting factor
TERRA	TElomeric Repeat containing RNA
TERT	Telomerase reverse transcriptase
TGF-β	Transforming growth factor beta
TIN2	TRF1 interacting protein 2
TNF-α	Tumor necrosis factor alpha
TM (Tm)	Melting temperature
TPE	Telomere position effect
TR ou TERC	Telomerase RNA component
TRD	Transcriptional repression domain
TRF2	Telomere Repeat Factor
TSA	Trichostatin A
USF	Upstream Stimulatory Factor
VDJ	Variable Diversity Joining
WT1	Wilms' Tumor 1
WRN	Werner protein
Xbp1	X-box binding protein1

CHAPTER 1

General introduction

In tissues of a multicellular organism, each cell has a limited lifespan. As cells divide, most of them gradually lose terminal DNA sequences of the chromosomes, which are capped with specialized DNA-protein structure called telomeres. The telomeres are crucial for maintaining the integrity of genetic information but also for the stability of the genome. As telomere erosion occurs at each replication round, telomeres will progressively shorten until cell death is induced. Nevertheless, some cells, such as stem and germ cells, require a mechanism to counteract telomere attrition. These cells possess telomerase, a highly regulated specific enzyme that maintains telomere length. This enzyme is not expressed in adult tissues but is reactivated in about 85% of cancers. For that reason, inhibition of telomerase in cancer cells has become an important point of interest in the anti-cancer strategies. In particular, regulation of its catalytic subunit hTERT, which is crucial for the telomerase activity, is a subject of intense investigation.

This study focuses on the hTERT gene regulation in both normal and cancer cells. For better understanding of the importance of telomeres and telomerase in the protection of genome integrity, we review the telomere structure in general. Then, the composition of the telomerase complex and its mechanism of action are discussed. Our interest focuses on new knowledge in telomerase regulation, and in particular regulation of hTERT expression is developed. As CpG methylation plays an important role in the regulation of hTERT expression, we describe how this occurs in normal and pathological situations. Furthermore, how transcriptional silencing occurs through DNA methylation is elaborated, and modes of detection and clinical implications of DNA methylation are summarized. The final paragraph pays particular attention to the B-cell specific PAX5 factor that might be involved in hTERT regulation in differentiating lymphocytes.

1. Telomeres and the maintenance of the genome integrity

The ends of chromosomes are formed by DNA nucleoprotein complexes termed telomeres. The telomere cap structure is essential to stabilize the chromosomes and thereby conserve the genetic information and maintain genome stability (Greider, 1996; Blackburn, 1997). In addition, telomeres anchor the chromosomal extremity to the nuclear matrix and assist chromosome alignment, which is essential for accurate segregation during meiosis (De Lange, 1992; Kirk *et al.*, 1997; Smith and De Lange, 1997; Smilenov *et al.*, 1999). The

special chromatin structure formed by telomeres allows cells to discriminate chromosome ends from intrachromosomal double stranded breaks (DSBs), and therefore prevent degradation, recombination, and/or fusion by cellular repair systems such as homologous recombination (HR) and non-homologous DNA end-joining (NHEJ) (Lundblad, 2000; Chan and Blackburn, 2002; Verdun and Karlseder, 2007). When the telomere extremities are damaged, cells can acquire structural chromosomal abnormalities, undergo a permanent cell cycle arrest called senescence, or die by apoptosis (Shay and Wright, 2001; Yaswen and Stampfer, 2002). Both aging and cancer phenotypes can be driven by these genomic changes (Klapper *et al.*, 2001; Pandita, 2002).

1.1 Telomere structure

The telomeric sequences were characterized for the first time in *Tetrahymena thermophila* in 1978 (Blackburn and Gall, 1978). Telomeric sequences and lengths depend on chromosomes and species (Baird *et al.*, 2003). In man, telomeres are made up of an average of 5 to 15 kb of $(TTAGGG)_n$ repeats and telomere-binding proteins. The telomere structure involves a lasso-like structure, termed t-loop, with a three-stranded DNA displacement loop, called D-loop (Figure 1) (Griffith *et al.*, 1999; Munoz-Jordan and Cross, 2001; Nikitina and Woodcock, 2004; de Lange, 2004). Specific proteins associated to telomeres are crucial for forming and maintaining the protective cap structure. In addition to protect chromosomal integrity, telomeres can also influence the expression of genes. Indeed, telomeres are subject to epigenetic regulation that silences expression of nearby genes, which is called telomere position effect (TPE) (Baur *et al.*, 2001; Koering *et al.*, 2002; Garcia-Cao *et al.*, 2004; Pedram *et al.*, 2006). Neverthess, it was recently shown that mammalian telomeres are not only silent genomic regions, but they can transcribe into TElomeric Repeat containing RNA (TERRA) molecules (Azzalin *et al.*, 2007; Schoeftner and Blasco, 2008). They constitute a novel class of mammalian RNAs present both in the nucleus of telomerase positive and negative cells. Mammalian TERRA molecules contain UUAGGG repeat sequences and range in size from 100 bases up to >9 kb. These RNA molecules have been suggested to have a role in organizing telomere architecture.

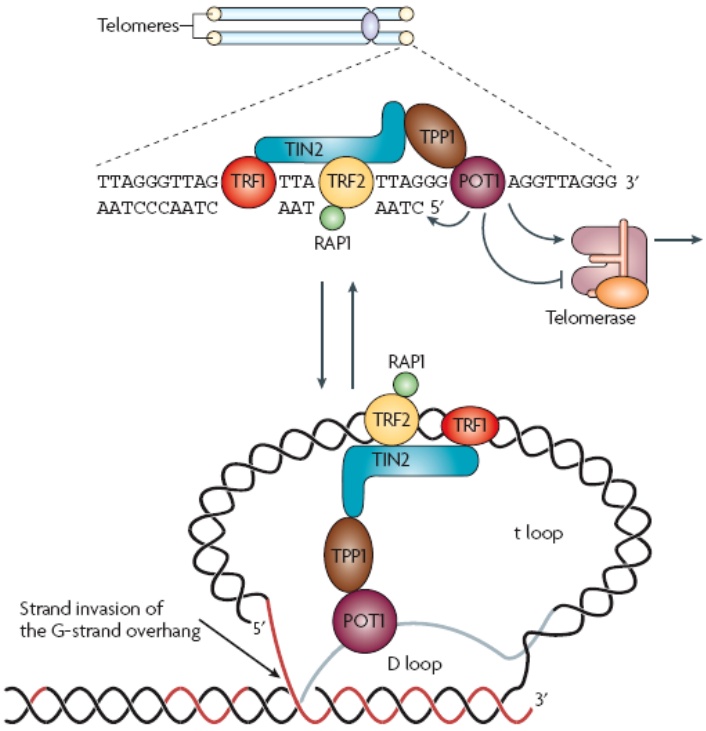


Figure 1. Telomere structure. Telomeres cap mammalian chromosomes and are composed of TTAGGG repetitive sequences that terminate in a 3′ single-stranded (ss) overhang. Telomeric DNA is associated with the six-protein shelterin complex (TRF1, TRF2, RAP1, TIN2, TPP1 and POT1). The ss overhang can invade the double-stranded region of the telomere to form a protective telomere (t) loop with a ss displacement (D) loop at the invasion site (cited from Deng *et al.*, 2008).

A large number of proteins have been found to be associated with telomeric DNA. Three proteins have been identified to bind directly and specifically to telomeric DNA. These proteins are TRF1, TRF2 (Telomere Repeat Factor) and POT1 (Protection of Telomere) (Kim Sh *et al.*, 2002). TRF1 and TRF2 bind as homodimers to double stranded telomeric repeats (Broccoli *et al.*, 1997), where they assemble the six-protein (TRF1/TRF2/RAP1/TIN2/TPP1/POT1) shelterin complex (Figure 1) (de Lange, 2005). POT1 associates with the 3'single stranded overhang through its oligonucleotide binding fold motif (Baumann and Cech, 2001; Baumann *et al.*, 2002). All these proteins can be found on telomeres at any time. TRF2 has been shown to be essential for the formation of the t-loop structure (Stansel *et al.*, 2001; Amiard *et al.*, 2007).

The three proteins, TRF1, TRF2 and POT1, primarily interact together and then cooperate directly or indirectly with other proteins to modulate telomere structure, function and length. TRF1 recruits numerous proteins to the telomere. TRF1 is modified by the poly(ADP-ribose) polymerase (PARP) Tankyrase 1 and 2 (Kaminker *et al.*, 2001; Cook *et al.*,

2002). The ADP-ribosylation of TRF1 inhibits its ability to bind telomeres (Smith *et al.*, 1998). TRF1 also directly binds TIN2 (TRF1 interacting protein 2), an association that appears to protect TRF1 from tankyrase. TIN2 has been proposed to regulate the access of telomerase to the telomeres (Kim *et al.*, 1999; Ye *et al.*, 2004b) and appears to bring together TRF1 and POT1 through the TPP1 bridge protein (TPP1 was previously called PTOP/PIP1/TINT1). A third direct TRF1 interacting factor is PINX1, a protein that binds to TERT, the telomerase catalytic subunit (see hTERT part 2.1.2) (Zhou and Lu, 2001). TRF2 also has numerous interacting factors. For instance, the TRF2 protein makes a complex with the RAP1 protein (Li *et al.*, 2000), which interacts with different proteins as the DNA repair complex (Zhu *et al.*, 2000) and the nucleotide base excision repair endonuclease (Zhu *et al.*, 2003). Moreover, TRF2 can interact with PARP2, that will modulate its activity (Dantzer *et al.*, 2004).

Both TRF1 and TRF2 proteins interact with a number of proteins involved in DNA repair or checkpoint control as the DNA damage sensing protein ATM, whose kinase activity is thought to be inhibited at the telomere locus (Karlseder *et al.*, 2004), but also interact with the BLM helicase (Lillard-Wetherell *et al.*, 2004), the protein WRN (Opresko *et al.*, 2002), and the NHEJ protein Ku (Hsu *et al.*, 2000; Song *et al.*, 2000). Finally, the TRF1 and TRF2 complexes are linked through binding of TIN2 factor, thereby stabilizing their levels and localization at telomeres and modulating their function (Kim *et al.*, 2004; Ye *et al.*, 2004b). Consequently, both telomere length and capping can be influenced by perturbations to either TRF1 and TRF2, or their associated proteins POT1, RAP1 or TIN2 (Van Steensel B. and De Lange, 1997; Baumann and Cech, 2001; Colgin *et al.*, 2003; Loayza and De Lange, 2003; Iwano *et al.*, 2004).

1.2 Mechanisms of telomere dysfunction

Dysfunctional telomeres are unable to exert their chromosome end-protective functions. The most common mechanism of telomere disruption is their progressive erosion due to the end-replication problem (Figure 2). This problem predicts that each round of DNA replication is accompanied by telomere shortening due to the failure of DNA polymerase to fully synthesize the extreme terminus of DNA strands. Semi-conservative DNA replication is accomplished by a cooperation between leading and lagging strand DNA syntheses. The direction of the leading strand DNA synthesis is the same as that of the replication fork

movement. Consequently, leading strand synthesis is processive and replicates one strand of the original DNA from an RNA primer until the end. On the contrary, the lagging strand DNA synthesis, which is in a direction opposite to replication fork movement, requires short pieces of newly synthesized DNA fragments from RNA primers, named Okasaki fragments. These fragments are then ligated to form a continuous DNA strand. Most RNA primers are replaced with DNA from an upstream Okasaki fragment, but the terminal RNA primer is never replaced with DNA (Figure 2) (Dhaene *et al.*, 2000). Telomeres are then shortened with 50-100 bp at each round of replication, for a total lifetime loss of approximately 2-4 kb. This shortening of telomeres acts as a mitosis counter that determines the maximum number of cell divisions and thus limits the proliferative capacity of any cell type.

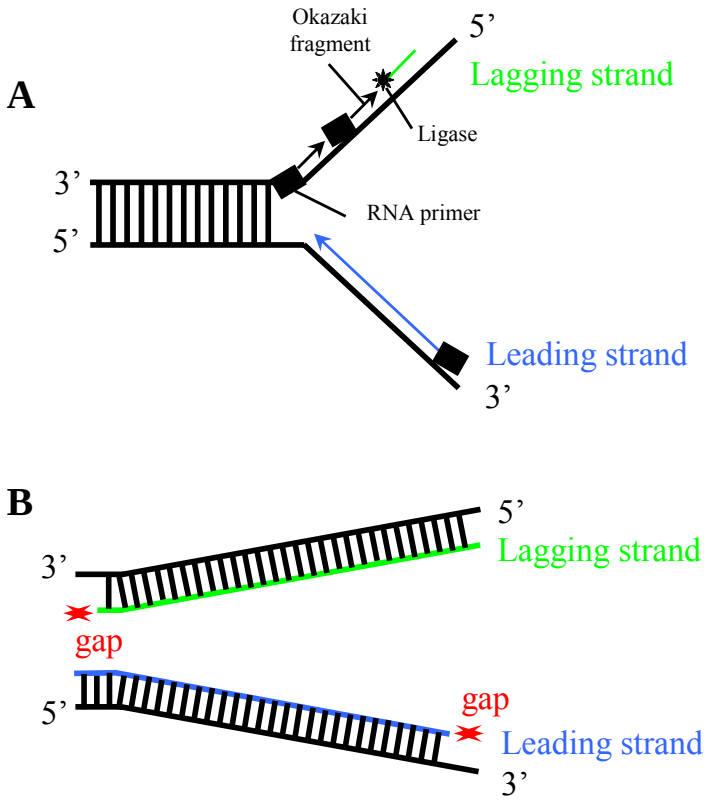


Figure 2. The "end-replication problem". A. As the replication fork opens, lagging strand synthesis proceeds from 3' to 5' as the overall result of removal of RNA primers and ligation of the individual 5' to 3' synthesized Okazaki fragments, B. After removal of the terminal RNA primers, gaps remain at the 5' end of the lagging and leading strand which can not be filled (cited from Dhaene *et al.*, 2000).

1.3 Consequences of telomere dysfunction

Normal somatic cells in tissues have a limited lifespan and undergo a process called replicative senescence, in which cells stop to proliferate (Figure 3) (Harley *et al.*, 1990; Wright *et al.*, 1996a; Harley, 1997).

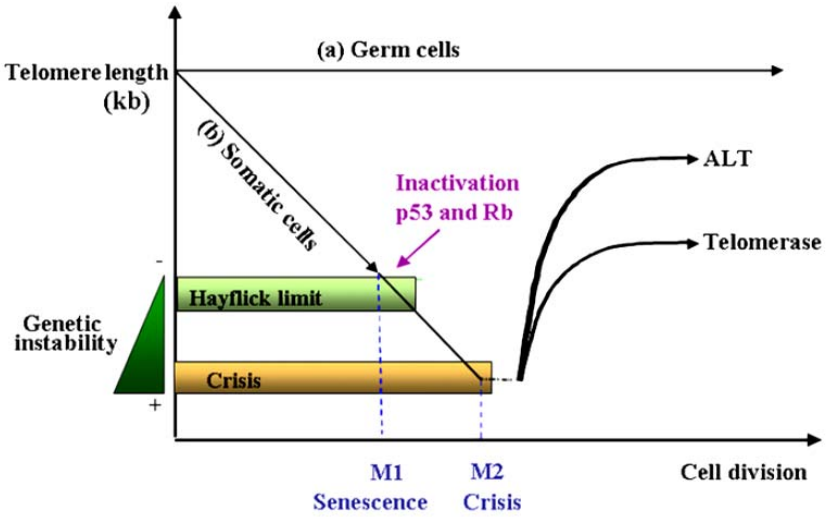


Figure 3. The telomere length evolution. As cells continue to divide, telomere length is lost until cells reach the first proliferative block, senescence. Bypass of senescence requires loss of both p53 and RB tumor suppressor pathways. After the loss of both pathways, cells continue to divide until they encounter the second proliferative block, crisis. Rare cells emerge from crisis and maintain their telomere length through activation of telomerase or the ALT mechanism. Germ cells maintain telomere length through telomerase activity (cited from Nittis *et al.*, 2008).

Senescence arises when cells contain some critically short telomeres and this is recognized by a DNA damage repair (DDR) signaling program (Campisi, 1997; Sedivy, 1998; di Fagagna *et al.*, 2003). Moreover, oxidative DNA damage or alterations in expression or function of the shelterin complex or the telomere-associated proteins can also induce telomere dysfunctions and engage DDR pathways (Rubio *et al.*, 2004). Indeed, dysfunctional telomeres can activate kinases, such as ATM (Denchi and de Lange, 2007; Guo *et al.*, 2007), which will phosphorylate downstream factors, like CHK1 and CHK2, that will consequently phosphorylate p53 (Gire *et al.*, 2004). Phosphorylation of p53 results in the stimulation of the expression of the cyclin-dependent kinase inhibitor p21. The p21 protein inhibits cell cycle progression by inhibiting cyclin-dependent kinases that phosphorylate and inactivate the retinoblastoma protein (RB). In fact, dysfunctional telomeres can lead to two tumor suppressive activities through p53 activation: cellular senescence (permanent cell cycle arrest) but also apoptosis (programmed cell death). When critical cell-cycle checkpoint functions are

lost (p53), cultured cells with short telomeres can escape senescence and continue to divide until they enter crisis. At this point, telomeres drastically shorten, chromosome end fusions and massive cell death occurs (Shay *et al.*, 1991; Wright and Shay, 1992; Hande *et al.*, 1999). Rare immortalized cells overcome the crisis after abnormal activation of telomerase or a telomerase-independent mechanism (alternative lengthening of telomeres described below) (Wright *et al.*, 1989; Murnane *et al.*, 1994; Bryan *et al.*, 1997; Duncan and Reddel, 1997). These events lead to the cell becoming immortal by maintaining stable telomere lengths (Shay and Roninson, 2004). A malignant process can begin when the cells persist to divide.

A model of cancer development in human breast cancers has been developed by Deng and Chang (Figure 4) (Deng and Chang, 2007). Dysfunctional telomeres, like telomere shortening, can lead to an increase of genomic instability along with the transition from benign hyperplasia to malignant carcinoma (Chin *et al.*, 2004; Meeker and Argani, 2004). Moreover, genetic changes can favorize cancer cells with aggressive tumor characteristics, such as the ability to induce an angiogenic response, metastasize, and eventually resistance to chemotherapeutic drugs (Maser *et al.*, 2007).

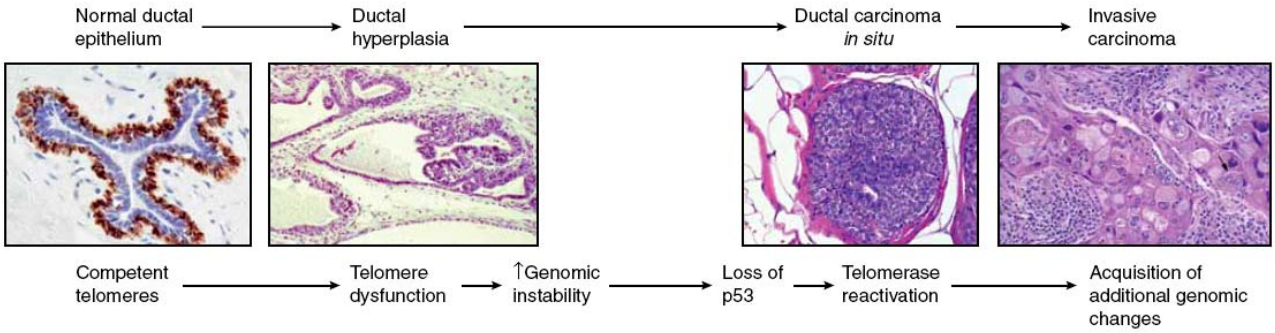


Figure 4. Speculative model of cancer development in human breast cancers. The model is based on available evidence, of how telomere dysfunction initiates genomic changes to promote the development of breast cancer. Loss of the p53-dependent DNA damage checkpoint is postulated to be important for tumor progression. Transition from normal ductal breast epithelium to invasive carcinoma correlates with the presence of dysfunctional telomeres and loss of p53 (cited from Deng and Chang, 2007).

1.4 Telomere maintenance

In human cells, one mechanism of telomere maintenance is named Alternative Lengthening of Telomeres (ALT). Epithelial tumors rarely activated ALT mechanism, contrary to neuroectodermal tumors (astrocytomas) or tumors derived from the mesenchyme (osteosarcomas, liposarcomas and glioblastomas), (Hakin-Smith *et al.*, 2003; Ulaner *et al.*,

2003; Montgomery *et al.*, 2004; Henson *et al.*, 2005; Costa *et al.*, 2006). However, the ALT pathway is not fully understood, it seems to involve telomere recombination (Dunham *et al.*, 2000) and is characterized by telomere length heterogeneity, ranging in size from 2 to 80 kb (Bryan and Reddel, 1997; Reddel, 2003).

ALT cells are characterized by the presence of ALT-associated promyelocytic leukemia bodies (APBs). APBs are nuclear structures containing telomeric DNA, telomeric proteins (TRF1, TRF2, TIN2, RAP1), promyelocytic leukemia (PML) protein, as well as proteins involved in DNA repair proteins (MRE11, RAD50, NBS1, RAD51 and RAD52) (Yeager *et al.*, 1999; Henson *et al.*, 2002; Wu *et al.*, 2000; Jiang *et al.*, 2007). However, it has been shown that APBs are not always required for ALT to occur contrarily to telomere recombination (Fasching *et al.*, 2005; Marciniak *et al.*, 2005). ALT cells are also marked out by the presence of extrachromosomal telomeric DNA (ECTRs, extrachromosomal telomeric repeats), including t-circles (Cesare and Griffith, 2004; Fasching *et al.*, 2007). APBs could have the role to sequester linear DNA away from DNA repair proteins that detect DSBs (Fasching *et al.*, 2007).

However, the mechanism frequently used by human tumor cells as well as several normal cells involves a macromolecular complex capable of maintaining telomere length. This complex is known as telomerase.

2. The human telomerase

Most cells maintain telomeres using telomerase. Telomerase is expressed in embryonal cells and in adult germline cells (Kim *et al.*, 1994a; Wright *et al.*, 1996b), but is undetectable in normal somatic tissues except for proliferative cells of renewing tissues such as basal epidermal cells, lymphocytes, and other hematopoietic cells (Broccoli *et al.*, 1995; Hiyama *et al.*, 1995c; Chiu *et al.*, 1996) (Forsyth *et al.*, 2002; Mason, 2003). In the absence of telomerase, normal human cells in culture have a finite life span and undergo cellular senescence normally after 40 to 70 population doublings. Telomerase is reactivated in more than 85-90% of all human cancers (Kim *et al.*, 1994a; Avilion *et al.*, 1996; Shay and Gazdar, 1997; Meeker and Argani, 2004).

Telomerase elongates critically short telomeres, stabilizes the length of other telomeres, and permits continued cell division (Bodnar *et al.*, 1998; Ouellette *et al.*, 2000; Steinert *et al.*, 2000; Stewart *et al.*, 2003). It also protects telomeres from NHEJ (Zhu *et al.*, 1999; Chan and

Blackburn, 2003). However, numerous non-canonical functions of telomerase have been revealed. For instance, telomerase has been implicated in DNA damage response (Masutomi *et al.*, 2005; Kedde *et al.*, 2006), in promotion of cell growth (Smith *et al.*, 2003b; Geserick and Blasco, 2006), in stem cell proliferation (Sarin *et al.*, 2005; Choi *et al.*, 2008), and finally in inhibition of apoptosis (Fu *et al.*, 2000; Cao *et al.*, 2002; Forsythe *et al.*, 2002; Smith *et al.*, 2003a; Rahman *et al.*, 2005; Del Bufalo D. *et al.*, 2005; Santos *et al.*, 2006).

2.1 Telomerase components

Telomerase is a large nucleoprotein complex, with a mass over 1000 kDa (Schnapp *et al.*, 1998). *In vitro*, two components are essential for its activity: the highly conserved reverse transcriptase, TERT, and an associated template RNA, TR (also referred to as TERC or TER) (Greider and Blackburn, 1989; Feng *et al.*, 1995; Lingner *et al.*, 1997; Nakamura *et al.*, 1997; Tesmer *et al.*, 1999). However, *in vivo*, a number of other proteins composed the telomerase complex.

2.1.1 hTR : human Telomerase RNA component

hTR RNA is one of the 2 components essential in obtaining telomerase activity *in vitro* (Weinrich *et al.*, 1997). In 1998, the hTR gene was cloned and localized on the human chromosome 3q26.3 (Soder *et al.*, 1997; Zhao *et al.*, 1998). This single-copy gene does not contain any intron.

hTR structure and protein associations

In man, the mature hTR transcript is 451 nucleotides long without polyadenosine tail and carries a 5' tri-methyl rather than mono-methyl guanosine cap (Feng *et al.*, 1995; Fu and Collins, 2006). The template region of hTR that is complementary to the (TTAGGG)n telomere sequence comprises 11 nucleotides (5'-CUAACCCUAAC-3').

hTR possesses a stem-Hinge-stem-ACA (H/ACA) motif (Figure 5), which is required for cellular accumulation (Mitchell and Collins, 2000) and for telomerase activity *in vivo* (Fu and Collins, 2003). This motif is also present in small nucleolar (sno) RNAs and small Cajal body (sca) RNAs (Matera *et al.*, 2007). The H/ACA motif interacts with numerous proteins

like dyskerin, NHP2, NOP10, and GAR1 (Mitchell *et al.*, 1999b; Dragon *et al.*, 2000). Dyskerin, NHP2 and NOP10 form the H/ACA core proteins as they are crucial for the stability of hTR (Hoareau-Aveilla *et al.*, 2006; Fu and Collins, 2007; Walne *et al.*, 2007). They interact with the chaperone protein NAF1, which is consequently substituted by GAR1 (Darzacq *et al.*, 2006). Two other motifs identified as biogenesis box (BIO box) and CAB box are also needed for in vivo accumulation of hTR in Cajal bodies (Fu and Collins, 2003; Jady *et al.*, 2004; Fu and Collins, 2006; Fu and Collins, 2007). Finally, the CR4-CR5 domain of the hairpin is required for interaction with hTERT and thus for telomerase activity (Mitchell and Collins, 2000; Bachand and Autexier, 2001; Chen *et al.*, 2002).

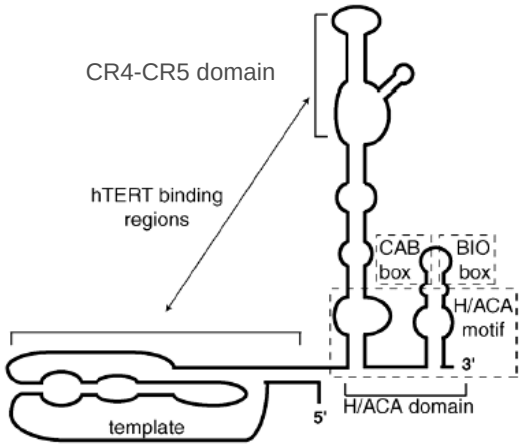


Figure 5. Motifs and domains of human telomerase RNA. Boxes or brackets indicate the locations of motifs involved in telomerase ribonucleoproteins biogenesis (the H/ACA motif and BIO box), hTERT binding or intranuclear localization (the CAB box) (cited from Collins, 2008).

Expression and tumorigenesis

RT-PCR analysis showed that hTR RNA is widely expressed in both tumor and non-tumor tissues such as testis, ovary, brain liver, small intestine, thymus, kidney, and prostate (Feng *et al.*, 1995; Yi *et al.*, 2001). Thus, it was concluded that hTR was not crucial for telomerase activation, even if the expression was shown to be up-regulated in cancer cells in comparison to normal cells (Heine *et al.*, 1998; Soder *et al.*, 1998; Atkinson *et al.*, 2005). In the mouse, the silencing of mTR expression leads to progressive telomere attrition over numerous generations because of a lack of telomerase activity (Blasco *et al.*, 1997). In addition, occurrence of chromosomal abnormalities like end-to-end fusions, and levels of apoptosis in highly proliferative tissues was found increased (Lee *et al.*, 1998; Rudolph *et al.*,

1999). In later generations, loss of telomere function and fusions occurred preferentially on chromosomes with critically short telomeres (Hemann *et al.*, 2001).

RT-PCR analyses on colon cancer showed that the hTR mRNA expression is correlated with telomerase activity (Yan *et al.*, 2001). Thus, this gene might be involved in telomerase-reactivation. Besides, a recent study showed that overexpression of both hTR and hTERT in cancer cells and in lung fibroblasts greatly increased telomerase activity and telomere length elongation, while independent overexpression of either hTR or hTERT has the same effect to a lesser extent (Wong and Collins, 2006; Cristofari and Lingner, 2006). The exact mechanisms by which TR promotes tumor growth remains unclear.

2.1.2 hTERT : human Telomerase Reverse Transcriptase

hTERT is the catalytic subunit of telomerase that harbors the reverse transcriptase activity. The human cDNA was isolated in 1997 (Kilian *et al.*, 1997; Meyerson *et al.*, 1997). The expression of hTERT is highly correlated with telomerase activity *in vitro* and *in vivo* (Meyerson *et al.*, 1997; Nakamura *et al.*, 1997). The hTERT expression is nearly imperceptible in the majority of differentiated somatic cells, which leads to inevitable telomeric attrition and subsequently cellular senescence. High levels of hTERT are detected in proliferative somatic cells like endometrial tissues or activated lymphocytes, but also in most immortalized and cancer cells.

The genomic sequence and the gene organization have been characterized by several groups (Cong *et al.*, 1999; Horikawa *et al.*, 1999; Takakura *et al.*, 1999; Wick *et al.*, 1999). The single-copy hTERT gene, which is composed of 16 exons and 15 introns spanning more than 40 kb, is localized very close to the telomere, on human chromosome 5p15.33 (Meyerson *et al.*, 1997; Bryce *et al.*, 2000). The hTERT promoter lacks traditional TATA and CAAT boxes. A consensus transcription start site has not been defined, however the various identified sites are all located 50 to 110 bp upstream of the translational start site of the gene.

The hTERT gene encodes a 127 kDa nuclear protein of 1132 amino acids (Meyerson *et al.*, 1997; Harrington *et al.*, 1997b). Four functional domains compose the hTERT protein: the N-terminal regulatory (R) domain, the RNA-binding (RB) domain, the reverse transcription (RT) domain, and the C-terminal dimerization domain (C) (Figure 6). hTERT protein also possesses a telomerase-specific motif (T). The RT domain, comprising 7 conserved reverse transcriptase motifs (1 and 2, A-E) is very important for the reverse transcriptase activity as

mutations in amino acids in these motifs switch off telomerase activity (Harrington *et al.*, 1997a; Weinrich *et al.*, 1997; Nakayama *et al.*, 1998). These motifs allow hTERT to recognize and subsequently reverse transcribe the hTR RNA template, leading to telomere elongation. Finally, substitutions in the C- and N-terminal domain also abrogate telomerase activity (Armbruster *et al.*, 2001; Banik *et al.*, 2002).

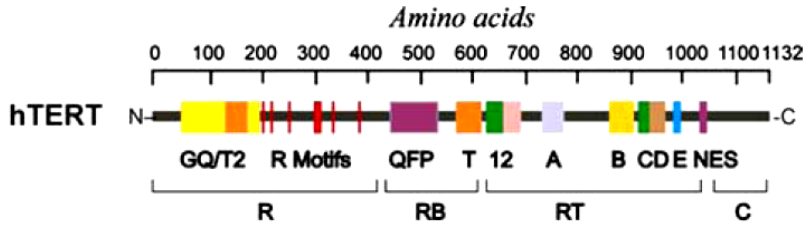


Figure 6. Working model for TERT domain architecture. The telomerase reverse transcriptase is divided into four major functional regions: the N-terminal regulatory (R) domain, the RNA-binding (RB) domain, reverse transcription (RT) domain, and the C-terminal dimerization domain (C) (cited from Dwyer *et al.*, 2007).

2.1.3 Auxiliary proteins

Numerous proteins or protein complexes bind to the telomerase complex and contribute to its activation or its stabilization. Auxiliary proteins of human telomerase include chaperone proteins, such as hsp90, p23, hsp70, p60, and hsp40. Hsp90 and p23 were found to bind hTERT protein, and promote assembly of the telomerase complex and are implicated in the *in vivo* activity of the telomerase complex (Holt *et al.*, 1999). In particular, the yeast hsp90 homolog has been shown to promote both telomerase DNA binding and nucleotide addition properties (Toogun *et al.*, 2008). Hsp40 and hsp70 also favor the accurate assembly of hTERT protein and hTR RNA into telomerase, by providing energy to the hsp90/p23 complex (Forsythe *et al.*, 2001).

The L22 and the hStau proteins are RNA-binding proteins associated with hTR RNA and hTERT protein (Le *et al.*, 2000). These two proteins might be involved in hTR transport, location, processing, and telomerase assembly (Le *et al.*, 2000).

The TEP1 protein was shown to bind hTR and hTERT and is associated with telomerase activity (Harrington *et al.*, 1997a; Beattie *et al.*, 2000).

As described previously, human telomerase interacts with the Dyskerin RNA binding protein, which is mutated in dyskeratosis congenita (DC) (Dokal, 2000). Dyskerin mutations lead to a decrease in telomerase activity, shorter telomeres and chromosome end fusions

(Dokal *et al.*, 1992; Mitchell *et al.*, 1999b; Kannan *et al.*, 2008). Dyskerin is involved in hTR processing and stabilization (Chen and Greider, 2004).

Heterogeneous nuclear ribonucleoproteins (hnRNPs) also interact with the telomerase complex. HnRNP A1 may help to place telomerase to the telomere extremity. Deficiency of hnRNP A1 leads to telomere shortening, whereas its restoration allows telomere elongation (LaBranche *et al.*, 1998). The complex hnRNP C1/C2 was found indispensable for telomerase activity as it directly binds to hTR RNA and co-localizes with telomeric proteins TRF1 and TRF2 (Ford *et al.*, 2000). Finally, hnRNP D might also interact with telomerase (Eversole and Maizels, 2000).

Other factors have been found to recruit and activate human telomerase at the 3' end of telomeres, such as homologs of the yeast Est1p protein: EST1A and EST1B. The overexpression of EST1A influences telomere length and capping (Snow *et al.*, 2003; Reichenbach *et al.*, 2003).

2.2 Mechanism of telomere maintenance by telomerase

The assembly of active telomerase involves the stabilization of hTR and its functional association with hTERT. The initial ribonucleoprotein (RNP) assembly of TR is thought to induce a conformational change of TR that stimulate TERT binding (Prathapam *et al.*, 2005; O'Connor and Collins, 2006; Stone *et al.*, 2007). A similar hierarchical RNP assembly mechanism is suggested for human telomerase in Figure 7.

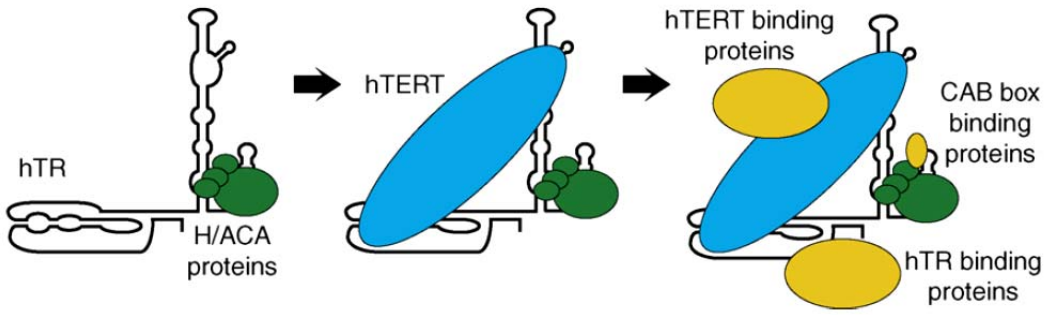


Figure 7. An ordered series of RNP assembly steps to product functional telomerase. First, assembly of hTR with the H/ACA-motif binding proteins (in green) dyskerin, NHP2, NOP10, and GAR1 is essential. RNP assembly on the hTR H/ACA motif would be predicted to influence the relative orientation of the hTR binding surfaces for hTERT (in blue), potentially promoting hTR–hTERT interaction. The numerous proteins that regulate human telomerase function (in yellow) may interact with the complex already formed (cited from Collins, 2008).

The association of hTR with the Dyskerin protein would control the position of the two hTR regions that bind hTERT (Mitchell *et al.*, 1999a; Mitchell *et al.*, 1999b; Vulliamy *et al.*, 2001; Chen and Greider, 2004). The hTERT association with telomerase RNP could be an hsp90-dependant process (Holt *et al.*, 1999; Forsythe *et al.*, 2001; Kim *et al.*, 2005). Finally, the hsp90/p23 complex is proposed to adjust and stabilize the telomerase structure in an active form (Keppler *et al.*, 2006). The nucleolar protein PinX1 and the two hTERT-interacting proteins NAT10 and the GNL3L are suggested to inhibit TERT–TR interaction (Lin and Blackburn, 2004) (Fu and Collins, 2007). Moreover, PinX1 could alternatively decrease RNP activity (Zhou and Lu, 2001; Banik and Counter, 2004).

The association of telomerase complex with telomeres requires either a pre-formed active complex or on the independent assembly of hTERT and telomerase RNP on telomeres (Holt *et al.*, 1997; Tomlinson *et al.*, 2006; Jady *et al.*, 2006). Elongation is then performed by hTERT until the end of the template region. The complex can subsequently translocate and reposition on the newly synthesized DNA, in order to continue telomere elongation. The lagging strand is then synthetized by the DNA polymerase complex (Figure 8).

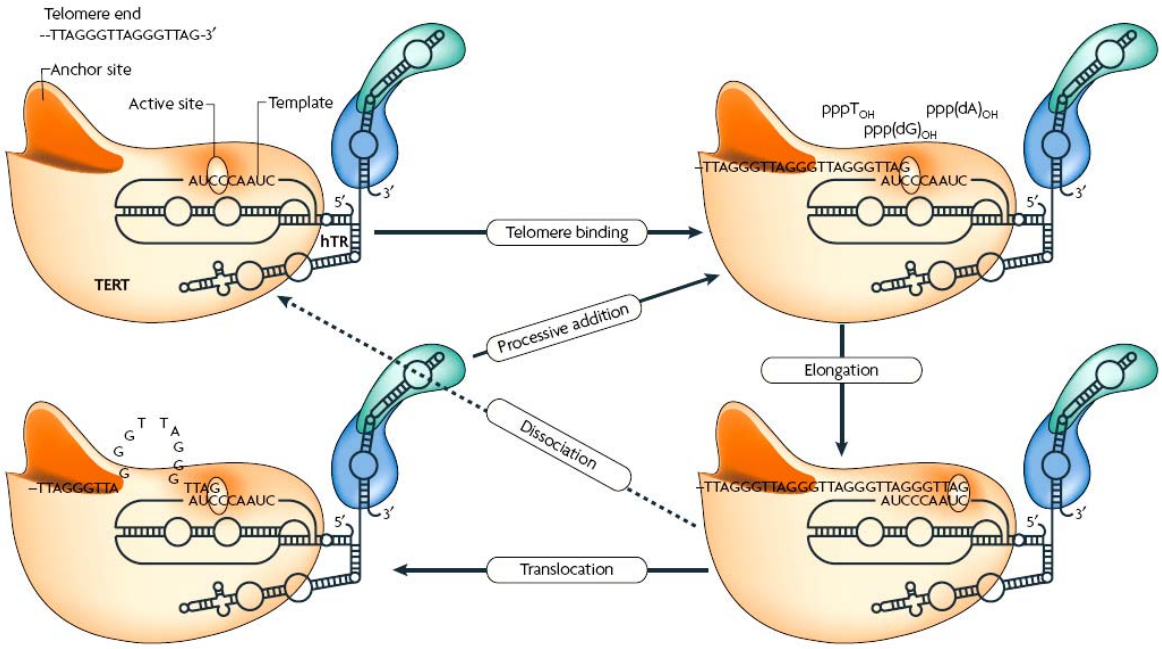


Figure 8. Mechanism of telomere elongation by telomerase. Two protein structures are schematically illustrated: the larger one represents telomerase reverse transcriptase (TERT), with shaded regions depicting the telomere substrate anchor site and the catalytic dNTP binding and template alignment site. The smaller protein structure represents dyskerin and other members involved in hTR processing and assembly into active telomerase. The sequence of the hTR template region is shown. The steps in telomere synthesis are (i) telomere binding, (ii) elongation, in which six nucleotides (GGTTAG) are sequentially added to the telomere, and (iii) translocation, in which the heteroduplex is shifted back by six nucleotides, positioning the enzyme for another round of elongation, that is, processive addition of GGTTAG. The process can be interrupted (dotted arrow). The exact motions and structures of the single-stranded and heteroduplexed regions of the telomere relative to the TERT anchor and active sites are not known (cited from Harley, 2008).

The human telomerase enzyme appears to be a dimer with two hTRs and two hTERTs subunits (Beattie *et al.*, 2001; Wenz *et al.*, 2001). The telomerase multimerization could enhance the processivity of the enzyme (Moriarty *et al.*, 2004).

3. Regulation of telomerase

As reported previously, telomerase is inactive in most somatic cells. In contrast, several normal cells and more than 70 % of immortalized human somatic cell lines or human cancers express high levels of telomerase. They also exhibit stable telomere length compared to the cells from which they originate. Thus, a telomere length maintenance prossess seems to be necessary for tumorigenesis or immortalization (Bryan and Reddel, 1997; Shay and Gazdar, 1997; Saldanha *et al.*, 2003). The regulation of the telomerase complex in expression or activation of its components is of particular interest. Telomerase can be regulated at different level such as its recruitment to the telomere terminus, accumulation of its components and its catalytic activation via correct assembly of the complex in an active conformation (Smogorzewska and De Lange, 2004). Most cells that lack telomerase activity also lack hTERT expression and, to some extent, hTR expression, whereas the auxiliary components appear to be widely expressed. *In vitro* experiments revealed that hTR and hTERT are sufficient to reconstitute telomerase activity. The activation of their expression is therefore considered as a crucial event (Weinrich *et al.*, 1997).

3.1 Regulation of telomerase at the telomere terminus

The shelterin complex might sequester the telomere terminus in the t-loop conformation, preventing accessibility of telomerase to telomere ends (Figure 9) (Griffith *et al.*, 1999).

More generally, all the components of shelterin act as negative regulators of telomerase: these include TRF1 and TRF2 (Smogorzewska *et al.*, 2000), RAP1 (Li *et al.*, 2000; Li and de Lange, 2003; O'Connor *et al.*, 2004), TIN2 (Ye and de Lange, 2004; Houghtaling *et al.*, 2004), and POT1/TPP1 (Loayza and De Lange, 2003; Liu *et al.*, 2004; Veldman *et al.*, 2004; Ye *et al.*, 2004a; Ye and de Lange, 2004). Moreover, the hnRNP proteins, except hnRNP C, are thought to connect human telomerase to telomeres, because they can associate with both hTR and telomeric repeats (Ford *et al.*, 2002; Fu and Collins, 2007).

POT1 could bind to the D-loop of the telomere, stabilizing the t-loop structure and thereby preventing telomerase access to the 3' terminus (Loayza *et al.*, 2004; Lei *et al.*, 2005; Kelleher *et al.*, 2005). Telomeres could be switched in an active state by opening the t-loop folding. Depletion of POT1 in human cells can result in telomere elongation (Veldman *et al.*, 2004; Ye *et al.*, 2004a). On the contrary, association of POT1 with TPP1 can increase the telomerase activity and its processivity (Figure 9) (Wang *et al.*, 2007; Xin *et al.*, 2007).

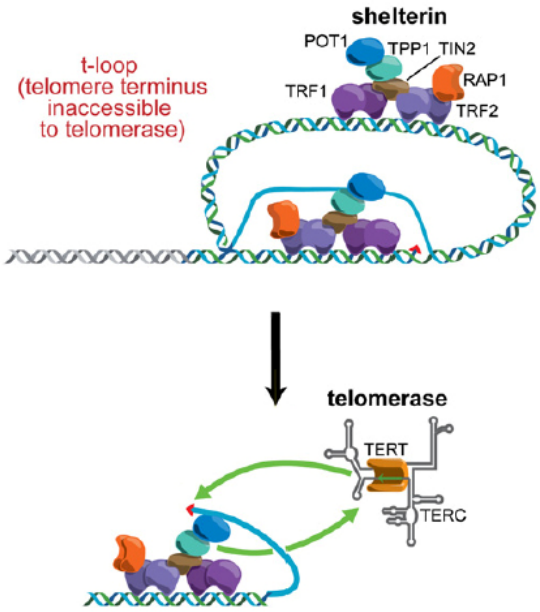


Figure 9. Possible mechanisms for Shelterin and POT1-TPP1 mediated regulation of telomerase. In mammalian cells, t loops are proposed to be non-permissive for telomerase activity due to sequestration of the telomere terminus (top). Opening of the t-loop could be in itself sufficient to allow telomerase to act (bottom) (cited from Bianchi and Shore, 2008).

A short telomere recruits less TRF1 factors than long telomere, that raise the chance of being elongated by telomerase (Ancelin *et al.*, 2002). In addition, two telomeric enzymes, TANK1 and 2, are shown to reduce the binding of TRF1 to telomere terminus (Smith and De Lange, 2000; Kaminker *et al.*, 2001; Cook *et al.*, 2002). PINX1, that interacts with TRF1, has also been proposed to be involved in the regulation of telomere length (Zhou and Lu, 2001).

Recently, PARP-1 has been proposed to inhibit telomerase activity as it can alter poly-ADP-ribosylation of TERT and the expression of TEP1 (Ghosh *et al.*, 2007). Finally, the newly discovered TERRAs were shown to be able to inhibit telomerase activity *in vitro*, possibly through matching with the template region of TR (Schoeftner and Blasco, 2008).

3.2 Regulation of the telomerase component: hTR

hTR expression might be regulated through several mechanisms, and the transcriptional regulation is emerging as the essential one. The promoter sequences of hTR contain numerous transcription factor binding sites that have been shown to be bound by activators and repressors (Zhao *et al.*, 1998). A minimal promoter region is localized 272 bp upstream of the transcriptional start site. This region encompasses Sp1-Sp3 binding sites in addition to a CCAAT box (Zhao *et al.*, 2000) (Figure 10). Only one site conferred a positive regulation on the promoter. Three other sites, located downstream of the CCAAT box, appeared to be repressive (Zhao *et al.*, 2003). Moreover, the MDM2 protein is found to transcriptionally repress the Sp1 activity. Activation of hTR also occurs through binding of RB, which is able to displace Sp1 from MDM2 (Zhao *et al.*, 2000; Johnson-Pais *et al.*, 2001). Recently, MDM2 was also found to directly inhibit transcriptional activity by binding hTR promoter (Zhao *et al.*, 2005). The CCAAT box seems to be recognized by the NF-Y transcriptional complex and appears as essential for the hTR transcription. In addition, induction of hTR expression was observed under hypoxic condition probably throughout the hypoxia response element (HRE) site (Anderson *et al.*, 2006).

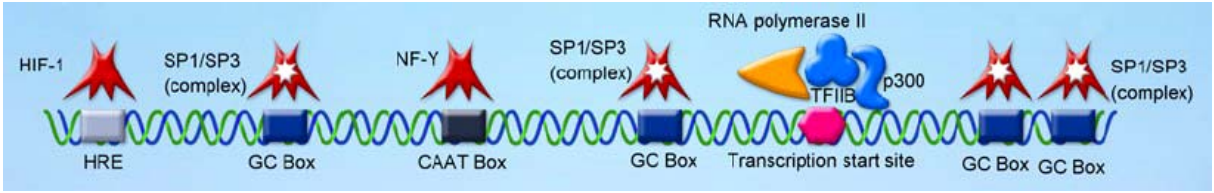


Figure 10. Transcriptional regulation of the hTR core promoter. Summary of known regulators of hTR transcription. The hTR core promoter contains binding sites for a number of transcription factors including Sp1 and HIF-1, which are positive regulators of hTR transcription and Sp3, which represses hTR transcription. Binding of the NF-Y complex to the CAAT box sequence is essential for transcriptional activation of hTR, however many regulators mediate their effects through Sp1 or Sp3 binding at the GC boxes (cited from Cairney and Keith, 2008).

The presence of a CpG island has suggested the possibility of hTR regulation by DNA methylation (see DNA methylation part 4). However, levels of DNA methylation are apparently not linked to hTR expression in both normal and tumor tissues or cell lines (Hoare *et al.*, 2001; Guilleret *et al.*, 2002b).

3.3 Regulation of the catalytic subunit of the telomerase: hTERT

hTERT expression is the limiting factor for telomerase activity (Meyerson *et al.*, 1997; Ramakrishnan *et al.*, 1998; Liu *et al.*, 2000) and its expression is sufficient to induce *in vitro* telomerase activity and avoid telomere erosion in fibroblasts (Bodnar *et al.*, 1998; Vaziri and Benchimol, 1998; Ramirez *et al.*, 2001). Genomic characterization of the hTERT gene has revealed that a minimum promoter region is essential for transcriptional activation. This includes the proximal 283 bp region upstream of the initiation ATG codon (Cong *et al.*, 1999; Horikawa *et al.*, 1999; Takakura *et al.*, 1999; Wick *et al.*, 1999). Several groups have found specific sites in the hTERT promoter sequence involved in transcriptional activation and repression (Li *et al.*, 1999; Wu *et al.*, 1999; Gunes *et al.*, 2000; Misiti *et al.*, 2000; Xu *et al.*, 2000; Poole *et al.*, 2001; Xu *et al.*, 2001; Ducrest *et al.*, 2002; Mauro and Foster, 2002). In addition, the hTERT promoter presents abundant CpG sites, suggesting a possible role of DNA methylation in its transcription regulation. Therefore, in the regulation of hTERT gene expression genetic, epigenetic, and post-transcriptional mechanisms are implicated.

3.3.1 Regulation at the genetic level

Identification of transcription factors involved in the control of hTERT transcription has been the centre of numerous investigations. The presence of sites for multiple activators and repressors suggests a complex system of regulation (Figure 11). Among these sites, several GC boxes for Sp1 transcription factor binding and two E-boxes (CACGTG) were identified within the hTERT promoter. E-boxes are able to bind the basic helix-loop-helix/leucine zipper transcription factors Myc/Max/Mad. Max can form heterodimers with Myc and Mad proteins, resulting in gene activation (Myc/Max) or repression (Mad/Max). Myc and Mad have antagonistic effects, and their expression is usually inverse. c-Myc is more highly expressed in proliferating and neoplastic cells (Luscher, 2001).

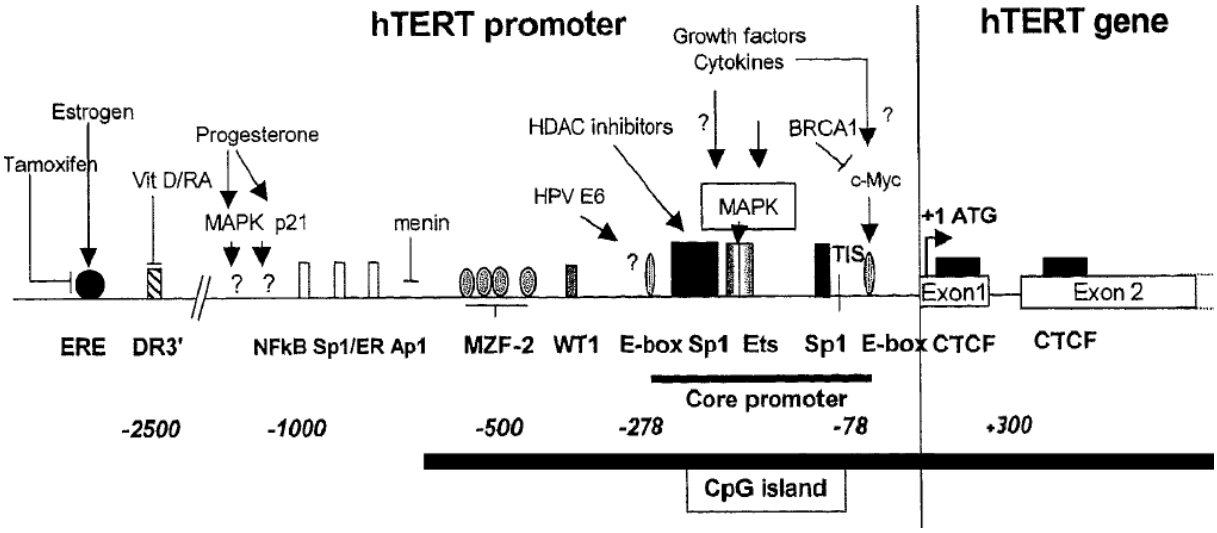


Figure 11. Regulatory sequences in the hTERT minimal promoter and the exonic proximal region (cited from Pendino *et al.*, 2006).

Factors downregulating hTERT transcription

In normal cells, inhibitors might repress hTERT expression and their action might be blocked in tumor cells, resulting in telomerase activation (Ducrest *et al.*, 2002). However, the inhibition can be partial and cell-type specific. Correspondingly, Mad is mostly activated in differentiating and non-proliferating cells (Luscher, 2001) and does not seem capable of totally suppressing hTERT expression (Poole *et al.*, 2001; Ducrest *et al.*, 2002). The WT1 factor downregulates hTERT transcription, but only in Wilm's tumor cells (Oh *et al.*, 1999). Other factors such as USF1 and USF2 can inhibit hTERT expression through direct binding at the E-box sites in oral cancer cells (Chang *et al.*, 2005). Another transcription factor, MZF-2 significantly limits hTERT transcription but it is suggested to play a minor role in hTERT regulation (Fujimoto *et al.*, 2000). Likewise, E2F-1 can bind to the hTERT promoter at two sites and inhibit hTERT transcription in cancer cells, whereas it allows its activation in normal cells (Crowe *et al.*, 2001; Won *et al.*, 2002a). The COUP-TIFII transcription factor, which plays a major role in development and cell differentiation, was shown to inhibit telomerase activity through reducing hTERT transcription by E-box binding in telomerase-positive cells (Wang *et al.*, 2004). The CBFA1 transcription factor was recently shown to repress hTERT transcription in human mesenchymal stem cell populations in order to favor

cell differentiation (Isenmann *et al.*, 2007). The transcription factor activator protein (AP-1), which is expressed in both cancer and normal cells and induces cell proliferation, apoptosis, differentiation, appears to be a constitutive transcriptional repressor of the hTERT gene (Takakura *et al.*, 2005). Viral regulators, such as NFX1-91, were shown to repress hTERT expression in high-risk human papillomavirus (HPV) E6 or c-Myc-expressing keratinocytes. NFX1-91 interacts with hTERT promoter and with the corepressor Sin3A/Histone deacetylase to directly repress hTERT transcription (Xu *et al.*, 2008). Moreover, NFX1-91 can be degraded by the HPV E6/E6AP complex, upon which a hTERT derepression occurs (Gewin *et al.*, 2004).

However, the repression of hTERT transcription by all these inhibitors cannot explain the absence of hTERT expression in most telomerase-negative cells. Importantly, the CCCTC-binding factor (CTCF) was shown to be a specific repressor of telomerase-negative normal cells. CTCF directly binds to a region situated in the first two exons of hTERT and thus blocks transcription, whereas this binding is lacking in telomerase-positive cells (Renaud *et al.*, 2005). CTCF, which is ubiquitously expressed, is a methylation-sensitive factor with versatile regulatory functions (Ohlsson *et al.*, 2001). CTCF is able to employ diverse combinations of its 11 zinc fingers to target various DNA sequences (Filippova *et al.*, 1996). This factor is involved in transcriptional activation of the amyloid beta-protein precursor promoter (Vostrov and Quitschke, 1997), silencing of c-myc (Filippova *et al.*, 1996), insulation of β-globin gene (Bell and Felsenfeld, 2000; Farrell *et al.*, 2002) and imprinting control of the H19 region (Kanduri *et al.*, 2000). The mechanism of CTCF inhibition could involve inhibition of either the transcription–initiation complex or the transcription elongation. Hypermethylation of the two first exons of the hTERT gene in most telomerase-positive cells blocks CTCF binding (Guilleret and Benhattar, 2004).

Repression of hTERT transcription is not always due to a direct effect of inhibitors. A more general repressor could be p53. Over-expression of p53 can lead to a rapid decresase of hTERT expression in a Sp1-dependent manner (Xu *et al.*, 2000; Kanaya *et al.*, 2000), but inhibition of p53 activity did not induce hTERT expression (Lin and Elledge, 2003). It was proved that p53 can form a complex with Sp1, which disturbs the transcriptional activity of Sp1 and leads to transcriptional repression (Xu *et al.*, 2000; Kanaya *et al.*, 2000). Likewise, p16 was shown to repress telomerase activity through transcriptional inhibition of hTERT in malignant glioma (Saito *et al.*, 2004). Transforming growth factor beta (TGF-β), which restrains cell proliferation and stimulates cell differentiation, represses hTERT transcription in

normal and cancer cells (Li *et al.*, 2006b). The mechanisms of hTERT repression are controversial: while some studies demonstrated that TGF-β repressed hTERT transcription via indirect down-regulation of c-Myc expression (Hu *et al.*, 2006), others reported direct interaction of Smad3 and c-Myc disturbing c-Myc activity (Li *et al.*, 2006a; Lacerte *et al.*, 2008). TNF-α (Tumor necrosis factor alpha) was also found to indirectly inhibit hTERT transcription in normal and leukemic human myeloid cells (Beyne-Rauzy *et al.*, 2005).

Factors activating the hTERT transcription

Numerous transcription factors able to activate hTERT transcription have been identified, including c-Myc, Sp1, AP-2 and AP-4. In particular, c-Myc binds to the two E-boxes on the hTERT promoter and activates the transcription in a dose-dependent manner (Ducrest *et al.*, 2002; Wang *et al.*, 1998; Zou *et al.*, 2005). However, several studies found that Myc and hTERT expression levels are not necessarily tightly correlated in cancer cells. It remains unclear whether endogenous binding of c-Myc on the hTERT promoter plays a critical role in the regulation of hTERT transcription *in vivo*. C-Myc and Sp1 could also be indirectly activated by Survivin, a member of the inhibitor-of-apoptosis family, inducing up-regulation of telomerase through hTERT activation (Endoh *et al.*, 2005). AP-2 was identified as a transcriptional activator of the hTERT promoter (Cong *et al.*, 1999) and, of particular interest, it exhibited tumor-specific binding to the core promoter region (Deng *et al.*, 2007). Although this study examined only one tumor type (lung cancer), this may partly explain tumor specific hTERT transcription. The involvement of HIF-1 in the activation of hTERT expression in tumor hypoxia has been demonstrated *in vitro* (Nishi *et al.*, 2004; Yatabe *et al.*, 2004). Hormones and growth factors are also involved in regulating gene hTERT expression (Bayne and Liu, 2005). Estrogen activates hTERT transcription via binding to the estrogen-responsive element (ERE) in the hTERT promoter (Kyo *et al.*, 1999; Misiti *et al.*, 2000; Nanni *et al.*, 2002). Progesterone is also able to promote hTERT transcription in progesterone-receptor-positive breast cancer cells (Wang *et al.*, 2000). Additionally, the epidermal growth factor (EGF) is implicated in hTERT activation (Maida *et al.*, 2002). Nevertheless, the activation of hTERT by these factors seems to rather indirect, and often cell-specific. For instance, the hALP protein (human N-acetyltransferase-like protein) modifies the activity of histone acetylation and induces telomerase activity via transactivation of the hTERT promoter (Lv *et al.*, 2003). The HER2/Neu, Ras and Raf oncoproteins can also

induce hTERT transcription through the ETS transcription factor ER81 and the mitogen-activated protein (MAP) kinase pathway in normal cells (Goueli and Janknecht, 2004). Bmi-1 overexpression allowed the escape from cellular growth control mechanisms, such as the p53 and the RB pathways (Pardal *et al.*, 2003; Valk-Lingbeek *et al.*, 2004), and subsequently induces telomerase activation through the activation of hTERT gene transcription (Dimri *et al.*, 2002). Moreover, the telomerase transcriptional elements-interacting factor (TEIF) might be a transcriptional activator of hTERT (Tang *et al.*, 2004). The binding of USF1/2 heterodimer to the E-boxes in the hTERT promoter always occurs, but it leads to promoter activation exclusively in hTERT-positive cells (Goueli and Janknecht, 2003). As previously reported, the HPV E6 protein induces hTERT transcription, which is dependent upon Myc binding sites (Veldman *et al.*, 2003; Liu *et al.*, 2005). Recently, another splice variant of NFX1, NFX1-123, was shown to activate hTERT expression and telomerase activity in HPV E6-expressing cells (Katzenellenbogen *et al.*, 2007). Finally, the latent membrane protein 1 (LMP1) of Eptein-Barr Virus was found to activate the hTERT promoter and increase telomerase activity in B lymphocytes (Terrin *et al.*, 2008).

Tollefsbol and Andrews propose that other regulatory elements distant from the 5' flanking region of the promoter could also be implicated in hTERT regulation. Thus, the expression of hTERT in aging cells and tumorigenesis could be due to a collective effect of binding of all these different factors, which may be under methylation control (Tollefsbol and Andrews, 2001).

The numerous factors involved in the hTERT transcriptional regulation are summerized Table 1, but how exactly hTERT is activated in telomerase-positive cells and repressed in telomerase-negative ones is not yet clear. Moreover, the endogenous-hTERT mRNA levels detected in telomerase-positive cell lines are very low (0.2 to 6 copies per cell) (Ducrest *et al.*, 2001; Yi *et al.*, 2001). In contrast, a high level of transcription is obtained after transfection of the hTERT core promoter, which can be as strong as the SV40 promoter, in telomerase-positive cell lines (Cong *et al.*, 1999).

All these findings suggest a complex system of transcriptional regulation of the telomerase catalytic subunit hTERT. Moreover, the hTERT mRNA and protein are also subject to other mechanisms of regulation, adding to its complexity.

Table 1. Recapitulative table of the transcription factors that bind the hTERT 5'-regulatory region.

Transcription factors	Role	Number of binding sites		Reference
		in the 5'-regulatory region	in the core promoter (-283 to +1)	
p53	**Repressor**	2	0	(Xu *et al.*, 2000; Kanaya *et al.*, 2000)
Mad1	**Repressor**	2	2	(Oh *et al.*, 2000)
MZF-2	**Repressor**	4	0	(Fujimoto *et al.*, 2000)
WT1	**Repressor**	1	0	(Oh *et al.*, 1999)
TGFβ	**Repressor**	-	-	(Yang *et al.*, 2001; Li *et al.*, 2006a)
Menin/JunD/NF-κB	**Repressor**	2	-	(Lin and Elledge, 2003)
COUP-TIFII	**Repressor**	3	2	(Wang *et al.*, 2004)
E2F-1	**Repressor** in cancer cells	2	2	(Crowe *et al.*, 2001)
USF1/2	**Repressor**	2	2	(Chang *et al.*, 2005)
AP-1	**Repressor**	2	0	(Takakura *et al.*, 2005)
CBFA1	**Repressor**	2	0	(Isenmann *et al.*, 2007)
NFX1-91	**Repressor**	1	1	(Gewin *et al.*, 2004; Xu *et al.*, 2008)
CTCF	**Repressor**	2 in the exonic region		(Renaud *et al.*, 2005)
Smad3	**Repressor**	1	1	(Hu *et al.*, 2006; Li *et al.*, 2006a)
E2F-1	**Activator** in normal cells	2	2	(Won *et al.*, 2002a; Alonso *et al.*, 2006)
Estrogen	**Activator**	2	0	(Kyo *et al.*, 1999; Misiti *et al.*, 2000; Nanni *et al.*, 2002)
Sp1	**Activator**	14	9 (+ 1 in the exonic region)	(Kyo *et al.*, 2000)
c-Myc	**Activator**	2	2	(Wu *et al.*, 1999; Kyo *et al.*, 2000; Zou *et al.*, 2005)
Bmi-1	**Activator**			(Dimri *et al.*, 2002; Pardal *et al.*, 2003; Valk-Lingbeek *et al.*, 2004)
USF1/2	**Activator**	2	2	(Goueli and Janknecht, 2003)
hALP	**Activator**	Potential binding to +90 to -120		(Lv *et al.*, 2003)
ER81	**Activator**	2	0 (+ 2 in the exonic region)	(Goueli and Janknecht, 2004)
HIF-1	**Activator**	2	2	(Nishi *et al.*, 2004; Yatabe *et al.*, 2004; Anderson *et al.*, 2006)
TEIF	**Activator**	Potential binding to +90 to -120		(Tang *et al.*, 2004)
AP2	**Activator**	17	9 (+ 5 in the exonic region)	(Cong *et al.*, 1999; Horikawa *et al.*, 1999; Takakura *et al.*, 1999; Wick *et al.*, 1999; Deng *et al.*, 2007)
AP4	**Activator**	9	3 (+ 1 in the exonic region)	(Cong *et al.*, 1999)
CCAC	**Activator**	1	0	(Wick *et al.*, 1999)
c-Ets-2	**Activator**	2	2	(Horikawa *et al.*, 1999)
c-Myb	**Activator**	2	0	(Horikawa *et al.*, 1999; Takakura *et al.*, 1999; Wick *et al.*, 1999)
CREB/ATF	**Activator**	1	0	(Cong *et al.*, 1999)
NFκB/T3Rα	**Activator**	1	0	(Cong *et al.*, 1999)
HPV E6	**Activator**	2	2	(Veldman *et al.*, 2003; Liu *et al.*, 2005)
NFX1-123	**Activator**	-	-	(Katzenellenbogen *et al.*, 2007)
LMP1	**Activator**	-	-	(Terrin *et al.*, 2008)

3.3.2 Regulation at the epigenetic level

DNA methylation

Several groups have examined the methylation status of the hTERT CpG island promoter. It was initially expected that methylation of the hTERT promoter induces gene silencing. However, some reports indicated no significant correlation between hTERT expression and methylation status as hTERT CpG island was found hypomethylated in many telomerase-negative and telomerase-positive cell lines and tumors (Devereux *et al.*, 1999; Dessain *et al.*, 2000; Lopatina *et al.*, 2003; Shin *et al.*, 2003). Other groups have observed a positive correlation between hTERT methylation and hTERT expression. Hypermethylation of the hTERT promoter was reported in hTERT-positive cancer cells or tissues, while no methylation was found in normal hTERT-negative cells (Guilleret *et al.*, 2002a; Nomoto *et al.*, 2002) (see DNA methylation alterations and cancer part 4.3).

Moreover, in tumor cell lines with an hTERT hypermethylated promoter, treatment with the demethylating agent 5-aza-2'-deoxycytidine promoted hTERT promoter demethylation up to 95%, with a strong decrease of hTERT mRNA expression (Guilleret and Benhattar, 2003). This suggests a role of DNA methylation in the regulation of hTERT expression (Kumakura *et al.*, 2005). These unusual correlations between DNA methylation and hTERT expression in normal and cancer cells has generated confusion among telomerase researchers.

The mechanisms involved were clarified using a methylation cassette assay. Selective demethylation within the core promoter around the transcription start site was shown to significantly increase hTERT transcriptional activity (Renaud *et al.*, 2007). In parallel, all telomerase-positive cancer cell lines examined exhibited hypomethylation around the transcription start site despite the presence of hypermethylation in more upstream regions (Zinn *et al.*, 2007). ChIP assay revealed that both active and inactive chromatin marks are present across the hTERT promoter. The active chromatin mark around the transcription start site was strongly associated with unmethylated DNA. These data suggest that the absence of methylation in association with active chromatin marks around the transcription start site allows the expression of hTERT, indicating that the hTERT DNA methylation pattern is consistent with the classical dynamics of gene expression. In our regulation model (Renaud *et al.*, 2007), DNA methylation exhibits a dual role in hTERT transcriptional regulation: hTERT methylation prevents the CTCF inhibitor from binding, but the core promoter requires partial hypomethylation to allow hTERT transcription (Figure 12).

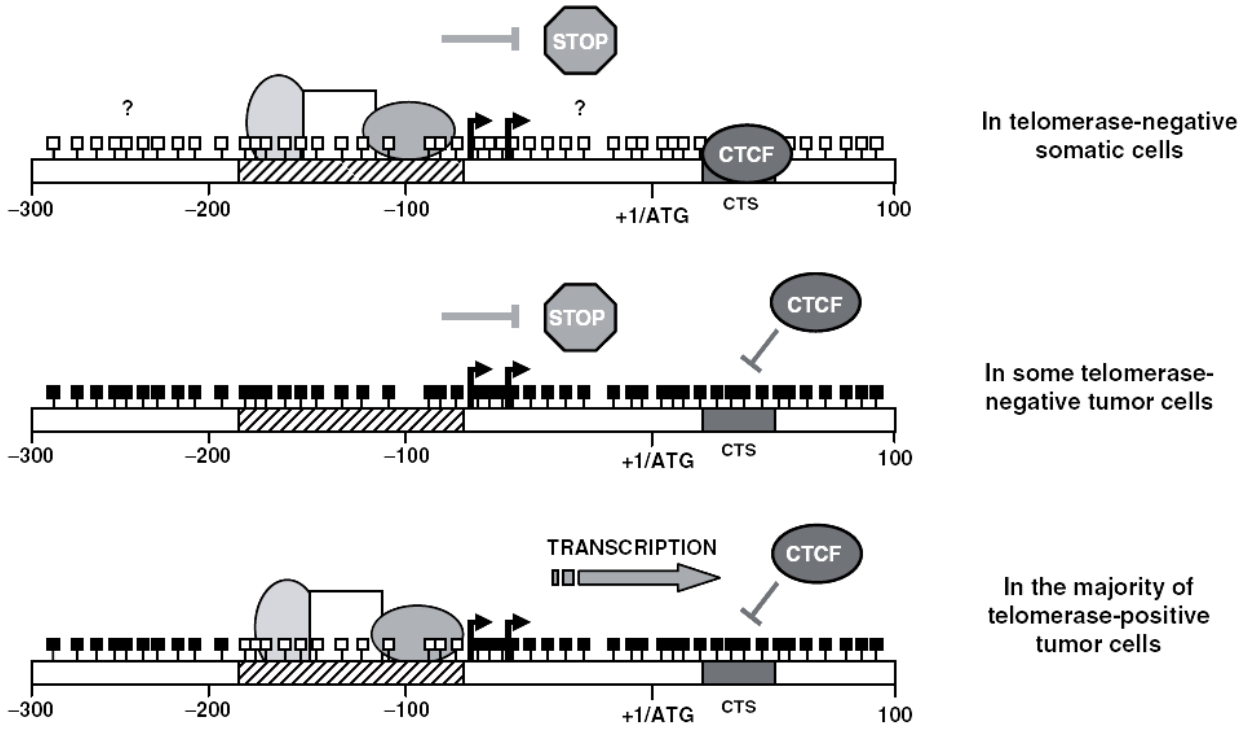


Figure 12. Proposed model of hTERT transcriptional regulation. In telomerase-negative somatic cells, CTCF binds to unmethylated CTCF binding site and inhibits hTERT transcription. Full methylation of the hTERT minimal promoter and exon1 in some telomerase-negative tumor cells cannot lead to hTERT transcription. A majority of the tumor CTCF sites within the hTERT gene are methylated, thus preventing CTCF binding. Partial demethylation of the hTERT promoter region with the formation of an active transcriptional complex can lead to hTERT transcription and telomerase activity. CTS stands for CTCF target sites. The hatched box represents the region A showed as unmethylated in cancer cell lines and tumor tissues. Question marks represent the two regions surrounding the region A, and that might have a strong influence on the hTERT promoter activity, most probably methylation-sensitive binding sites to transcription factors. Empty squares represent unmethylated CpG sites, and solid squares represent methylated CpG sites (cited from Renaud *et al.*, 2007).

The DNA packaging

The chromatin structure of the native hTERT locus was also examined. It is likely that histone deacetylation, leading to chromatin condensation, is implicated in hTERT repression in telomerase-negative normal cells (Cong and Bacchetti, 2000; Nakamura *et al.*, 2001a; Takakura *et al.*, 2001; Hou *et al.*, 2002; Wang and Zhu, 2003; Lopatina *et al.*, 2003). In telomerase-negative cells, the inhibition of histone deacetylase by Trichostatine A (TSA) led to open chromatin structure and hTERT transcription (Wang and Zhu, 2003). Interestingly, the Sp1 factor, despite its transcriptional activation of hTERT, might also recruit histone deacetylase (HDAC) and induce gene silencing of hTERT in normal cells (Won *et al.*, 2002b).

The c-Myc/Max complex was found to be associated with acetylated histones, resulting in enhanced hTERT expression in proliferating leukemia cells (Xu *et al.*, 2001). In contrast, the Mad1/Max complex was found associated with deacetylated histones and decreased hTERT expression. Recently, the presence of trimethylated H3-K4, which is likely due to the histone methyltranferase SMYD3, is associated with transcription of the hTERT gene in telomerase-proficient tumor cells (Atkinson *et al.*, 2005; Liu *et al.*, 2007). Trimethylated H3-K4 indeed induced recrutement of HAT, providing access of transcriptional activators, such as Sp1 and c-Myc, to the hTERT promoter (Kyo *et al.*, 2008). By contrast, H3K4 can be demethylated by lysine-specific demethylase 1 (LSD1) and this can block hTERT transcription in normal and cancer cells (Zhu *et al.*, 2008).

3.3.3 Regulation at the post-transcriptional and post-translational level

Telomerase activity has been shown to be regulated by alternative splicing of hTERT (Ulaner *et al.*, 1998; Fan *et al.*, 2005). At least 7 alternatively spliced variants (4 insertions and 3 deletions) can co-exist and their expression level depends on the tissue type (Ulaner *et al.*, 1998; Wick *et al.*, 1999; Ulaner *et al.*, 2000). However, the 4 insertions and the β deletion (182 bp) result in premature termination and nonfunctional proteins. Only a full length mRNA encodes a protein with catalytic activity, which is unexpectedly less present than the β-spliced variant (Kilian *et al.*, 1997; Collins and Mitchell, 2002). Interestingly, the α deletion (36 bp) has been shown to considerably inhibit telomerase activity (Colgin *et al.*, 2000; Yi *et al.*, 2000), because of an inactive complex forming between truncated hTERT mRNA and hTR (Lai *et al.*, 2001). The γ deletion (189 bp) is also believed to be catalytically inactive (Hisatomi *et al.*, 2003). Recently, other alternative spliced of hTERT forms were detected in lung and colon, and could also regulate telomerase activity (Saeboe-Larssen *et al.*, 2006).

Furthermore, the expression patterns of hTERT alternative splice variants can be different from the corresponding adjacent normal tissues, such as in ovary, kidney, uterine, and breast cancer (Ulaner *et al.*, 2000; Yokoyama *et al.*, 2001; Zaffaroni *et al.*, 2002; Fan *et al.*, 2005). For instance, malignant thyroid tumors exhibit a high level of full-length hTERT transcript (Anderson *et al.*, 2006; Wang *et al.*, 2008). Moreover, the presence of alternative splicing of hTERT has been correlated with the deficient telomerase activity (Fujiwara-Akita *et al.*, 2005; Ohyashiki *et al.*, 2005; Zaffaroni *et al.*, 2005). Interestingly, hypoxic conditions can induce a switch in the hTERT splice pattern in favor of the active isoform (Anderson *et*

al., 2006). These results support a role for hTERT splice-variants in the regulation of telomerase activity.

Post-translational processing of the hTERT protein might also regulate its activity. Phosphorylation of hTERT by protein kinase C (PKC), protein phosphatase 2A (PP2A), Akt or c-Abl tyrosine kinase are involved in regulation of telomerase activity (Li *et al.*, 1998; Kharbanda *et al.*, 2000; Yu *et al.*, 2001; Jagadeesh and Banerjee, 2006; Anderson *et al.*, 2006). PKC has been shown to modulate telomerase activity via a phosphorylation of hTERT in human breast, head, and neck cancer cells (Li *et al.*, 1998; Chang *et al.*, 2006; Anderson *et al.*, 2006). Depletion of PKC affects the hTERT-hsp90 interaction, suggesting that hTERT phosphorylation is necessary for assembly and activation of the telomerase complex (Chang *et al.*, 2006; Anderson *et al.*, 2006). Finally, phosphorylation is likely to allow transfer of hTERT protein from the cytoplasm to the nucleus, subsequently allowing telomerase assembly (Aisner *et al.*, 2002).

Interactions between transcriptional factors and epigenetic regulators need to be further explored to better understand regulation of the hTERT transcription. Likewise, transport and post-translational modifications of hTERT protein, as well as assembly and recruitment of the telomerase complex also require more investigations to completely clarify the telomerase activation mechanism.

3.4 Telomerase as a biomarker of cancer

Numerous molecular markers for common cancers have been suggested, such as lung, breast, and colon, but telomerase activity is the most universal one since its overall prevalence is estimated at 85% tumors (Kim, 1997; Shay and Gazdar, 1997; Dhaene *et al.*, 2000). Unfortunately, telomerase activity is also observed in some normal cells, such as proliferative progenitor cells in self-renewing tissues and activated lymphocytes (Hiyama *et al.*, 1995c; Hiyama *et al.*, 1996b; Wright *et al.*, 1996b), but also in several benign tumors such as fibroadenoma of the breast (Hiyama *et al.*, 1996a), hyperplastic nodule/adenoma of the thyroid (Matthews *et al.*, 2001), and colon adenoma (Hiyama *et al.*, 1996b). For instance, the occurrence of activated lymphocytes in leukemia complicates the identification of telomerase-positive cells derived from neoplastic clones. However, the telomeres of neoplastic cells from acute leukemia are generally shorter than those in the corresponding normal cells because of

active cell division. Accordingly, telomere length needs to be determined in addition to the measurement of telomerase activity because it reflects the mitotic history of the disease (Pendino *et al.*, 2006). In patients with inflammatory diseases, the sensitivity of telomerase activity detection reached only 70% mainly because of activated lymphocyte contamination (Hiyama *et al.*, 1998). For the same reason, the detection of telomerase in body fluids such as cervical smear, scraping samples or in native urine, is of limited use in spite of its potential as a non invasive marker of cancer (Orlando *et al.*, 2001; Jarboe *et al.*, 2002).

Likely, some normal tissues or precancerous lesions can also exhibit telomerase activity and induce telomerase activity without the presence of neoplastic cells. This could be the case in organs like lung, esophagus, stomach, colon, liver, bladder, prostate, head, uterus, and skin. On the other hand, detection of telomerase activity may be a valuable diagnostic marker for breast cancer as it occurs in most invasive breast cancer tissues and carcinoma in situ samples. Moreover, the sensitivity and specificity of telomerase activity were significantly better than those of cytology (Hiyama *et al.*, 2000).

Telomerase could also be a prognostic indicator as telomerase activity levels increased along with cancer progression in gastric and colon adenocarcinomas (Hiyama *et al.*, 1995b; Chadeneau *et al.*, 1995; Tahara *et al.*, 1999; Tatsumoto *et al.*, 2000). Poor prognosis is associated with telomerase activity in patients with gastric cancer (Hiyama *et al.*, 1995b), lung cancer (Marchetti *et al.*, 1999), breast cancer (Clark *et al.*, 1997), and neuroblastoma (Hiyama *et al.*, 1995a; Poremba *et al.*, 1999; Streutker *et al.*, 2001).

In blood of cancer patients, circulating DNA or RNAs of tumor cells can be present, and therefore the detection of hTERT mRNA in the blood could be used as a powerful and noninvasive cancer biomarker (Chen *et al.*, 2000; Dasi *et al.*, 2001; Shin *et al.*, 2002). However, as previsouly mentioned, hTERT mRNA as well as telomerase activity are known to be upregulated in activated lymphocytes, which seriously limits the use of this biomarker in the patients with various types of inflammation such as autoimmune disease and infection (Hiyama *et al.*, 1995c; Hiyama *et al.*, 1998).

The *in situ* detection of telomerase-positive cells could be very usefull, allowing morphological identification of telomerase-positive cells and discrimination of cancerous and noncancerous cells. For instance, hTERT detection can be performed through *in situ* hybridization or immunohistochemistry. However, technical difficulties have been reported because of the low amount of hTERT mRNA or protein per cell or because of the lack of specificity of hTERT antibodies (Kumaki *et al.*, 2001; Hiyama *et al.*, 2001; Kumaki *et al.*,

2002; Wu *et al.*, 2006; Anderson *et al.*, 2006). Moreover, the stabity of the enzyme, and the hTERT mRNA, or the presence of alternate splicing variants can also lead to false negative results.

3.5 Telomerase as a target for anti-cancer therapeutics

The main interest of targeting telomerase is that it is ubiquitously and specifically expressed in cancer cells, including the putative cancer stem cell (Cortez-Gonzalez and Zanetti, 2007). The low or transitory level of telomerase in normal tissues, including normal stem cells, potentially offers tumor specific features to anti-telomerase drugs and low normal tissue toxicity. Different approaches have been explored in order to develop anti-cancer therapeutics, which might target either the telomerase positive-cells (immunotherapy, gene therapy), the components of telomerase (hTR, hTERT), or directly the telomeres (G-quadruplex inhibitors). Other strategies consist in targeting telomerase expression and regulation (transcriptional factor inhibitors).

Both hTERT and hTR promoters are promising in telomerase gene therapy, for which suicide gene therapy and oncolytic viral therapy approaches have been developed (Figure 13) (Keith *et al.*, 2004; Keith *et al.*, 2007; Cairney and Keith, 2008).

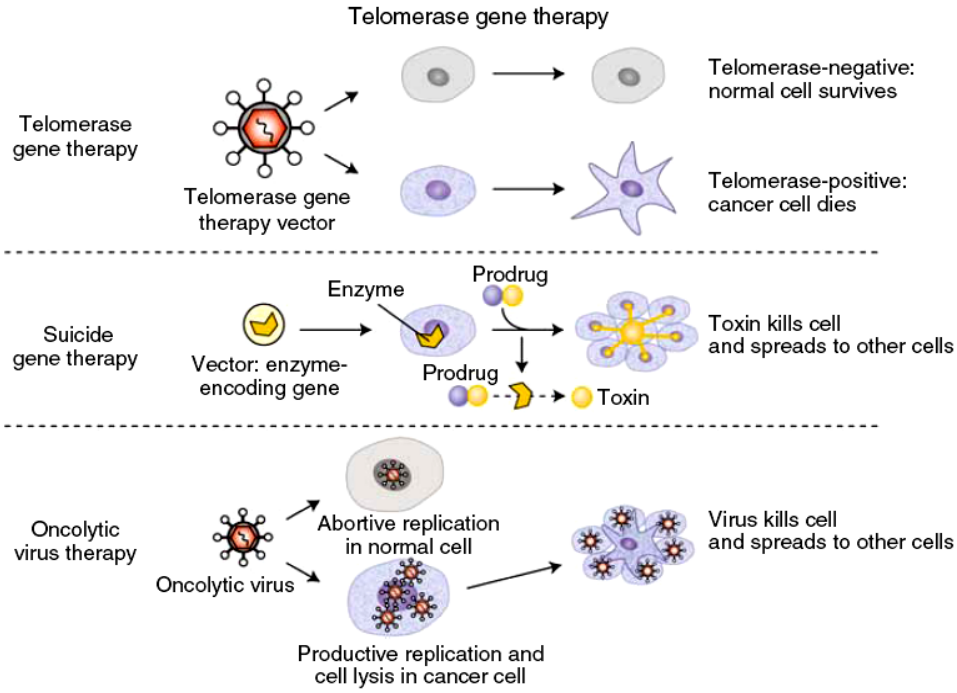


Figure 13. Different telomerase gene therapy approaches (cited from Shay and Keith, 2008)**.**

Telomerase-targeting adenoviral suicide gene therapy is based on the deliverery of a suicide gene therapy construct (Ad-hTR-NTR). The following steps are the activation of the nitroreductase suicide gene by the hTR promoter, and finally the activation the prodrug CB into a cytotoxic alkylating agent (Plumb *et al.*, 2001; Bilsland *et al.*, 2003). On the other hand, the telomerase-specific oncolytic virus approach utilizes manipulated adenoviruses that have developped properties allowing telomerase-positive cancer cells to be specifically destroyed (Keith *et al.*, 2004; Keith *et al.*, 2007). The hTERT promoter can control the replication of adenovirus and allow a selective dissemination of the virus within tumor cells, whereas infection of normal somatic cells does not induce toxic effects (Fujiwara *et al.*, 2007). For example, Telomelysin is a telomerase-specific replication-competent adenovirus (TRAD), which has recently advanced to clinical trials as it induces anti-tumor effects on numerous human cancer cells.

Telomerase (hTERT) immunotherapy is based on two general approaches. The first one uses autologous dendritic cells transfected with hTR component to activate cytotoxic T cells and kill telomerase-positive tumor cells. This approach has resulted in a therapeutic cancer vaccine called GRNVAC1, which recently entered into a clinical trial on prostate cancer patients (Su *et al.*, 2005). The second approach concerns a peptide vaccine named GV1001, derived from the hTERT active site. It is indicated for the treatment of pancreas, liver and lung cancer (Brunsvig *et al.*, 2006; Bernhardt *et al.*, 2006).

To target hTERT, nucleoside inhibitors such as AZT or AZGTP were planned to inhibit reverse transcriptase activity via blocking the incorporation of dNTPs (Fletcher *et al.*, 2001). The hTERT antisense oligodesoxynucleotides decrease telomerase activity and limited cell growth in bladder cancer cells, but no other studies confirmed the results (Kraemer *et al.*, 2003). Ribozymes have been successful in endometrial, breast and ovarian carcinoma (Ludwig *et al.*, 2001; Saretzki *et al.*, 2001). Dominant negative hTERT proteins, catalytically inactive, were also shown to inhibit telomerase activity (Hahn *et al.*, 1999; Herbert *et al.*, 1999; Zhang *et al.*, 1999a).

To target hTR, GRN163L inhibitor is an oligonucleotide complementary to hTR template that competitively inhibits telomerase (Herbert *et al.*, 2005; Dikmen *et al.*, 2005; Gellert *et al.*, 2006). GRN163L demonstrated anti-tumorigenic properties in hematological and solid tumor models, but also in potential cancer stem cells, which are believed to be chemotherapy-resistant. GRN163L is clinically tested in association with usual paclitaxel-carboplatin chemotherapy (Shay and Keith, 2008).

In summary, telomerase-targeting therapies are a promising approach to treat cancer. However, telomerase-negative tumors would be refractory to these therapies. As these maintain telomerase length through ALT pathway, new anti-cancer therapies should be developed to target these tumors (Stewart, 2005).

4. DNA methylation

Epigenetic inheritance rests on gene expression levels and is independent of the DNA sequence, in contrast to genetic inheritance. The most common epigenetic event in the mammalian genome is DNA methylation. In vertebrates, DNA methylation results in the addition of a methyl group (CH3) (Figure 14) on a cytosine preceding a guanosine, the CpG dinucleotide, by the DNA methyltransferase enzyme (DNMT) (Jones, 1999).

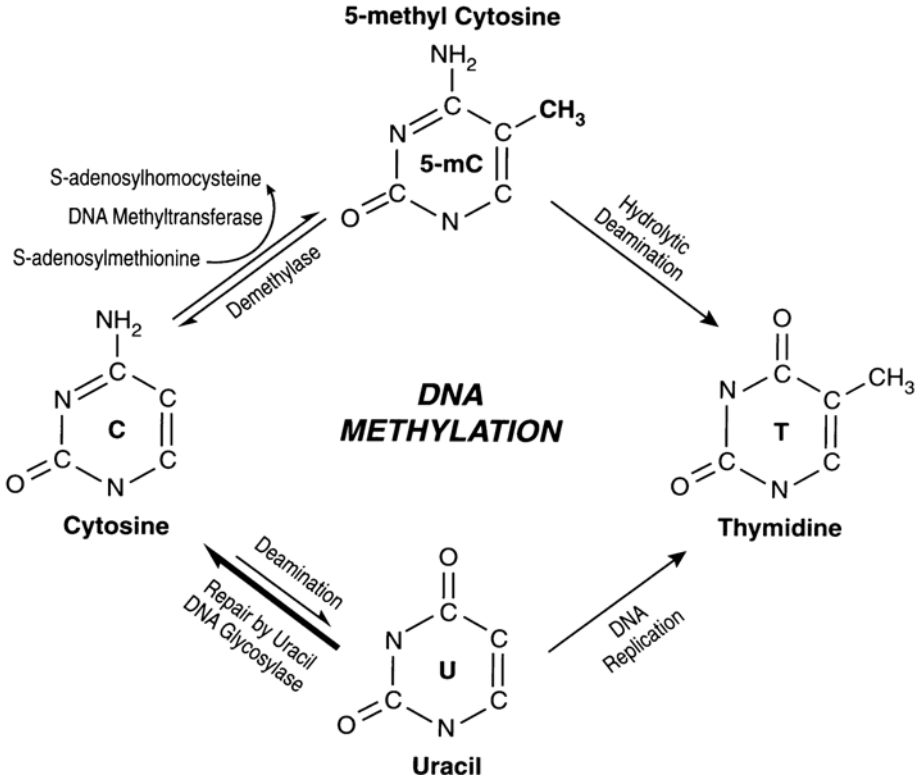


Figure 14. Schematic representation of the biochemical pathway for cytosine methylation, demethylation and mutagenesis of cytosine and 5-methylcytosine (cited from Singal and Ginder, 1999)**.**

Distribution of CpGs and CpG islands

About 70-80% of the CpG are methylated and these are randomly dispersed along the DNA sequence. These methylated regions are typical of non coding DNA. This highly methylated DNA replicates later than unmethylated DNA (Bestor, 1990). Late replicating DNA is characteristic of inactive chromatin (Bird and Wolffe, 1999). This mechanism participates to the transcription inhibition of repeat elements, inserted viral sequences, and transposons.

Small regions of DNA (0.5 to 5 kb), termed CpG islands, have precise characteristics. They are GC rich (60% to 70%), have a CpG/GpC ratio of at least 0.6, and are generally unmethylated (Cross and Bird, 1995). CpG islands characterize the promoter regions of half of human genes, including housekeeping genes and tissue-specific genes. The promoters of CpG islands are normally unmethylated in all tissues with the exeption of non-transcribed genes on the inactive X-chromosome and imprinted autosomal genes, of which one of the parental alleles is repressed through methylation (Bird, 2002). CpG islands more rarely are located in the core of the gene, or even in the 3'-region, and these are more prone to methylation (Nguyen *et al.*, 2001).

4.1 Proteins that mediate DNA methylation

DNA methylation within a gene promoter CpG island correlates with its transcriptional silencing (Bird, 2007). Only methylation within and/or around the promoter region is associated with gene silencing (Jones, 1999; Singal *et al.*, 2002). DNA methylation by itself does not directly repress transcription, but can prevent binding of transcriptional activators and can induce formation of inactive chromatin through proteins that specifically bind to methylated DNA, leading to transcriptional repression (Kass *et al.*, 1997; Bird and Wolffe, 1999).

4.1.1 Chromatin structure and histone code

The chromatin in mammalian cells is represented by a series of nucleosomes arranged in a compact configuration. The nucleosome consists of 146 bp DNA wrapped around a protein octamer containing two molecules of each histone H2A, H2B, H3, and H4 (Richmond

and Davey, 2003). At sites where transcription takes place, this chromatin structure becomes more "open" and accessible to transcription factors. Certain amino acids of histone proteins can be chemically modified by acetylation or ubiquitination of lysine, methylation of lysine or arginine, and phosphorylation of serine (Bird and Wolffe, 1999; Spotswood and Turner, 2002; Peterson and Laniel, 2004).

The histone code affects chromatin arrangement by modifying contacts between different histones and between histones and chromatin. Histone modifications allow the compartmentalization of the DNA into domains, such as silent heterochromatin and active euchromatin (Martin and Zhang, 2005). Moreover, the influence of histone code on the chromatin structure also allows a regulation of several main mechanisms like replication, transcription, DNA repair, and chromosome condensation (Kouzarides, 2007).

Histone acetylation, catalyzed by histone acetyltransferase (HAT), plays a central role in the formation of permissive chromatin and is associated with active transcription (Wolffe, 1996). High level of H3 acetylation is likely to be associated with gene promoters and conserved non-coding sequences (Roh *et al.*, 2005; Roh *et al.*, 2006). Acetylation has the potential to affect chromatin structure by disrupting inter-nucleosomal interactions (Shogren-Knaak and Peterson, 2006; Shia *et al.*, 2006). The acetylation of lysine residues on N-terminal histone tails reduces the positive charge of the histones and subsequently decreases its attraction with DNA. Thus, histone H3 and H4 acetylation results in decondensation of the chromatin to permit binding of transcription factors to DNA (Krajewski, 2002). Active chromatin marks comprise H3K9 acetylation, H4 acetylation, but also dimethylation at H3K4 and trimethylation at H3K4, H3K36, or H3K79, which lead to chromatin decondensation (Chambeyron and Bickmore, 2004; Barski *et al.*, 2007; Okitsu and Hsieh, 2007).

Histone deacetylases (HDACs) can remove acetyl groups from lysines of histones, which represses transcription by decreasing the accessibility of DNA for transcription factors (Strahl and Allis, 2000). Histone methyltransferases (HMTs) are also recruited to silence euchromatin. Condensed heterochromatin is indeed enriched in trimethylation of H3K9, H3K27, and H4K20 (Kouzarides, 2007). Methylated H3K9 represents a binding site for the heterochromatin protein 1 (HP1), which is known to repress gene transcriptionally. Interestingly, trimethylation of H3K9 has also been involved in the regulation of telomere length in mice since HMT null mice seem to have abnormally long telomeres (Garcia-Cao *et al.*, 2004).

Moreover, histone demethylases, as RBP2 (Christensen *et al.*, 2007) or LSD1 (Shi *et al.*, 2004) allow specific H3K4 demethylation, which induces transcriptional silencing.

4.1.2 Methyl binding proteins

The DNA methylation pattern is believed to be interpreted by a conserved family of proteins, the methyl-CpG binding domain (MBD) family (Wade, 2001; Jaenisch and Bird, 2003). Several proteins, which share a common MBD domain, have been identified including MeCP1, MeCP2, MBD1, MBD2, MBD4 and Kaiso (Prokhortchouk and Hendrich, 2002; Hendrich and Tweedie, 2003). With exception of the MBD domain, the sequence of MBD proteins is quite different, suggesting different functions. They also exhibit different ability to bind to methylated DNA. These proteins are ubiquitous: numerous cell types express multiple MBD proteins (Hendrich and Bird, 1998). They are known to complex with different proteins involved in transcriptional silencing (Figure 15). Downregulation of MBD proteins has been shown to allow recovery of transcriptional expression without altering DNA methylation status (Lopez-Serra *et al.*, 2008). MBD proteins allow a crosstalk between DNA methylation and recruitment of a gene silencing machinery, but can not change DNA methylation patterns (Lopez-Serra *et al.*, 2006).

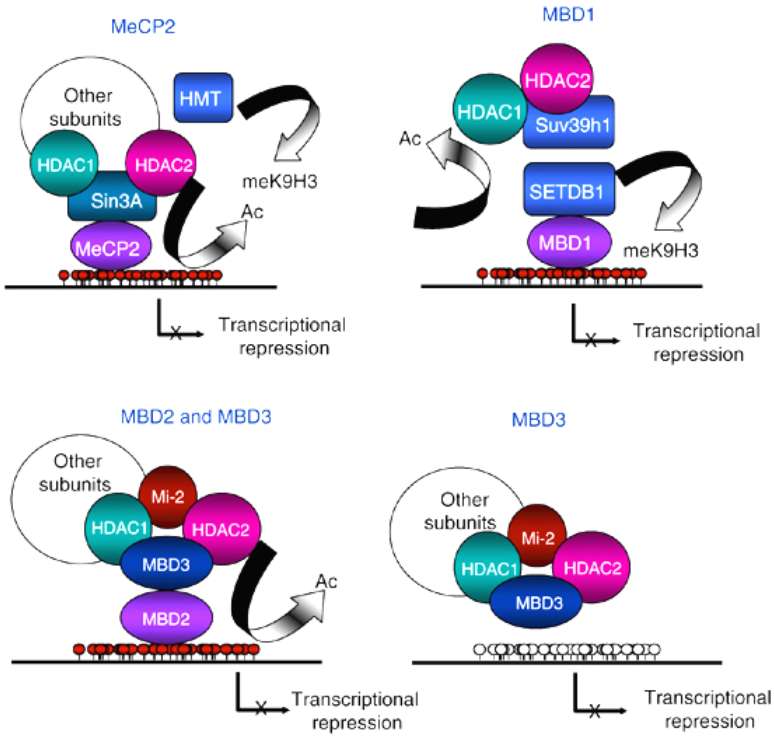


Figure 15. Mechanisms of epigenetic silencing by MBD proteins. Red and white circles represent methylated and unmethylated CpGs, respectively (cited from Lopez-Serra and Esteller, 2008).

MeCP2

The MeCP2 multidomain protein is the first of the member of the family of MBD proteins discovered (Lewis *et al.*, 1992; Nan *et al.*, 1993). MBD proteins bind to methylated DNA through a domain called Methylated Binding Domain (MBD), and repress transcription using the Transcriptional Repression Domain (TRD) (Ballestar *et al.*, 2003; Fraga *et al.*, 2003). MeCP2 binds a single methylated CpG dinucleotide irrespective of the DNA sequence (Lewis *et al.*, 1992; Nan *et al.*, 1993). MeCP2 is able to associate in a complex, comprising of HDACs and the Sin3a transcriptional co-repressor, which leads to transcriptional repression (Nan *et al.*, 1998; Jones *et al.*, 1998) (Bird and Wolffe, 1999). This discovery allowed to link DNA methylation and histone modifications. The TRD domain of MeCP2 is likely to associate with preinitiation complex assembly, via binding with TFIIB (Kaludov and Wolffe, 2000). More recently, MeCP2 has been shown to bind to the histone H3K9 methyltransferase (Fuks *et al.*, 2003), which leaves a repressive mark on chromatin (Lachner and Jenuwein, 2002). MeCP2 null mutant mice are viable and fertile (Chen *et al.*, 2001; Guy *et al.*, 2001), and have a phenotype similar to that of the Rett Syndrome, a neurological disorder of women caused by mutation of MeCP2 (Amir *et al.*, 1999; Chen *et al.*, 2001; Guy *et al.*, 2001).

MBD1

MBD1 was first shown to be a part of the MeCP1 complex (Cross *et al.*, 1997). MBD1 comprises the MBD and the TRD domains, but also several zinc-coordinating CXXC sequences, such as those discovered in DNA methyltransferase I (Cross *et al.*, 1997). MBD1 exhibits a high affinity for heavily methylated sequences (Ng *et al.*, 2000; Fujita *et al.*, 2000). The transcriptional repression mediated by MBD1 is likely to be HDAC dependent (Ng *et al.*, 2000). The third CXXC motif binds to DNA irrespective of the methylation and can repress transcription from unmethylated DNA (Fujita *et al.*, 2000), but MBD1 can also inhibit transcription of a promoter methylated gene through its MBD domain (Jorgensen *et al.*, 2004). MBD1 has been demonstrated to form a complex with the histone H3K9 methylase SETDB1, and CAF-1 (chromatin assembly factor 1) (Sarraf and Stancheva, 2004), allowing the preservation of histone methylation patterns during DNA replication.

MBD2

The MBD2 protein can interact with a single methyl CpG dinucleotide, but no specific sequence has been identified (Bird and Wolffe, 1999). MBD2 has a TRD domain, which

significantly overlaps with the MBD domain, contrary to the domains of MBD1 and MeCP2 (Boeke *et al.*, 2000). The silencing induced by MBD2 is sensitive to HDAC inhibitors (Ng *et al.*, 1999).

MBD2 was initially observed to be connected with a part of the NuRD complex, and this association creates a new complex called MeCP1 (Feng and Zhang, 2001; Fatemi and Wade, 2006). MeCP1 was the first complex revealed to bind to methylated DNA and repress transcription (Meehan *et al.*, 1989). Interestingly, MeCP1 interacts with at least 10 methylated CpGs (Meehan *et al.*, 1989), although MBD2 only requires a single CpG (Hendrich and Bird, 1998). The density of methyl-CpGs probably allows differential targeting of specific genes. Moreover, protein/DNA complexes detected *in vitro* can be different depending on the cell type (Hendrich *et al.*, 2001).

MBD2 can be fully transcribed (MBD2a), but can also be truncated at its N-terminal (MBD2b) because of an alternative start codon. MBD2 can also be expressed as a testis-specific variant (MBD2t), lacking the C-terminal region (Hendrich and Bird, 1998).

Contrary to MeCP2, MBD2 null mutant mice have a minimal phenotype (Hendrich *et al.*, 2001). They exhibit normal methylation patterns without change in genomic imprinting (Hendrich *et al.*, 2001). MBD2 deficiency is correlated with modification in the expression level of certain cytokines crucial to T-lymphocyte differentiation (Hutchins *et al.*, 2002), and with a decreased incidence of colon tumors promoted by mutation of the APC gene (Sansom *et al.*, 2003b).

MBD3

MBD2 and MBD3 share about 70% of sequence similarity (Hendrich and Bird, 1998). Surprisingly, mammalian MBD3, contrarily to Xenopus MBD3 and all other members of this family, does not recognize methylated DNA (Hendrich and Bird, 1998). MBD3 is a part of the transcriptional repressor Mi-2–NuRD complex, which contains several proteins such as HDACs and a chromatin remodelling ATPase (Hendrich and Bird, 1998; Zhang *et al.*, 1999b). MBD3 is crucial to normal mammalian development as MBD3 knockout mice are not viable (Hendrich and Bird, 1998; Hendrich *et al.*, 2001).

MBD4

MBD4 recognizes methylated DNA, although its main role is in DNA repair mechanisms because it functions as a mismatch-specific DNA N-glycosylase, which can efficiently repair the methyl-CpG/TpG mismatches that can occur by spontaneous

deamination (Hendrich and Bird, 1998; Hendrich *et al.*, 1999). This enzyme is likely to remove the whole base creating an abasic site, which can be repair through base and nucleotide excision repair enzymes (Kress *et al.*, 2006; Barreto *et al.*, 2007). MBD4 is also implicated in the transcriptional inhibition of CDKN2A (p16) and MLH1 expression in a methylation-dependent manner (Kondo *et al.*, 2005).

Redundancy role of the MBDs

MBD knockout animals do not exhibit dramatic phenotypes, contrarily to DNA methyltransferase null mutant mice which fail to survive (Jaenisch and Bird, 2003). Only MBD3-null mutants fail to develop (Hendrich *et al.*, 2001). The phenotypes of the MeCP2 (Chen *et al.*, 2001) and MBD2 (Hendrich *et al.*, 2001) null mutant mice suggest that the loss of MeCP2 or MBD2 is partially compensated by other MBD proteins (Brero *et al.*, 2005). About 50% of the sites identified to be linked by MeCP2 were detected associated with MBD2 after depletion of MeCP2. On the other hand, other proteins different from MBD proteins could interpret DNA methylation. It has been shown that a strong connection exists between a single MBD protein and specific methylated regions, suggesting that several genes are regulated by the only one MBD proteins (Magdinier and Wolffe, 2001). A genome-wide study confirmed that several genes appeared to be linked with a single MBD, while it revealed that other genes can bind to different MBD proteins (Ballestar *et al.*, 2003).

MBD and cancers

In human cancer cell lines, the binding of MBD protein to hypermethylated promoters of tumor suppressor genes has been associated with transcriptional silencing (Lopez-Serra and Esteller, 2008). The nature of MBD bound to tumor suppressor genes such as CDKN2A or DAPK1, seems to be tumor type and gene specific (Lopez-Serra *et al.*, 2006).

Some of the polymorphisms in the sequence of MBD proteins have been associated with cancer risk, as polymorphisms in MBD1 increase the risk of lung cancer (Jang *et al.*, 2005). Downregulation of MeCP2 can stop the development of prostate cancer, while its expression promotes the progression of the cancer (Bernard and Eilers, 2006). In breast cancer, MeCP2 is highly expressed and is associated with oestrogen receptor positivity (Muller *et al.*, 2003).

MBD2 has been shown to repress aberrantly methylated tumor suppressor genes such as CDKN2A (p16) in a colon cancer cell line (Sato *et al.*, 2002) or GSTP1 (glutathione S-transferase P1) in breast cancer cell line (Lin and Nelson, 2003). Depletion of MBD2 was also shown to stimulate GSTP1 expression, and to inhibit progression of human lung and colorectal cell lines and human cancer xenografts (Campbell *et al.*, 2004). It has been revealed that Mbd2-deficient mice are resistant to intestinal tumor growth (Sansom *et al.*, 2003a).

4.1.3 DNA methyltransferases (DNMTs)

In normal cells, DNA methylation patterns are dynamic: unmethylated sequences can be converted into methylated CpG and methylation can also be lost during development. Methylation can be *de novo* (when CpGs are unmethylated on both DNA strands) or maintenance (when CpGs are methylated on one DNA strand). After active demethylation of embryonic DNA, *de novo* methylation begins on most CpG sites except on CpG islands (Kafri *et al.*, 1992). After implantation, the majority of the genome is methylated and demethylation of tissue-specific genes occurs in the tissues where they are expressed (Razin and Cedar, 1991).

In mammalian cells, the DNA methyltransferases identified are DNMT1, DNMT1b, DNMT1o, DNMT1p, DNMT2, DNMT3a, DNMT3b, and DNMT3L (Okano *et al.*, 1999; Robertson, 2002). DNMT1 is a *de novo* and maintenance methyltransferase. DNMT3a and DNMT3b are also *de novo* methyltransferases that are regulated by DNMT3L, which stimulates their catalytic activity (Suetake *et al.*, 2004). DNMT3L identifies unmethylated lysine 4 on histone H3 and recruits or activates DNMT3a2 leading to *de novo* DNA methylation (Jia *et al.*, 2007). Replication of homo-methylated DNA produces hemi-methylated DNA in which one strand of the DNA remains methylated and the newly synthesized is unmethylated (Figure 16). Hemimethylated DNA can become fully methylated by maintenance methyltransferase DNMT1. Addition of methyl groups to cytosines by DNMT is also involved in transcriptional regulation, genome stability, imprinting, and X-chromosome inactivation (Riek *et al.*, 2001; Judson *et al.*, 2002).

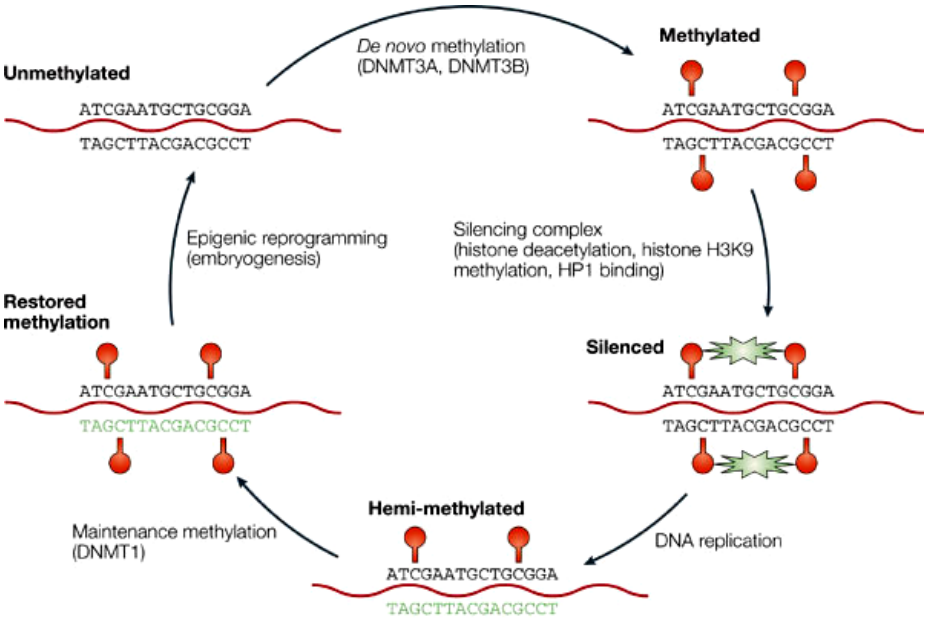


Figure 16. The DNA methylation machinery. In early embryogenesis, DNA is largely devoid of methylation (top left). Post implantation, *de novo* methylation begins, mediated primarily by DNA (cytosine-5-)-methyltransferases DNMT3a and DNMT3b (top). When methylation affects CpG islands, methyl-binding proteins trigger a silencing cascade (bottom right). After DNA replication, newly synthesized DNA (in green) is unmethylated. DNMT1 rapidly scans the old DNA strand. This results in faithful replication of methylation patterns (bottom left) and the maintenance of silencing. Adult patterns of methylation are erased by epigenetic reprogramming in early embryogenesis (cited from Issa, 2004).

The importance of DNMT enzymes has been revealed in null mutant mice which are not viable (Robertson, 2002). Interestingly, Dicer-deficient mice have low DNMT expression and a global DNA methylation failure, which can induce abnormal telomere recombination and elongation (Benetti *et al.*, 2008).

The mechanism of demethylation is not entirely clarified yet. DNA demethylation may occur through a demethylase activity of a protein such as the MBD2b 5-methylcytosine glycosylase, although its role is controversial (Bhattacharya *et al.*, 1999; Patra *et al.*, 2008). Otherwise, DNA can passively be demethylated by several rounds of replication in the absence of maintenance methyltransferase activity (Szyf, 2003). Indeed, purified MBD2 alone is unable to demethylate DNA: only cellular extracts containing MBD2 show demethylase activity (Patra *et al.*, 2001; Patra *et al.*, 2002; Patra and Bettuzzi, 2007).

Mutations in methylation related genes are linked to human disease. Mutations in the methyltransferase gene DNMT3b are found in patients with the ImmunoDeficiency Centromere (ICF) syndrome (Hansen *et al.*, 1999). ICF syndrome is a rare autosomal

recessive disorder, characterized by the presence of variable immunodeficiency, chromosomal instability (Chr 1, 9 and 16), and mild facial anomalies.

The mRNA and the protein level of the three DNMTs were found moderately over-expressed in several types of tumor cells (De Marzo *et al.*, 1999; Robertson *et al.*, 1999). In mice, DNMT1 was found essential for the development of intestinal polyposis (Eads *et al.*, 2002).

In cancer cells, DNMT1 seems to be responsible for most of the DNA methylation, in particular the maintenance of abnormal promoter methylation. Recently, lack of DNMT1 activity in human cancer cells was found to induce a mitotic disaster (Chen *et al.*, 2007). However, the three active DNMTs are thought to collaborate in order to maintain the methylated state and inhibit gene transcription (Liang *et al.*, 2002). DNMTs can also bind to different proteins that repress gene expression and thus can coordinate repression (Rountree *et al.*, 2000; Robertson *et al.*, 2000; Bachman *et al.*, 2001; Burgers *et al.*, 2002). For instance, DNMTs can directly associate with HDAC inducing their binding to gene promoters (Ling *et al.*, 2004; Espada *et al.*, 2004). Interestingly, DNMTs and both protein complexes PRC1 and PRC2 (Polycomb repressive complex) might coordinately stabilize silencing at polycomb target genes (Li *et al.*, 2007).

4.2 Mechanisms of transcriptional repression by DNA methylation

Different possible mechanisms have been proposed to explain gene silencing by DNA methylation. DNA methylation might hinder the binding of transcription factors such as AP-2, c-Myc, E2F, and NFkB, which bind to sequences containing CpG dinucleotides (Tate and Bird, 1993). Alternatively, transcriptional repressors might bind to methylated DNA and induce gene silencing. Methyl groups on the major groove of DNA do indeed create new functional moieties allowing novel DNA interactions. In this way, DNA methylation can affect histone modification and chromatin structure by bringing about a general deacetylation of histones H3 and H4 (Irvine *et al.*, 2002; Hashimshony *et al.*, 2003). It can also prevent methylation at H3K4 and induce methylation of H3K9 (Okitsu and Hsieh, 2007), leading to compaction of the chromatin and subsequent inhibition of transcription. In gene silencing, methylation is thus likely to be dominant over chromatin mechanisms. Indeed, inhibition of HDAC by Trichostatin A can induce re-expression of abberantly silenced hypermethylated

genes only if demethylating drugs, such as 5-azacytidine, first demethylate their promoters (Cameron *et al.*, 1999).

4.3 DNA methylation alterations and cancer

While gene mutations are very frequent in cancer, it is well established that epigenetic alterations play an important role in loss of gene expression (Jones and Laird, 1999; Baylin and Herman, 2000). Cancers generally show loss of methylation in most regions where CpG dinucleotides are usually methylated, and gain of methylation in CpG islands in promoter regions (Feinberg and Vogelstein, 1987; Jones, 2002; Feinberg, 2004; Feinberg *et al.*, 2006). These losses and gains result in a decrease in overall methylation levels. Moreover, genome hypomethylation and CpG island hypermethylation precede malignancy, indicating that they are actively involved in the generation of the malignant state.

4.3.1 DNA hypomethylation

Hypomethylation is observed in solid tumors such as metastatic hepatocellular carcinoma (Lin *et al.*, 2001), cervical cancer (Kim *et al.*, 1994b), prostate cancer (Bedford and van Helden, 1987), breast cancer (Jackson *et al.*, 2004) and also in hematological malignancies such as B-cell chronic lymphocytic leukemia (Ehrlich, 2002). Hypomethylation contributes to carcinogenesis through activation of normally silenced genes, such as imprinted genes or genes on the X chromosome, latent retrotransposons, but also through chromosome instability (Wilson *et al.*, 2007). Moreover, hypomethylation gradually increases with tumor grade (Gama-Sosa *et al.*, 1983; Narayan *et al.*, 1998). A correct level of DNA methylation in the pericentromeric regions of the chromosome is essential for stability and accurate replication of DNA. On chromosomes 1 and 16, these regions are anormally hypomethylated and unstable in several tumors such as breast and ovarian cancers, and sporadic Wilms tumors (Narayan *et al.*, 1998; Qu *et al.*, 1999). Moreover, hypomethylation due to DNMT1 depletion was found to promote early lesions in the colon, with DNMT3b involved in initial adenoma formation (Yamada *et al.*, 2005; Lin *et al.*, 2006). Hypomethylation also induces IGF2 expression through loss of imprinting in cancers, such as colon cancer (Cui *et al.*, 2002; Liou *et al.*, 2007). An abnormal expression of synuclein-γ gene, usually restricted to neurons, is also induced by hypomethylation in breast and ovarian cancer (Gupta *et al.*, 2003).

4.3.2 DNA hypermethylation

While global hypomethylation is detectable in the cancer genome, abnormal hypermethylation of CpG islands located in cancer-related genes such as tumor suppressor genes is observed, leading to a loss of gene function (Figure 17). Inactivation of both alleles of the gene is required to induce phenotypic consequences in a tumor (Knudson *et al.*, 2001).

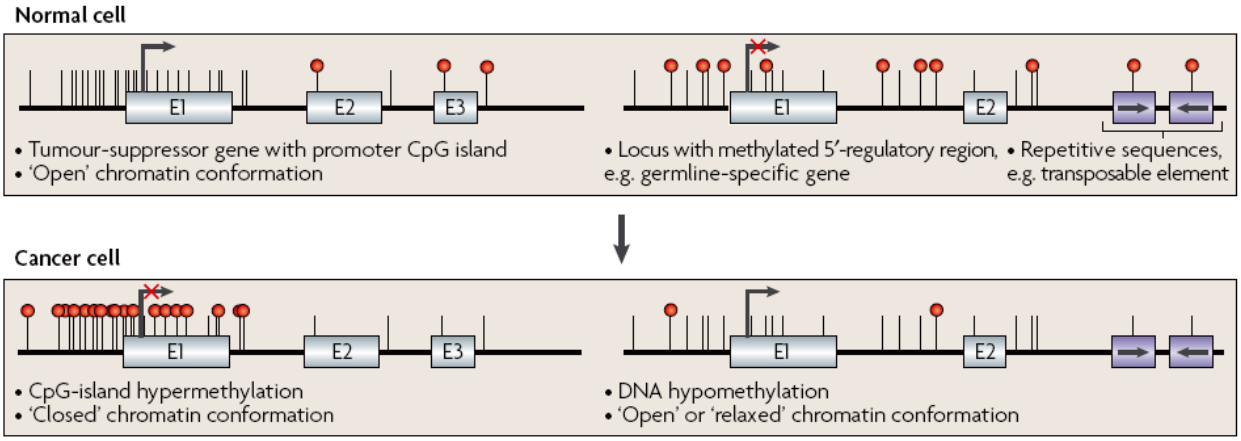


Figure 17. Altered DNA-methylation patterns in tumorigenesis (cited from Esteller, 2007)**.**

Epigenetic gene silencing predisposes to mutational events during tumor progression. This was first shown for the mismatch-repair gene MLH1, which is frequently hypermethylated in sporadic cases of colorectal, endometrial and gastric cancer withv microsatellite instability (Kane *et al.*, 1997; Herman *et al.*, 1998; Esteller *et al.*, 1999a; Fleisher *et al.*, 1999), where transcriptional inactivation of MLH1 by promoter hypermethylation is the main cause of microsatellite instability. Likewise, promoter hypermethylation of the MGMT methyltransferase gene, with as a consequence gene silencing, interferes with the removal of promutagenic O^6-methylguanine, which is then read as an adenine by the DNA polymerases, generating G to A mutations (Esteller *et al.*, 1999b; Nakamura *et al.*, 2001b; Park *et al.*, 2001; Wolf *et al.*, 2001). This illustrates how epigenetic events can induce genetic lesions that are crucial in the development of cancer. The glutathione S-transferase P1 GSTP1 and the familial breast cancer gene BRCA1 can also be silenced by promoter hypermethylation in human cancers, leading to potential DNA lesions (Esteller *et al.*, 1998; Esteller *et al.*, 2000a; Esteller *et al.*, 2001). Many genes have been

found to undergo hypermethylation in cancer. These genes can be related to regulation of the cell cycle (CDKN2A, CDKN2B, RB), DNA repair (BRCA1, MGMT), apoptosis (DAPK), angiogenesis, metastasis, differentiation, drug resistance, signal transduction, or transcription factor, and detoxification (Das and Singal, 2004; Teodoridis *et al.*, 2004; Esteller, 2005b). Although some genes are methylated in many different cancers, other genes are methylated in specific type of cancers. The mechanisms leading to specific DNA methylation in specific cancer have yet to be elucidated.

To evaluate the importance of CpG island hypermethylation in cancers, demethylating drugs such as 5-azacytidine can be used to reactivate the silenced genes and restore protein expression in cultured cancer cells (Esteller *et al.*, 2000b; Merlo *et al.*, 1995; Herman *et al.*, 1995; Herman *et al.*, 1998).

In addition, it has been suggested that tumors may acquire a CpG island methylator phenotype (CIMP). The CIMP phenotype involves the concerted hypermethylation of numerous CpG islands. This was first reported in colorectal cancer (Toyota *et al.*, 1999a), but since described in several other types of cancer, including glioblastoma, gastric, liver, pancreatic, esophageal and ovarian cancer, as well as acute lymphocytic and myelogenous leukemia (Kim *et al.*, 2003; Issa, 2004).

4.3.3 Mechanisms of aberrant CpG island methylation

Two models have been proposed to explain how CpG islands become methylated in cancer (Baylin *et al.*, 1998; Jones, 1999; Tycko, 2000). First, protective factors, which prevent CpG island methylation, might be lost. These factors, such as structural proteins (Zardo and Caiafa, 1998) or transcription factors (Brandeis *et al.*, 1994) could compete for the binding sites of methyltransferase within the CpG island. The second mechanism suggests that epigenetic lesions of cancer cells may originate from a deficiency in one of the components of the epigenetic machinery.

Some defects in epigenetic control will target identified putative DNA hypermethylation target consensus sequences (Feltus *et al.*, 2003). Furthermore, it has been suggested that microRNAs might be involved in epigenetic silencing in cancer cells (Kawasaki and Taira, 2004; Morris *et al.*, 2004; Mattick and Makunin, 2005).

4.4 Clinical implications of gene silencing in cancer

Contrary to gene silencing through mutation, the epigenetic changes that induce transcriptional inhibition are potentially reversible. The molecular modifications associated with gene silencing in cancers could be used as markers for risk evaluation, diagnosis, but also prognosis. Development of new strategies to reverse gene silencing must be investigated for preventing and treating neoplastic diseases.

4.4.1 Epigenetic therapy

Demethylating drugs, such as 5-azacytidine, decitabine (5-aza-2'-deoxycytidine), 1-β-D-arabinofurasonyl-5-azacytosine or dihydro-5-azacytidine, can reactivate transcription of genes due to promoter methylation (Arnold *et al.*, 2003; Costa *et al.*, 2004; Teodoridis *et al.*, 2004; Esteller, 2005a). 5-aza-2′-deoxycytidine is integrated into the DNA after phosphorylation, whereas 5-azacytidine is preferentially integrated into RNA (Santini *et al.*, 2001). These DNA demethylating agents have shown significant antitumoral activity, when they are given to patients at low doses. Both 5-azacytidine and 5-aza-2′-deoxycytidine are elective treatments for the myelodisplastic syndrome, a pre-leukemic disease (Mack, 2006). The main drawback of DNA demethylating agents is their lack of specificity, as they cause global hypomethylation, and it is therefore impossible to reactivate a selected specific gene (Villar-Garea and Esteller, 2003)..

Promising agents for the epigenetic therapy of cancer also comprise HDAC inhibitors, which can reactivate the transcriptional expression of tumor-suppressor genes, such as CDKN1A (p21). However, these inhibitors provoke pleiotropic effects such as differentiation, cell-cycle arrest and apoptosis, but might also induce undesirable consequences (Teodoridis *et al.*, 2004). Nevertheless, clinical trials of HDAC inhibitors were shown to be well tolerated in humans. For instance, the deacetylase inhibitor vorinostat (suberoylanilide hydroxamic acid, SAHA) has been accepted for the treatment of cutaneous T-cell lymphoma (Thompson, 2006).

Furthermore, it has been observed that associations of demethylating agents and HDAC inhibitors have synergetic effects that successfully reverse epigenetic gene silencing (Keen *et al.*, 2003; Primeau *et al.*, 2003). Combination trials are currently in progress in the clinic.

Epigenetic alterations in cancer cells also present significant perspectives for cancer diagnosis or for response to therapy.

4.4.2 Epigenetic changes as biomarkers of cancer

Advantages of DNA methylation as a biomarker

The chemical stability of DNA makes it a highly attractive biomarker. Indeed, DNA is not as prone to degradation as RNA or protein and can be isolated from frozen or even from formalin-fixed paraffin-embedded tissues (Bian *et al.*, 2002). DNA methylation patterns are quite stable and do not vary in response to short-term events, contrary to gene-expression profiles. Furthermore, the methylation signal is suitable for detection of low-concentration markers. Aberrant promoter hypermethylation in whatever type of cancer is located within the same region of a relevant gene, in contrast to genetic markers. The detection of hypermethylation is a positive signal that can be perceived in the context of a constellation of normal cells.

Cancer detection

The success of cancer treatments frequently depend on an early diagnosis. As some promoter CpG islands are hypermethylated in cancer but not in normal cells, unique CpG island methylation profiles can define each neoplasia (Esteller *et al.*, 2001; Paz *et al.*, 2003). For instance, hypermethylation of the GSTP1 gene is sufficient to be informative in about 90% of the prostate cancers (Cairns *et al.*, 2001), but generally, a larger panel of markers is needed. Methylation-sensitive oligonucleotide microarray can be used to differentiate mantle cell lymphoma from grades I/II follicular cell lymphoma (Shi *et al.*, 2003). Abnormal CpG-island hypermethylation is a promising marker to detect cancer cells in biological fluids and biopsy samples, where tumor-derived DNA can also be released (Laird, 2003). The major sources of cell-free DNA in studies are serum or plasma, but samples obtained by exfoliative cytology, endoscopic brush techniques, punction, as well as urine, saliva, bronchoalveolar lavage and sputum samples, can also be used (Laird, 2005).

Another important finding has been that CpG island hypermethylation of tumor-suppressor genes occurs early in tumorigenesis. For example, CpG-island hypermethylation of CDKN2A and MGMT is already present in colorectal adenomas. Likewise MLH1 methylation is detected in atypical endometrial hyperplasia (Esteller, 2005a). Moreover, an

increased number of methylated genes is observed with tumor progression (Gallagher *et al.*, 2005).

Disease prognosis

As DNA methylation can be correlated with cancer progression, it is worthy to determine its prognostic relevance. A correlation between hypermethylation of CpG islands and clinical outcome for numerous types of cancer, such as gastric, colon, lung, prostate, and ovarian cancer has been described (Maruyama *et al.*, 2002; Maeda *et al.*, 2003; Graziano *et al.*, 2004; Toyooka *et al.*, 2004; Wei *et al.*, 2006). For instance, death-associated protein kinase (DAPK), CDKN2A and epithelial membrane protein 3 (EMP-3) hypermethylation have been related to tumor aggressivity in lung, colorectal, and brain cancer patients (Esteller, 2005b). Furthermore, methylation can be a stronger predictor of survival and tumor recurrence than age or tumor stage (Brock *et al.*, 2003; Clement *et al.*, 2006).

The methylation profile could also have a predictive role in the response to a chemotherapeutic agent. The occurrence of MGMT hypermethylation was shown to be linked with improved survival in glioma patients treated with alkylating agents (Esteller *et al.*, 2000a); (Hegi *et al.*, 2005), and the same may be true for lymphoma (Esteller *et al.*, 2002). MGMT is known to reverse the addition of alkyl groups to the guanine base, which is the target of alkylating chemotherapeutic drugs, such as BCNU (carmustine), ACNU (nimustine), procarbazine, streptozotocin, and temozolamide. Hypermethylation of other DNA-repair genes has been identified by CpG-island microarrays to predict drug-responsiveness (Glasspool *et al.*, 2006).

4.5 Methods for the evaluation of DNA methylation

4.5.1 Analysis of genome-wide methylation content

High-performance liquid chromatography (HPLC) and high-performance capillary electrophoresis (HPCE) provide powerful techniques to study global DNA methylation by quantification of 5-methylcytosine (Fraga *et al.*, 2002) (Figure 19). Other techniques using the *Sss*I DNA methyltransferase or the anti 5-MC antibodies can also be used to analyze the genome-wide methylation content.

4.5.2 Techniques for gene-specific methylation analysis

The analysis of DNA methylation of precise sequences was initially based on the use of methylation-sensitive restriction enzymes that can differentiate methylated recognition sites from unmethylated sites in regions of interest. The main drawbacks of this approach are an incomplete restriction-enzyme digestion and a restriction in the regions which can be analysed. An important progress in cancer epigenetics has been the treatment of DNA with sodium bisulfite, which allows the transformation of unmethylated cytosines to uracils but leaves methylated cytosines unaffected (Figure 18). However, a complete conversion is essential to avoid misinterpretation of the methylation content.

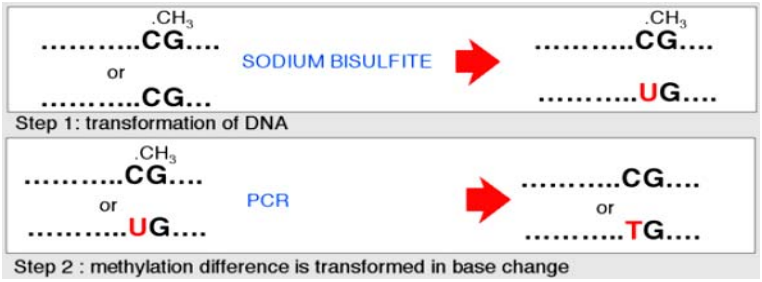


Figure 18. Principle of sodium bisulfite conversion. Standard molecular biology techniques erase DNA methylation information. The solution is to modify DNA in a methylation-dependent way before amplification. By treating the genomic DNA with sodium bisulfite, unmethylated cytosines convert to uracil residues. The converted DNA is no longer self-complementary, and amplification of either the top or the bottom DNA strand requires different primers (cited from Patra *et al.*, 2008).

Bisulfite genome sequencing (Frommer *et al.*, 1992) is considered as the gold standard for gene-specific methylation analysis because it provides the methylation status of every cytosine residue within the target sequence. However, the experimental approach is laborious and time-consuming and not quantitative. More recently, the pyrosequencing method allows to analyse methylation in real time (Uhlmann *et al.*, 2003).

Methylation-specific PCR (MSP) is a widely used technique (Herman *et al.*, 1996) that employs two pairs of primers in separate reactions to specifically amplify methylated and unmethylated molecules, after modification of the DNA by sodium bisulfite. The sensitivity of MSP is very high, methylated molecules can be detected down to a level of 0.1% in the total population. MSP is rapid and easy to execute. Nevertheless, the information obtained by MSP is only qualitative and this method may not be appropriate for all types of tissue. The major drawbacks of MSP are its susceptibility to false positives, and the limited number of

CpG examined as the analysis is restricted to CpG residues complementary to the primers used (Rand *et al.*, 2002).

MSP has been made quantitative by the addition of fluorescent TaqMan probes, which allows to detect MSP products in real time, such as in the MethyLight approach (Eads *et al.*, 2000). MethyLight is sensitive and does not suffer from signal from non-specific amplification. The quantitative analysis of methylated alleles (QAMA) is an additional sensitive technique derived from MSP (Zeschnigk *et al.*, 2004). In QAMA analysis, the same primer set is used to amplify both methylated and unmethylated alleles after bisulfite reaction. The methylation status is evaluated by two different fluorescent TaqMan probes specific for methylated and unmethylated DNA. Likewise, ConLight-MSP uses a fluorescent probe to detect unconverted DNA and therefore prevents overestimation of DNA methylation (Rand *et al.*, 2002). Alternatively, HeavyMethyl analysis avoids binding of primers to unmethylated DNA by a blocker oligo (Cottrell *et al.*, 2004).

High resolution melting analysis (HRM) has been shown to be a sensitive and specific technique for the analysis of methylation (Wojdacz and Dobrovic, 2007). One primer set and a fluorescent intercalating dye are used to amplify both methylated and unmethylated templates after bisulfite modification. The principle of the methylation-sensitive HRM analysis (MS-HRM) is the accurate monitoring of the fluorescence of the DNA duplex as a function of the temperature. A comparaison between the melting profiles of unknown samples and the profiles of standards allows to evaluate the amount of methylation. Another method named SMART-MSP, for Sensitive Melting Analysis after Real Time-MSP, relies upon a combination of a real-time MSP analysis and an HRM analysis (Kristensen *et al.*, 2008).

Many other techniques require specific steps after the non-discriminative PCR amplification of the modified DNA. Methylation-sensitive single-strand conformation analysis (MS-SSCA) resolves the differences between methylated and unmethylated alleles by denaturation of the PCR product followed by electrophoresis in a non-denaturing polyacrylamide gel (Bianco *et al.*, 1999). This technique is easy to handle, semi-quantitative and can be applied even on DNA from microdissected formalin-fixed paraffin-embedded tissues (Bian *et al.*, 2001).

The methylation sensitive dot blot assay (MS-DBA) utilizes dot blot analysis with two probes specific for methylated and unmethylated DNA. MS-DBA is a sensitive, specific, and quantitative method applicable to frozen or fixed tissues (Clement and Benhattar, 2005).

After non-discriminatory amplification of sequences, methylation-specific denaturing gradient gel electrophoresis (MS-DGGE) differentially detects methylated DNA molecules on the basis of differences in thermal stability caused by differences in base sequence after bisulfite treatment (Aggerholm *et al.*, 1999). However, a cautious design of the primers is essential to guarantee optimal melting behavior of the amplified product.

Finally, combined bisulfite restriction analysis (COBRA) utilizes divergences in the sequences of recognition sites of restriction enzymes after bisulfite treatment of methylated and unmethylated DNA (Xiong and Laird, 1997). COBRA relies on a full digestion to correctly detect methylation, and is limited to restriction sites containing CpG within the sequence of interest.

4.5.3 Global CpG island methylation analysis

Restriction landmark genomic scanning (RLGS) allows the examination of thousands of unselected CpG islands in the genome within a single gel (Costello *et al.*, 2000). Genomic DNA digested with methylation-sensitive restriction enzymes is radioactively labeled and then run in a two-dimensional gel, resulting in a complex pattern of spots. When an enzymatic site is methylated, it is not cleaved and a spot will be missing (Figure 19). The limitation of RLGS comes from the occurrence of digestion sites within CpG islands, which is not systematic.

Other important methods for detecting abnormal DNA methylation include methylation-sensitive arbitrary primed PCR (Gonzalgo and Jones, 1997), methylated CpG-island amplification (MCA) (Toyota *et al.*, 1999b) and amplification of intermethylated sites (AIMS) (Frigola *et al.*, 2002). No sequence information is required before amplification and DNA templates are first enriched for methylated sequences. Therefore, the amplification of CpG islands and gene-rich regions are favored (Frigola *et al.*, 2002). However, validation of the results by bisulfite genomic sequencing is necessary and these should be carefully interpretated because of the PCR background coming from repetitive sequences.

New technologies employing CpG-island and promoter microarrays allow to efficiently analyze CpG-island methylation at a genome-wide scale. A recent method, related to the differential methylation hybridization (DMH) (Huang *et al.*, 1999), is the HELP assay (HpaII tiny fragment enrichment by ligation-mediated PCR). This method requires the cutting of the DNA samples with a methylation-sensitive restriction enzyme (MspI) or its methylation-insensitive isoschizomer (HpaII) and subsequent hybridization to a genomic DNA microarray (Khulan *et al.*, 2006). This assay is a simple method that has revealed numerous tissue-specific and differentially methylated sequences (Khulan *et al.*, 2006).

Interestingly, McrBC digestion is a new tool to enrich unmethylated DNA by using McrBC enzyme that predominantly cuts methylated DNA. This method is used to identify densely methylated regions at genomic level but has moderate resolution (Lippman *et al.*, 2005; Irizarry *et al.*, 2008).

Techniques using the ChIP-on-chip approach as MBD affinity purification relies on immunoprecipitated DNA using antibodies against MBD proteins, which have a high affinity for methylated cytosines (Figure 19) (Lopez-Serra *et al.*, 2006).

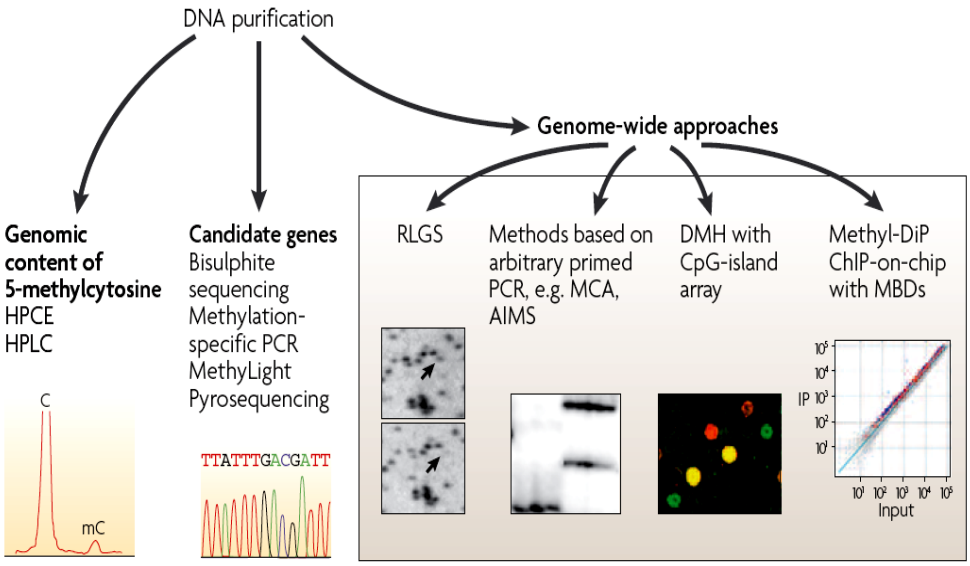


Figure 19. Techniques for studying epigenetic changes in cancer. The overall DNA 5-methylcytosine content can be determined using high-performance capillary electrophoresis (HPCE) or high-performance liquid chromatography (HPLC), or the DNA methylation of specific candidate genes can be detected with methylation-sensitive methods. Recently, several genome-wide approaches to detecting DNA methylation have been developed as RLGS, restriction landmark genomic scanning; MCA, methylated CpG-island amplification; AIMS, amplification of intermethylated sites; DMH, differential methylation hybridization. For global profiling, chromatin immunoprecipitation (ChIP) is combined with DNA arrays (ChIP-on-chip) to detect patterns across the genome (cited from Sawan *et al.*, 2008).

Recently, a direct method to immunoprecipitate unmethylated DNA has been developed using a CXXC-domain column (CXXC affinity purification CAP) (Illingworth *et al.*, 2008). On the other hand, in the MedIP assay (methylated DNA immunoprecipitation), DNA immunoprecipitated with anti 5-MC antibody can be used as a probe for hybridization to genomic microarrays, allowing a rapid analysis of numerous CpG sites (Weber *et al.*, 2005; Keshet *et al.*, 2006). One needs to be aware of PCR biases because of the whole-genome amplification step after immunoprecipitation.

An alternative approach is the analysis of CpG methylation in specific subsets of genomic regions through the use of the Illumina technology (Golden Gate), originally elaborated for SNP detection (Fan *et al.*, 2006). The ratio of the methylated and unmethylated PCR products is determined at single CpG sites.

5. Lymphoid system

Adaptive immunity allows the development of specific antigens throughout the humoral response mediated by B-lymphocytes and cell-mediated immunity. Additionally, differents cells are implicated in the cellular immunity such as cytotoxic T-lymphocytes, activated macrophages, activated natural killer (NK) cells.

5.1 Lineage commitment

5.1.1 Differentiation of hematopoietic stem cells

Lymphocytes originate from hematopoietic stem cells (HSCs), which have the properties of self-renewal and multilineage differentiation. HSCs are characterized by high amounts of the c-kit receptor (CD117), and the lack of cell-surface proteins expressed on differentiated cells. HSC give rise to either the lymphoid or erythro-myeloid lineages, leading to the emergence of common lymphoid progenitors (CLPs) or common myeloid (CMP) progenitors in the bone marrow (Kondo *et al.*, 1997; Akashi *et al.*, 2000; Traver *et al.*, 2000). CLPs express Flt3, that is important for multilineage potency (Sitnicka *et al.*, 2002), and can only differentiate to B, T, NK, and dendritic cells (DC) because they lack self-renewal capacity (Traver *et al.*, 2000; Akashi *et al.*, 2000). Intrinsic as well as extrinsic factors are involved in the control of early hematopoiesis. Environmental factors such as secreted growth

factors (cytokines), hormones, and the other cell-cell interaction (Martinez-Agosto *et al.*, 2007) and sequence-specific DNA binding proteins like transcription factors are able to both promote and suppress lineage-specific genes, blocking of the cell destiny to one lineage (Nutt and Kee, 2007; Rothenberg, 2007; Iwasaki and Akashi, 2007).

5.1.2 Differentiation of T-lymphocytes

T cell differentation can lead to formation of CD4 (helper) and CD8 (cytotoxic) T cells in the thymus. The ability of T cells to recognize foreign antigens is mediated by the T cell receptor (TCR), which requires rearrangement of its beta chain. A step called beta-selection eliminates T cells with a defective T cell receptor because of defective gene rearrangement.The TCR requires both CD8 and CD4 co-receptors to guarantee the specificity of the TCR for an antigen. The earliest T cells express neither CD4 nor CD8, but at this stage thymocytes upregulate both CD4 and CD8, becoming double positive cells. Then a negative selection occurs to eliminate autoreactive thymocytes and finally mature to single-positive (CD4+CD8- or CD4-CD8+).

CD8 T cells kill cells infected with pathogens, while B cells are able to generate specific antibodies to facilitate the elimination of these pathogens. CD4 T cells promote the roles of CD8 T cells and B cells. Upon contact with an antigen, naive T cells are activated and develop into effector cells (Spits, 2002). After elimination of the antigen, few cells differentiate into long-lived memory T cells to induce a faster immune response, upon renewed contact with the same antigen.

5.1.3 Differentiation of B-lymphocytes

When B lymphocytes differentiate they acquire a functional membrane-bound Ig through sequential rearrangement of the immunoglobulin (Ig) genes and expression of B-cell-specific proteins. The primary differentiation step engages DNA rearrangements joining the diversity (D) region segments and joining (J) region segments of the Ig heavy chain genes. This is followed by DNA recombination of the variable (V) region with the DJ segment, leading to the formation of the pre-B-cell receptor (pre-BCR), which is an important checkpoint of the transition from the pro-B to the pre-B cells (Figure 20).

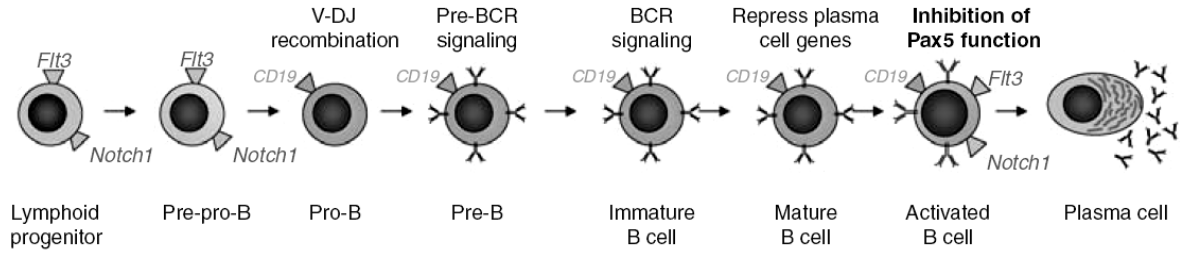


Figure 20. Simplified model of the stage of B-cell development (cited from Holmes *et al.*, 2008).

Successful light-chain gene rearrangement leads to competent BCR complexes that induce positive selection of immature IgM^+ B cells. These immature B cells migrate to the peripheral lymphoid organs (Meffre *et al.*, 2000), where they will become mature B cells that circulate through blood and lymphoid organs (Peschon *et al.*, 1994). After antigen contact, mature B-cell differentiation can occur through stimulation with T-cell independent antigens, inducing a rapid development of the plasma cell (Weber *et al.*, 2005). In contrast, the response to T-cell dependent antigens results in the formation of germinal centre (GC), where B cells are co-stimulated by T-cells. In the GC, the activated B-cells first give rise to B-lymphoblasts (Heyzer-Williams and Heyzer-Williams, 2005). Moreover, in the GC, somatic hypermutation of the variable domains of Ig genes (Pascual *et al.*, 1994; Neuberger and Milstein, 1995; Kelsoe, 1996) allows a selection of B cells with the best affinity for further expansion and differentiation to become plasma cells and memory B cells. Secondary DNA rearrangement of B-cells can take place via a class switch mechanism and thus induce production of large amounts of IgG, IgA or IgE antibodies, creating a rapid secondary response.

5.2 The PAX5 factor in the B cell development

5.2.1 PAX5 and control of B-lineage

The PAX5 transcription factor, also known as B-cell-specific activator protein (BSAP), is the esssential B-lineage commitment factor that locks the fate of early progenitors to the B cell pathway. PAX5 allow to start and maintain the B-cell transcription program (Mikkola *et al.*, 2002). The other B-cell transcription factors, E2A and EBF, can induce the activation of B-cell-specific genes and V(D)J recombination, but in the absence of PAX5

cannot constrain B cell progenitors to develop into B cells. Moreover, the PAX5$^{-/-}$ pro-B cells, as well as HSC, were shown to exhibit self-renewal capacity and multilineage potential (Schaniel *et al.*, 2002a; Schaniel *et al.*, 2002b). They can give rise *in vitro* to functional NK cells, DC, macrophages, osteoclasts, and granulocytes (Figure 21) (Nutt *et al.*, 1999). However, exogenous expression of PAX5 can rescue the development to the mature B cell stage, while its inactivation induces the loss of B-cell identity and function (Horcher *et al.*, 2001). Moreover, the PAX5 inactivation in pro-B cells can induce *in vivo* T cell development (Mikkola *et al.*, 2002).

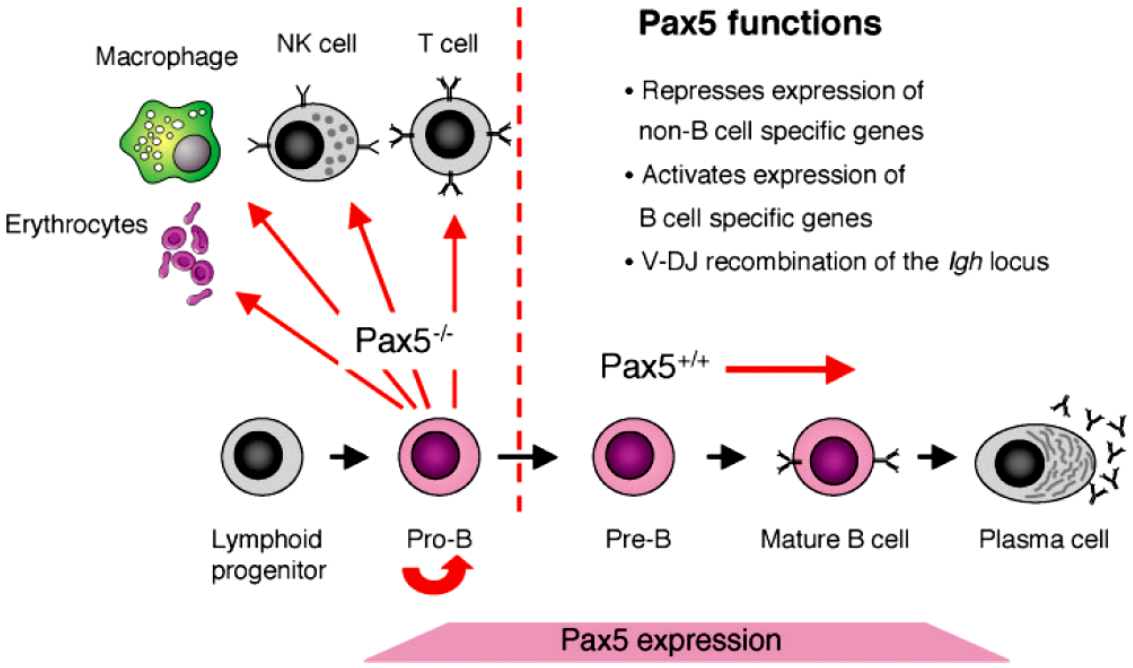


Figure 21. PAX5 is essential for commitment to the B cell lineage (cited from Carotta and Nutt, 2008)**.**

PAX5 exhibits a dual function that allows to control the B-lineage commitment. PAX5 promotes B-cell-specific genes by facilitating signal transduction from the pre-BCR and BCR and at the same time suppresses other lineage-specific genes (Nutt *et al.*, 1999) (see PAX5 target genes part 5.2.4).

In the terminal differentiation of B cells, the plasma cell regulators, such as Blimp1 (B lymphocyte induced maturation protein) and Xbp1 (X-box binding protein1) are expressed, allowing the control of the plasma cell transcriptional program. PAX5 keeps Blimp1 under control in mature B cells (Delogu *et al.*, 2006), while Blimp binds to and represses the PAX5 gene in plasma cells (Shaffer *et al.*, 2002).

5.2.2 PAX5 regulation

The Paired box (PAX) family comprises of 9 members that have in common a conserved 128 amino acid DNA-binding domain called the paired domain, which contains two conserved helix-turn-helix motifs connected by a linker (Czerny *et al.*, 1993; Xu *et al.*, 1995; Xu *et al.*, 1999). PAX proteins, which associate with degenerate DNA consensus sequences, can play the role of transcriptional activators and repressors (Kozmik *et al.*, 1992; Busslinger *et al.*, 1996; Dorfler and Busslinger, 1996; Nutt *et al.*, 1998). This suggests that other interacting proteins are likely to be necessary to target the PAX proteins to specific binding sites. PAX proteins are known as essential regulators in tissue- and differentiation stage-specific transcription (Underhill, 2000).

The PAX5 gene contains two distinct promoters. The TATA-containing upstream promoter allows to transcribe the exon 1A, while the TATA-less downstream promoter is associated with exon 1B. Both splice variants are identical from exon 2 to 10. In the mouse, the TATA-promoter of PAX5 is predominantly inactivated by DNA methylation. PAX5A mRNA is expressed in pro-B, pre-B, or mature B cells, but not in terminally differentiated plasma cells nor in adult testis. Weak activity of the TATA-promoter was also found in the developing central nervous system. The TATA-less promoter on the other hand, is mainly inactivated by histone deacetylation in the terminally differentiated B cell lines. PAX5B transcripts are present in embryos, adult testis, spleen, and B cell lines, suggesting that the TATA-less promoter is active in all PAX5 postive cells.

5.2.3 PAX5 expression

In normal and neoplastic B cells

The PAX5 gene is normally detected during B cell development, from pro-B cells up to the mature B cell stage, but not in plasma cells (Figure 21) (Barberis *et al.*, 1990; Nutt *et al.*, 1997). PAX5 expression can be used for the diagnosis purposes as a pan-B cell marker (Torlakovic *et al.*, 2002).

PAX5 can also be a lineage-specific marker in B-cells neoplasms (Zhang *et al.*, 1996; Torlakovic *et al.*, 2002). Recently, a large study revealed that PAX5 is expressed in 91.5% B-cell non-Hodgkin lymphomas (B-NHL) and in 85.7% Hodgkin lymphomas (HL), but nor in

of T cell lymphomas (Mhawech-Fauceglia *et al.*, 2007). This result was confirmed an earlier large study (Torlakovic *et al.*, 2002). It is also expressed in a subset of myeloid and acute myeloid leukemias (Tiacci *et al.*, 2004). PAX5 can also be helpful as an immunohistologic marker to discriminate undifferentiated neoplasms (Jensen *et al.*, 2007).

In other normal and neoplastic tissues

PAX5 was furthermore shown to be expressed in adult brain tissue, in the embryonic midbrain and in adult testis of the mouse (Adams *et al.*, 1992). In contrast, another study showed no expression of PAX5 in the adult testis, but a focal expression of PAX5 in normal adult brain tissue (Torlakovic *et al.*, 2006). Moreover, PAX5 was highly expressed in neuroendocrine carcinomas such as Merkel cell carcinoma (MCC) and small cell carcinoma (SCC) (Dong *et al.*, 2005; Torlakovic *et al.*, 2006; Mhawech-Fauceglia *et al.*, 2007), but not in medulloblastoma, neuroblastoma and astrocytomas, (Jensen *et al.*, 2007; Mhawech-Fauceglia *et al.*, 2007), contrary to previous results (Kozmik *et al.*, 1995; Stuart *et al.*, 1995; Baumann Kubetzko *et al.*, 2004). PAX5 is also expressed in some T-cell acute lymphocytic leukemias, but not in T cell lymphomas (Zhang *et al.*, 1996). In addition, PAX5 has been detected in a small number of breast carcinomas, endometrial adenocarcinomas, urothelial carcinomas of the bladder (Mhawech-Fauceglia *et al.*, 2007), and in superficial transitional cell carcinoma of the bladder (Babjuk *et al.*, 2002).

5.2.4 PAX5 target genes

PAX5 is required for the production of normal antibody-producing B-cells (Urbanek *et al.*, 1994; Nutt *et al.*, 1997). Accordingly, activated PAX5 target genes code for crucial components of the (pre)BCR signaling cascade, such as the receptor signaling chain Igα, also called CD79a and mb-1 (Fitzsimmons *et al.*, 1996; Nutt *et al.*, 1997), the stimulatory coreceptor CD19 (Kozmik *et al.*, 1992; Nutt *et al.*, 1998), and the central adaptor protein BLNK (Schebesta *et al.*, 2002).

The CD79a gene encodes the transmembrane molecule Ig α which, together with the Ig β (B29) protein, forms a heterodimer mediating signal transduction through the pre-B- and B-cell receptors (Borst *et al.*, 1996). Likely, the CD19 gene codes for a protein with two extracellular immunoglobulin-like domains (Stamenkovic and Seed, 1988; Tedder and Isaacs,

1989), which is implicated in the activation or inhibition of proliferation, depending on the stage of B-cell differentiation. CD19 mRNA is weakly expressed in B-lymphoid cells, and follows PAX5 expression. The BLNK gene is a direct PAX5 target, which encodes a cytoplasmic linker protein that allows progression from pro-B to pre-B cells. The pre-B cell receptor signaling lies on the bridge BLNK protein (SLP-65) for associating the Syk kinase to its downstream effector pathways, such as intracellular calcium signaling, protein kinase C, and different MAPK pathways (Fu *et al.*, 1998; Ishiai *et al.*, 1999). In agreement with a central role in pre-BCR signaling, BLNK gene mutations arrest B cell development at the pro-B to pre-B cell transition in humans (Minegishi *et al.*, 1999).

PAX5 also facilitates expression of the Ig chain by promoting the second V_H-DJ_H recombination step of the IgH gene (Nutt *et al.*, 1997; Fuxa *et al.*, 2004). PAX5 is able to change the chromatin structure by removing the H3-K9 methylation in the V_H locus, inducing accessibility of V_H genes for the recombinase machinery V_H-to-DJ_H recombination (Urbanek *et al.*, 1994; Nutt *et al.*, 1997).

PAX5 also contributes to the regulatory network by activating the transcription factor gene Lef1 and N-myc in pro-B cells (Kozmik *et al.*, 1992) and by preserving Ebf1 expression in committed B lymphocytes (Fuxa *et al.*, 2004; Roessler *et al.*, 2007).

On the other hand, PAX5 is known to suppress expression of several receptors essential for multipotency such as Flt3 receptor (Holmes *et al.*, 2006), but also the macrophage colony-stimulating factor (M-CSF) receptor (Tagoh *et al.*, 2006) and the Notch1 receptor, which is essential for T cell development (Souabni *et al.*, 2002).

Identification of new targets

Recently, cDNA microarray technology was used to identify PAX5 target genes (Delogu *et al.*, 2006; Schebesta *et al.*, 2007; Pridans *et al.*, 2008). One hundred seventy PAX5-activated genes were identified by gene-expression profiling of wild-type and PAX5-deficient pro-B cells. These studies confirmed that many genes repressed by PAX5 are expressed in non-B cell lineages, or during plasma cell differentiation. Actuality, 110 genes were identified as PAX5-repressed genes coding for high number of proteins implicated in cell-cell communication, adhesion, migration, nuclear processes, and cell metabolism. PAX5 seems to have the role to activate secondary transcription factors that further strengthen the B cell program, by promoting the downstream transcriptional cascade.

5.2.5 PAX5 interactions

PAX5 is able to bind DNA through its N-terminal domain (Czerny *et al.*, 1993) and to regulate gene transcription via a C-terminal domain (Dorfler and Busslinger, 1996). The transcriptional activity of PAX5 is directed by interactions with different active proteins (Figure 22).

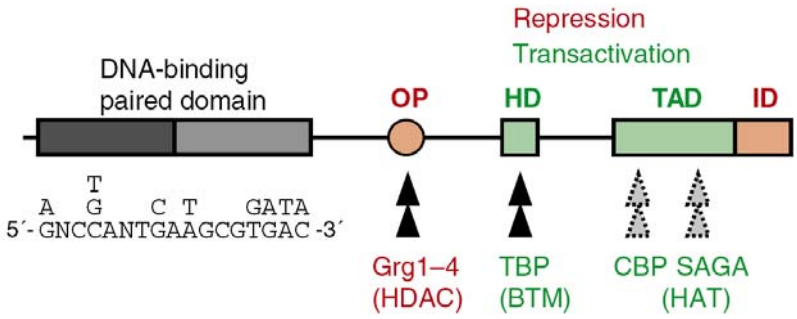


Figure 22. Functional domains and interacting proteins of PAX5 (cited from Cobaleda *et al.*, 2007).

The PAX5 homeodomain (HD) can bind the TATA-binding protein (TBP) of the basal transcription machinery but also the RB protein (Eberhard and Busslinger, 1999), while the transactivation domain (TAD) can link to histone acetyltransferases (HAT) such as the coactivator CREB-binding protein, CBP (Emelyanov *et al.*, 2002) or Spt–Ada–Gcn5 acetyltransferase (SAGA) chromatin remodeling complex (Barlev *et al.*, 2003). In fact, a ternary complex composed of PAX5, Daxx, and CBP was detected (Emelyanov *et al.*, 2002). On the contrary, Groucho corepressors, which interact with larger histone deacetylase (HDAC) complexes, can bind to the octapeptide motif of PAX5 (OP), inducing a repression function of PAX5.

PAX5 regulatory function is also affected by interactions with other transcription factors such as Ets proteins, Runx1, c-Myb and Id proteins (Holmes *et al.*, 2008). For instance, PAX5 regulates the B cell-specific CD79a promoter through the cooperative binding with different Ets family members like Ets-1, Fli-1, and GABPα (Fitzsimmons *et al.*, 1996). Therefore, the interactions with PAX5 allows Ets-1 to form better interactions with its low-affinity DNA Ets-binding site (Garvie *et al.*, 2001). Moreover, activation of CD79a gene by PAX5 is dependent on the demethylation of the Ets binding site (Maier *et al.*, 2003)

The binding of PAX5 to the CD79a promoter is restrained by Id proteins (Id1-3) (Roberts *et al.*, 2001). Likely, Id2 antagonizes PAX5 binding to the activation-induced cytidine deaminase (Aicda or AID) promoter (Gonda *et al.*, 2003). On the contrary, PAX5 interactions with Runx1 or c-Myb are shown to promote gene expression through cooperative DNA binding (Kishi *et al.*, 2002). PAX5 also associates with the PU.1 protein and has opposite activity on the Igk locus (Maitra and Atchison, 2000), while it cooperates to engage Groucho proteins and repress the Igh locus (Linderson *et al.*, 2004).

PAX5 plays a complex and pleiotropic role in B-cell transcriptional regulation. PAX5 can operate as an activator, repressor but also a docking protein, depending on the target gene sequence. PAX5 appears to require both acetylation and chromatin remodeling activities to promote transcription. It is possible that the main function of PAX5 is not to recruit the basal transcription machinery to the promoter but rather to transform the structure of local chromatin, enabling other sequence-specific factors to promote transcription.

5.2.6 Oncogenic action of PAX5

B-cell non-Hodgkin's lymphomas exhibit various abnormalities including numerous different translocations. For instance, the t(9;14) translocation brings the entire PAX5 gene under the control of strong promoters from the IGH locus, inducing an overexpression of PAX5 (Lida *et al.*, 1996; Morrison *et al.*, 1998). Both over and downregulation of PAX5 expression can lead to cancer (Cobaleda *et al.*, 2007). High levels of PAX5 mRNA were observed in some NHL, supporting the notion that deregulated PAX5 gene expression may contribute to B cell oncogenesis (Busslinger *et al.*, 1996; Krenacs *et al.*, 1998; Morrison *et al.*, 1998).

5.3 Telomeres and telomerase in lymphocyte differentiation

The adaptive immune response initiates the selection of the best antigen-binding naïve T and B lymphocyte(s) and finishs with a huge expansion of these selected lymphocytes, inducing an extensive number of cell divisions of these lymphocytes during the entire life. For this expansion, they need telomerase in order to escape the side effects of telomere attrition.

5.3.1 Telomeres in HSCs and T cell functions

HSCs are quiescent cells that display low levels of telomerase activity. The length of the HSC telomeres decreases, probably because of insufficient levels of telomerase activity to fully prevent telomere erosion. However, bone marrow stem cell population seems to have stronger telomerase activity whereas in peripheral blood, weak telomerase activity has been detected (Hiyama *et al.*, 1995c; Broccoli *et al.*, 1995).

Telomerase activity has been shown to be particularly regulated during T cell differentiation (Weng, 2002). In the thymus, high levels of telomerase activity are revealed in all subsets of T cells. However, the telomerase activity of immature CD4-CD8- and CD4+CD8+ cells is higher than single positive mature CD4 and CD8 T cells (Weng *et al.*, 1998). In the periphery, low telomerase activity is detected in mature naïve T cells, while rapid activation occurs upon stimulation of T cells (Weng *et al.*, 1996).

Clonal expansion of T cells results in telomere loss during the differentiation of naïve to memory T cells. A large study has shown that naïve T cells have longer average telomere length than memory T cells in both CD4 and CD8 T cells (Rufer *et al.*, 1999).

5.3.2 Telomeres in B cell functions

B cells exhibit notably longer telomeres than all other blood cell populations, such as T-cells, natural killer cells and monocytes (Martens *et al.*, 2002).

Contrary to T lymphocytes, no significant loss of telomere length is detected between naïve B cells and memory B cells (Son *et al.*, 2003), while B cells in the germinal centre of tonsils have longer telomeres compared to naïve and memory B cells (Weng *et al.*, 1997a).

Like T cells, telomerase is highly activated after antigen stimulation (Hiyama *et al.*, 1995c; Weng *et al.*, 1997a). Likely, low levels of telomerase activity are observed in naïve and memory B cells, in contrast to GC B cells that exhibit high telomerase activity (Norrback *et al.*, 1996; Hu *et al.*, 1997; Igarashi and Sakaguchi, 1997; Weng *et al.*, 1997b). The correlation between telomere length and telomerase activity levels in GC B cells support the hypothesis that telomerase compensates in GC B cells for the ensueing telomere shortening in memory B cells.

Expression levels of TERT RNA parallel telomerase activity levels in GC B cells (Norrback *et al.*, 2001; Hu and Insel, 1999). Moreover, the expression of hTR is correlated with overall telomerase activity at various stages during lineage development, differentiation, and activation (Igarashi and Sakaguchi, 1997; Weng *et al.*, 1997b).

B cells demonstrate a slower rate of telomere erosion than T lymphocytes, suggesting the use of more efficient mechanisms of telomere protection than in T cells during the lymphocytes differentiation (Son *et al.*, 2000).

5.3.3 Telomere and telomerase in B cell disorder

Significant telomerase activity is detected in most lymphomas (Norrback *et al.*, 1996; Trentin *et al.*, 1999; Ely *et al.*, 2000). However, the level of activity is relatively difficult to determine, since normal GC B-cells are likely to have higher telomerase activity than lymphomas and follicular hyperplasias display telomerase levels similar to those of lymphomas (Norrback *et al.*, 1996).

A large study of telomerase activity in various types of B-NHLs revealed that all B-NHLs contained telomerase activity, with the exception of low grade marginal zone B-cell lymphomas (Ely *et al.*, 2000). Accordingly, telomerase activity has been detected in about 93% of high-grade non-Hodgkin's lymphomas (MacNamara *et al.*, 2001). Moreover, GC derived lymphomas had the longest telomere length and the highest telomerase activity (Ladetto *et al.*, 2004). The telomerase activity was also shown to be positively correlated to the proliferation of the lymphoma (Ely *et al.*, 2000; Chiu *et al.*, 2003), and to the clinical aggressiveness of the B-NHLs. Patients with more aggressive tumors such as Burkitts lymphoma show longer telomeres and higher telomerase activity than the more indolent tumors (Remes *et al.*, 2000; Ohyashiki *et al.*, 2001; Kubuki *et al.*, 2005). Interestingly, it has been suggested that in Hodgkin's disease the telomeres can be preserved by a telomerase-independent mechanism (Brousset *et al.*, 1997; Brousset *et al.*, 1998).

In addition, a high level of telomerase activity was detected both in acute myeloblastic leukemia (AML) and acute lymphoblastic leukemia (ALL) (Broccoli *et al.*, 1995; Counter *et al.*, 1995; Zhang *et al.*, 1996; Ohyashiki *et al.*, 1997; Engelhardt *et al.*, 2000; Li *et al.*, 2000; Baumann *et al.*, 2002). The hTERT mRNA transcription has been highly associated in acute

leukemia with telomerase activity (Xu *et al.*, 1998; Ohyashiki *et al.*, 2001), which is also correlated with the prognosis in acute leukemias (Ohyashiki *et al.*, 1997).

6. Outline of the present investigation

The goal of our studies was to better understand the molecular mechanisms of the transcriptional regulation of the hTERT gene. CTCF has been identified as a key factor that blocks transcription in telomerase-negative normal cells. Mechanisms regulating hTERT transcription comprise methylation dependent and methylation-independent mechanisms. We decided to explore both mechanisms through identification of proteins involved in hTERT regulation, and to investigate the potential of hTERT methylation as a cancer biomarker.

In most telomerase-positive carcinoma cells from bladder, breast, colon and cervix, hypermethylation of the hTERT CpG island has been shown to block the inhibitory effect of CTCF. The methylation highly inhibits the transcription of the gene, although a short hypomethylated region allows a low transcription level of the gene. Our working hypothese was that MBD proteins could be involved in the partial repression of hTERT, when the gene is hypermethylated. In **chapter 2**, we described the identification of proteins that mediate hTERT repression in methylated telomerase-positive cells. To this end, we performed chromatin immunoprecipitation assays and depletion by transient or constitutive RNA interference.

However, small subset of telomerase-positive cells, such as ovarian or lymphoid, exhibit unmethylated hTERT promoter. In **chapter 3**, we aimed to identify the methylation-independent mechanism of hTERT transcriptional regulation in lymphoid cells. We particularly investigated potential tissue-specific factor(s), which allow hTERT transcription in non-neoplastic lymphoid tissues and B cell lymphomas. Binding sites of a B cell-specific transcription factor PAX5 were identified downstream of the ATG translational start site. We explore the implication of this factor in the regulation of hTERT in normal and tumoral telomerase-positive B cells by ectopic expression of PAX5 in telomerase-negative cells, siRNA knockdown of PAX5 expression and ChIP assays.

The results found in the two first chapters underline the difference of the hTERT methylation pattern between most carcinoma cells and normal telomerase-positive B cells.

Telomerase activity or hTERT expression, thought as universal biomarker, have been shown to be not specific enough for cancer detection due to lymphocyte infiltrations in tumors. Therefore, we aimed to investigate the hTERT methylation as a potential cancer biomarker. In the **chapter 4**, the hTERT methylation was evaluated in the context of metastasis detection in cerebrospinal fluids as an adjunction of the cytological diagnosis. In this purpose a new method of methylation analysis was developed.

In the **Chapter 5**, concluding remarks about our findings and perspectives for future works are discussed.

REFERENCES

Adams B, Dorfler P, Aguzzi A, Kozmik Z, Urbanek P, Maurer-Fogy I, *et al*. (1992). Pax-5 encodes the transcription factor BSAP and is expressed in B lymphocytes, the developing CNS, and adult testis. *Genes Dev,* **6,** 1589-1607.

Aisner DL, Wright WE and Shay JW. (2002). Telomerase regulation: not just flipping the switch. *Dig Dis Sci,* **12,** 80-85.

Akashi K, Traver D, Miyamoto T and Weissman IL. (2000). A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature,* **404,** 193-197.

Alonso MM, Fueyo J, Yung WK and Gomez-Manzano C. (2006). E2F1 and telomerase: alliance in the dark side. *Cell Cycle,* **5,** 930-935.

Amiard S, Doudeau M, Pinte S, Poulet A, Lenain C, Faivre-Moskalenko C, *et al*. (2007). A topological mechanism for TRF2-enhanced strand invasion. *Nat Struct Mol Biol,* **14,** 147-154.

Amir RE, Van dV, I, Wan M, Tran CQ, Francke U and Zoghbi HY. (1999). Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. *Nat Genet,* **23,** 185-188.

Ancelin K, Brunori M, Bauwens S, Koering CE, Brun C, Ricoul M, *et al*. (2002). Targeting assay to study the cis functions of human telomeric proteins: evidence for inhibition of telomerase by TRF1 and for activation of telomere degradation by TRF2. *Mol Cell Biol,* **22,** 3474-3487.

Anderson CJ, Hoare SF, Ashcroft M, Bilsland AE and Keith WN. (2006). Hypoxic regulation of telomerase gene expression by transcriptional and post-transcriptional mechanisms. *Oncogene,* **25,** 61-69.

Armbruster BN, Banik SS, Guo C, Smith AC and Counter CM. (2001). N-terminal domains of the human telomerase catalytic subunit required for enzyme activity in vivo. *Mol Cell Biol,* **21,** 7775-7786.

Arnold CN, Goel A and Boland CR. (2003). Role of hMLH1 promoter hypermethylation in drug resistance to 5-fluorouracil in colorectal cancer cell lines. *Int J Cancer,* **106,** 66-73.

Atkinson SP, Hoare SF, Glasspool RM and Keith WN. (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,** 7585-7590.

Avilion AA, Piatyszek MA, Gupta J, Shay JW, Bacchetti S and Greider CW. (1996). Human telomerase RNA and telomerase activity in immortal cell lines and tumor tissues. *Cancer Res,* **56,** 645-650.

Azzalin CM, Reichenbach P, Khoriauli L, Giulotto E and Lingner J. (2007). Telomeric repeat-containing RNA and RNA surveillance factors at mammalian chromosome ends. *Science,* **318,** 798-801.

Babjuk M, Kostirova M, Mudra K, Pecher S, Smolova H, Pecen L, *et al*. (2002). Qualitative and quantitative detection of urinary human complement factor H-related protein (BTA stat and BTA TRAK) and fragments of cytokeratins 8, 18 (UBC rapid and UBC IRMA) as markers for transitional cell carcinoma of the bladder. *Eur Urol,* **41,** 34-39.

Bachand F and Autexier C. (2001). Functional regions of human telomerase reverse transcriptase and human telomerase RNA required for telomerase activity and RNA-protein interactions. *Mol Cell Biol,* **21,** 1888-1897.

Bachman KE, Rountree MR and Baylin SB. (2001). Dnmt3a and Dnmt3b are transcriptional repressors that exhibit unique localization properties to heterochromatin. *J Biol Chem,* **276,** 32282-32287.

Baird DM, Rowson J, Wynford-Thomas D and Kipling D. (2003). Extensive allelic variation and ultrashort telomeres in senescent human cells. *Nat Genet,* **33,** 203-207.

Ballestar E, Paz MF, Valle L, Wei S, Fraga MF, Espada J, *et al*. (2003). Methyl-CpG binding proteins identify novel sites of epigenetic inactivation in human cancer. *EMBO J,* **22,** 6335-6345.

Banik SS and Counter CM. (2004). Characterization of interactions between PinX1 and human telomerase subunits hTERT and hTR. *J Biol Chem,* **279,** 51745-51748.

Banik SS, Guo C, Smith AC, Margolis SS, Richardson DA, Tirado CA, *et al*. (2002). C-terminal regions of the human telomerase catalytic subunit essential for in vivo enzyme activity. *Mol Cell Biol,* **22,** 6234-6246.

Barberis A, Widenhorn K, Vitelli L and Busslinger M. (1990). A novel B-cell lineage-specific transcription factor present at early but not late stages of differentiation. *Genes Dev,* **4,** 849-859.

Barlev NA, Emelyanov AV, Castagnino P, Zegerman P, Bannister AJ, Sepulveda MA, *et al*. (2003). A novel human Ada2 homologue functions with Gcn5 or Brg1 to coactivate transcription. *Mol Cell Biol,* **23,** 6944-6957.

Barreto G, Schafer A, Marhold J, Stach D, Swaminathan SK, Handa V, *et al*. (2007). Gadd45a promotes epigenetic gene activation by repair-mediated DNA demethylation. *Nature,* **445,** 671-675.

Barski A, Cuddapah S, Cui K, Roh TY, Schones DE, Wang Z, *et al*. (2007). High-resolution profiling of histone methylations in the human genome. *Cell,* **129,** 823-837.

Baumann Kubetzko FB, Di PC, Maag C, Meier R, Schafer BW, Betts DR, *et al*. (2004). The PAX5 oncogene is expressed in N-type neuroblastoma cells and increases tumorigenicity of a S-type cell line. *Carcinogenesis,* **25,** 1839-1846.

Baumann P and Cech TR. (2001). Pot1, the putative telomere end-binding protein in fission yeast and humans. *Science,* **292,** 1171-1175.

Baumann P, Podell E and Cech TR. (2002). Human Pot1 (Protection of telomeres) protein: Cytolocalization, gene structure, and alternative splicing. *Mol Cell Biol,* **22,** 8079-8087.

Baur JA, Zou Y, Shay JW and Wright WE. (2001). Telomere position effect in human cells. *Science,* **292,** 2075-2077.

Baylin SB and Herman JG. (2000). DNA hypermethylation in tumorigenesis: epigenetics joins genetics. *Trends Genet,* **16,** 168-174.

Baylin SB, Herman JG, Graff JR, Vertino PM and Issa JP. (1998). Alterations in DNA methylation: a fundamental aspect of neoplasia. *Adv Cancer Res,* **72,** 141-196.

Bayne S and Liu JP. (2005). Hormones and growth factors regulate telomerase activity in ageing and cancer. *Mol Cell Endocrinol*.

Beattie TL, Zhou W, Robinson MO and Harrington L. (2001). Functional multimerization of the human telomerase reverse transcriptase. *Mol Cell Biol,* **21,** 6151-6160.

Beattie TL, Zhou W, Robinson MO and Harrington L. (2000). Polymerization defects within human telomerase are distinct from telomerase RNA and TEP1 binding. *Mol Biol Cell,* **11,** 3329-3340.

Bedford MT and van Helden PD. (1987). Hypomethylation of DNA in pathological conditions of the human prostate. *Cancer Res,* **47,** 5274-5276.

Bell AC and Felsenfeld G. (2000). Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene. *Nature,* **405,** 482-485.

Benetti R, Gonzalo S, Jaco I, Munoz P, Gonzalez S, Schoeftner S, *et al*. (2008). A mammalian microRNA cluster controls DNA methylation and telomere recombination via Rbl2-dependent regulation of DNA methyltransferases. *Nat Struct Mol Biol,* **15,** 998.

Bernard S and Eilers M. (2006). Control of cell proliferation and growth by Myc proteins. *Results Probl Cell Differ,* **42,** 329-342.

Bernhardt SL, Gjertsen MK, Trachsel S, Moller M, Eriksen JA, Meo M, *et al*. (2006). Telomerase peptide vaccination of patients with non-resectable pancreatic cancer: A dose escalating phase I/II study. *Br J Cancer,* **95,** 1474-1482.

Bestor TH. (1990). DNA methylation: evolution of a bacterial immune function into a regulator of gene expression and genome structure in higher eukaryotes. *Philos Trans R Soc Lond B Biol Sci,* **326,** 179-187.

Beyne-Rauzy O, Prade-Houdellier N, Demur C, Recher C, Ayel J, Laurent G, *et al*. (2005). Tumor necrosis factor alpha inhibits hTERT gene expression in human myeloid normal and leukemic cells. *Blood*.

Bhattacharya SK, Ramchandani S, Cervoni N and Szyf M. (1999). A mammalian protein with specific demethylase activity for mCpG DNA. *Nature,* **397,** 579-583.

Bian YS, Osterheld MC, Fontolliet C, Bosman FT and Benhattar J. (2002). p16 inactivation by methylation of the CDKN2A promoter occurs early during neoplastic progression in Barrett's esophagus. *Gastroenterology,* **122,** 1113-1121.

Bian YS, Yan P, Osterheld MC, Fontolliet C and Benhattar J. (2001). Promoter methylation analysis on microdissected paraffin-embedded tissues using bisulfite treatment and PCR-SSCP. *Biotechniques,* **30,** 66-72.

Bianchi A and Shore D. (2008). How telomerase reaches its end: mechanism of telomerase regulation by the telomeric complex. *Mol Cell,* **31,** 153-165.

Bianco T, Hussey D and Dobrovic A. (1999). Methylation-sensitive, single-strand conformation analysis (MS-SSCA): A rapid method to screen for and analyze methylation. *Human Mutation,* **14,** 289-293.

Bilsland AE, Anderson CJ, Fletcher-Monaghan AJ, McGregor F, Evans TR, Ganly I, *et al*. (2003). Selective ablation of human cancer cells by telomerase-specific adenoviral suicide gene therapy vectors expressing bacterial nitroreductase. *Oncogene,* **22,** 370-380.

Bird A. (2002). DNA methylation patterns and epigenetic memory. *Genes Dev,* **16,** 6-21.

Bird A. (2007). Perceptions of epigenetics. *Nature,* **447,** 396-398.

Bird AP and Wolffe AP. (1999). Methylation-induced repression--belts, braces, and chromatin. *Cell,* **99,** 451-454.

Blackburn EH. (1997). The telomere and telomerase: nucleic acid-protein complexes acting in a telomere homeostasis system. A review. *Biochemistry (Mosc),* **62,** 1196-1201.

Blackburn EH and Gall JG. (1978). Tandemly Repeated Sequence at Termini of Extrachromosomal Ribosomal-Rna Genes in Tetrahymena. *Journal of Molecular Biology,* **120,** 33-53.

Blasco MA, Lee HW, Hande MP, Samper E, Lansdorp PM, DePinho RA, *et al*. (1997). Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. *Cell,* **91,** 25-34.

Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, *et al*. (1998). Extension of life-span by introduction of telomerase into normal human cells. *Science,* **279,** 349-352.

Boeke J, Ammerpohl O, Kegel S, Moehren U and Renkawitz R. (2000). The minimal repression domain of MBD2b overlaps with the methyl-CpG-binding domain and binds directly to Sin3A. *J Biol Chem,* **275,** 34963-34967.

Borst J, Jacobs H and Brouns G. (1996). Composition and function of T-cell receptor and B-cell receptor complexes on precursor lymphocytes. *Curr Opin Immunol,* **8,** 181-190.

Brandeis M, Frank D, Keshet I, Siegfried Z, Mendelsohn M, Nemes A, *et al*. (1994). Sp1 elements protect a CpG island from de novo methylation. *Nature,* **371,** 435-438.

Brero A, Easwaran HP, Nowak D, Grunewald I, Cremer T, Leonhardt H, *et al*. (2005). Methyl CpG-binding proteins induce large-scale chromatin reorganization during terminal differentiation. *J Cell Biol,* **169,** 733-743.

Broccoli D, Smogorzewska A, Chong L and De Lange T. (1997). Human telomeres contain two distinct Myb-related proteins, TRF1 and TRF2. *Nat Genet,* **17,** 231-235.

Broccoli D, Young JW and De Lange T. (1995). Telomerase activity in normal and malignant hematopoietic cells. *Proc Natl Acad Sci U S A,* **92,** 9082-9086.

Brock MV, Gou M, Akiyama Y, Muller A, Wu TT, Montgomery E, *et al*. (2003). Prognostic importance of promoter hypermethylation of multiple genes in esophageal adenocarcinoma. *Clin Cancer Res,* **9,** 2912-2919.

Brousset P, al ST, Chaouche N, Zenou RC, Schlaifer D, Chittal S, *et al*. (1997). Telomerase activity in reactive and neoplastic lymphoid tissues: infrequent detection of activity in Hodgkin's disease. *Blood,* **89,** 26-31.

Brousset P, Chaouche N, al ST, Zenou RC and Delsol G. (1998). Telomerase activity in Hodgkin's disease. *Leuk Lymphoma,* **30,** 189-192.

Brunsvig PF, Aamdal S, Gjertsen MK, Kvalheim G, Markowski-Grimsrud CJ, Sve I, *et al*. (2006). Telomerase peptide vaccination: a phase I/II study in patients with non-small cell lung cancer. *Cancer Immunol Immunother,* **55,** 1553-1564.

Bryan TM, Englezou A, la-Pozza L, Dunham MA and Reddel RR. (1997). Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. *Nat Med,* **3,** 1271-1274.

Bryan TM and Reddel RR. (1997). Telomere dynamics and telomerase activity in in vitro immortalised human cells. *Eur J Cancer,* **33,** 767-773.

Bryce LA, Morrison N, Hoare SF, Muir S and Keith WN. (2000). Mapping of the gene for the human telomerase reverse transcriptase, hTERT, to chromosome 5p15.33 by fluorescence in situ hybridization. *Neoplasia,* **2,** 197-201.

Burgers WA, Fuks F and Kouzarides T. (2002). DNA methyltransferases get connected to chromatin. *Trends Genet,* **18,** 275-277.

Busslinger M, Klix N, Pfeffer P, Graninger PG and Kozmik Z. (1996). Deregulation of PAX-5 by translocation of the Emu enhancer of the IgH locus adjacent to two alternative PAX-5 promoters in a diffuse large-cell lymphoma. *Proc Natl Acad Sci U S A,* **93,** 6129-6134.

Cairney CJ and Keith WN. (2008). Telomerase redefined: integrated regulation of hTR and hTERT for telomere maintenance and telomerase activity. *Biochimie,* **90,** 13-23.

Cairns P, Esteller M, Herman JG, Schoenberg M, Jeronimo C, Sanchez-Cespedes M, *et al*. (2001). Molecular detection of prostate cancer in urine by GSTP1 hypermethylation. *Clin Cancer Res,* **7,** 2727-2730.

Cameron EE, Bachman KE, Myohanen S, Herman JG and Baylin SB. (1999). Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nat Genet,* **21,** 103-107.

Campbell PM, Bovenzi V and Szyf M. (2004). Methylated DNA-binding protein 2 antisense inhibitors suppress tumourigenesis of human cancer cell lines in vitro and in vivo. *Carcinogenesis,* **25,** 499-507.

Campisi J. (1997). The biology of replicative senescence. *Eur J Cancer,* **33,** 703-709.

Cao Y, Li H, Deb S and Liu JP. (2002). TERT regulates cell survival independent of telomerase enzymatic activity. *Oncogene,* **21,** 3130-3138.

Carotta S and Nutt SL. (2008). Losing B cell identity. *Bioessays,* **30,** 203-207.

Cesare AJ and Griffith JD. (2004). Telomeric DNA in ALT cells is characterized by free telomeric circles and heterogeneous t-loops. *Molecular and Cellular Biology,* **24,** 9948-9957.

Chadeneau C, Hay K, Hirte HW, Gallinger S and Bacchetti S. (1995). Telomerase activity associated with acquisition of malignancy in human colorectal cancer. *Cancer Res,* **55,** 2533-2536.

Chambeyron S and Bickmore WA. (2004). Chromatin decondensation and nuclear reorganization of the HoxB locus upon induction of transcription. *Genes Dev,* **18,** 1119-1130.

Chan SW and Blackburn EH. (2002). New ways not to make ends meet: telomerase, DNA damage proteins and heterochromatin. *Oncogene,* **21,** 553-563.

Chan SW and Blackburn EH. (2003). Telomerase and ATM/Tel1p protect telomeres from nonhomologous end joining. *Mol Cell,* **11,** 1379-1387.

Chang JT, Lu YC, Chen YJ, Tseng CP, Chen YL, Fang CW, *et al*. (2006). hTERT phosphorylation by PKC is essential for telomerase holoprotein integrity and enzyme activity in head neck cancer cells. *Br J Cancer,* **94,** 870-878.

Chang JT, Yang HT, Wang TC and Cheng AJ. (2005). Upstream stimulatory factor (USF) as a transcriptional suppressor of human telomerase reverse transcriptase (hTERT) in oral cancer cells. *Mol Carcinog*.

Chen JL and Greider CW. (2004). Telomerase RNA structure and function: implications for dyskeratosis congenita. *Trends Biochem Sci,* **29,** 183-192.

Chen JL, Opperman KK and Greider CW. (2002). A critical stem-loop structure in the CR4-CR5 domain of mammalian telomerase RNA. *Nucleic Acids Res,* **30,** 592-597.

Chen RZ, Akbarian S, Tudor M and Jaenisch R. (2001). Deficiency of methyl-CpG binding protein-2 in CNS neurons results in a Rett-like phenotype in mice. *Nat Genet,* **27,** 327-331.

Chen T, Hevi S, Gay F, Tsujimoto N, He T, Zhang B, *et al*. (2007). Complete inactivation of DNMT1 leads to mitotic catastrophe in human cancer cells. *Nat Genet,* **39,** 391-396.

Chen XQ, Bonnefoi H, Pelte MF, Lyautey J, Lederrey C, Movarekhi S, *et al*. (2000). Telomerase RNA as a detection marker in the serum of breast cancer patients. *Clin Cancer Res,* **6,** 3823-3826.

Chin K, de Solorzano CO, Knowles D, Jones A, Chou W, Rodriguez EG, *et al*. (2004). In situ analyses of genome instability in breast cancer. *Nature Genetics,* **36,** 984-988.

Chiu CP, Dragowska W, Kim NW, Vaziri H, Yui J, Thomas TE, *et al*. (1996). Differential expression of telomerase activity in hematopoietic progenitors from adult human bone marrow. *Stem Cells,* **14,** 239-248.

Chiu KC, Fine M, Ikle D, Slovak ML and Arber DA. (2003). Telomerase activity and proliferation index in aggressive mature B-cell lymphoma: comparison to germinal center phenotypic markers. *Hum Pathol,* **34,** 1259-1264.

Choi JK, Southworth LK, Sarin KY, Venteicher AS, Ma WX, Chang W, *et al*. (2008). TERT promotes epithelial proliferation through transcriptional control of a Myc- and Wnt-related developmental program. *Plos Genetics,* **4**.

Christensen J, Agger K, Cloos PA, Pasini D, Rose S, Sennels L, *et al*. (2007). RBP2 belongs to a family of demethylases, specific for tri-and dimethylated lysine 4 on histone 3. *Cell,* **128,** 1063-1076.

Clark GM, Osborne CK, Levitt D, Wu F and Kim NW. (1997). Telomerase activity and survival of patients with node-positive breast cancer. *J Natl Cancer Inst,* **89,** 1874-1881.

Clement G and Benhattar J. (2005). A methylation sensitive dot blot assay (MS-DBA) for the quantitative analysis of DNA methylation in clinical samples. *Journal of Clinical Pathology,* **58,** 155-158.

Clement G, Braunschweig R, Pasquier N, Bosman FT and Benhattar J. (2006). Alterations of the Wnt signaling pathway during the neoplastic progression of Barrett's esophagus. *Oncogene,* **25,** 3084-3092.

Cobaleda C, Schebesta A, Delogu A and Busslinger M. (2007). Pax5: the guardian of B cell identity and function. *Nat Immunol,* **8,** 463-470.

Colgin LM, Baran K, Baumann P, Cech TR and Reddel RR. (2003). Human POT1 facilitates telomere elongation by telomerase. *Curr Biol,* **13,** 942-946.

Colgin LM, Wilkinson C, Englezou A, Kilian A, Robinson MO and Reddel RR. (2000). The hTERTalpha splice variant is a dominant negative inhibitor of telomerase activity. *Neoplasia,* **2,** 426-432.

Collins K. (2008). Physiological assembly and activity of human telomerase complexes. *Mech Ageing Dev,* **129,** 91-98.

Collins K and Mitchell JR. (2002). Telomerase in the human organism. *Oncogene,* **21,** 564-579.

Cong YS and Bacchetti S. (2000). Histone deacetylation is involved in the transcriptional repression of hTERT in normal human cells. *J Biol Chem,* **275,** 35665-35668.

Cong YS, Wen J and Bacchetti S. (1999). The human telomerase catalytic subunit hTERT: organization of the gene and characterization of the promoter. *Hum Mol Genet,* **8,** 137-142.

Cook BD, Dynek JN, Chang W, Shostak G and Smith S. (2002). Role for the related poly(ADP-Ribose) polymerases tankyrase 1 and 2 at human telomeres. *Mol Cell Biol,* **22,** 332-342.

Cortez-Gonzalez X and Zanetti M. (2007). Telomerase immunity from bench to bedside: round one. *J Transl Med,* **5,** 12.

Costa A, Daidone MG, Daprai L, Villa R, Cantu S, Pilotti S, *et al*. (2006). Telomere maintenance mechanisms in liposarcomas: Association with histologic subtypes and disease progression. *Cancer Research,* **66,** 8918-8924.

Costa FF, Verbisck NV, Salim AC, Ierardi DF, Pires LC, Sasahara RM, *et al*. (2004). Epigenetic silencing of the adhesion molecule ADAM23 is highly frequent in breast tumors. *Oncogene,* **23,** 1481-1488.

Costello JF, Fruhwald MC, Smiraglia DJ, Rush LJ, Robertson GP, Gao X, *et al*. (2000). Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. *Nat Genet,* **24,** 132-138.

Cottrell SE, Distler J, Goodman NS, Mooney SH, Kluth A, Olek A, *et al*. (2004). A real-time PCR assay for DNA-methylation using methylation-specific blockers. *Nucleic Acids Res,* **32,** e10.

Counter CM, Gupta J, Harley CB, Leber B and Bacchetti S. (1995). Telomerase activity in normal leukocytes and in hematologic malignancies. *Blood,* **85,** 2315-2320.

Cristofari G and Lingner J. (2006). Telomere length homeostasis requires that telomerase levels are limiting. *EMBO J,* **25,** 565-574.

Cross SH and Bird AP. (1995). CpG islands and genes. *Curr Opin Genet Dev,* **5,** 309-314.

Cross SH, Meehan RR, Nan X and Bird A. (1997). A component of the transcriptional repressor MeCP1 shares a motif with DNA methyltransferase and HRX proteins. *Nat Genet,* **16,** 256-259.

Crowe DL, Nguyen DC, Tsang KJ and Kyo S. (2001). E2F-1 represses transcription of the human telomerase reverse transcriptase gene. *Nucleic Acids Res,* **29,** 2789-2794.

Cui H, Onyango P, Brandenburg S, Wu Y, Hsieh CL and Feinberg AP. (2002). Loss of imprinting in colorectal cancer linked to hypomethylation of H19 and IGF2. *Cancer Res,* **62,** 6442-6446.

Czerny T, Schaffner G and Busslinger M. (1993). DNA sequence recognition by Pax proteins: bipartite structure of the paired domain and its binding site. *Genes Dev,* **7,** 2048-2061.

Dantzer F, Giraud-Panis MJ, Jaco I, Ame JC, Schultz I, Blasco M, *et al*. (2004). Functional interaction between poly(ADP-ribose) polymerase 2 (PARP-2) and TRF2: PARP activity negatively regulates TRF2. *Molecular and Cellular Biology,* **24,** 1595-1607.

Darzacq X, Kittur N, Roy S, Shav-Tal Y, Singer RH and Meier UT. (2006). Stepwise RNP assembly at the site of H/ACA RNA transcription in human cells. *Journal of Cell Biology,* **173,** 207-218.

Das PM and Singal R. (2004). DNA methylation and cancer. *J Clin Oncol,* **22,** 4632-4642.

Dasi F, Lledo S, Garcia-Granero E, Ripoll R, Marugan M, Tormo M, *et al*. (2001). Real-time quantification in plasma of human telomerase reverse transcriptase (hTERT) mRNA: a simple blood test to monitor disease in cancer patients. *Lab Invest,* **81,** 767-769.

De Lange T. (1992). Human telomeres are attached to the nuclear matrix. *EMBO J,* **11,** 717-724.

de Lange T. (2004). T-loops and the origin of telomeres. *Nat Rev Mol Cell Biol,* **5,** 323-329.

de Lange T. (2005). Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes Dev,* **19,** 2100-2110.

De Marzo AM, Marchi VL, Yang ES, Veeraswamy R, Lin X and Nelson WG. (1999). Abnormal regulation of DNA methyltransferase expression during colorectal carcinogenesis. *Cancer Res,* **59,** 3855-3860.

Del Bufalo D., Rizzo A, Trisciuoglio D, Cardinali G, Torrisi MR, Zangemeister-Wittke U, *et al*. (2005). Involvement of hTERT in apoptosis induced by interference with Bcl-2 expression and function. *Cell Death Differ,* **12,** 1429-1438.

Delogu A, Schebesta A, Sun Q, Aschenbrenner K, Perlot T and Busslinger M. (2006). Gene repression by Pax5 in B cells is essential for blood cell homeostasis and is reversed in plasma cells. *Immunity,* **24,** 269-281.

Denchi EL and de Lange T. (2007). Protection of telomeres through independent control of ATM and ATR by TRF2 and POT1. *Nature,* **448,** 1068-1071.

Deng WG, Jayachandran G, Wu G, Xu K, Roth JA and Ji L. (2007). Tumor-specific activation of human telomerase reverses transcriptase promoter activity by activating enhancer-binding protein-2beta in human lung cancer cells. *J Biol Chem,* **282,** 26460-26470.

Deng Y and Chang S. (2007). Role of telomeres and telomerase in genomic instability, senescence and cancer. *Lab Invest,* **87,** 1071-1076.

Deng YB, Chan SS and Chang S. (2008). Telomere dysfunction and tumour suppression: the senescence connection. *Nat Rev Cancer,* **8,** 450-458.

Dessain SK, Yu H, Reddel RR, Beijersbergen RL and Weinberg RA. (2000). Methylation of the human telomerase gene CpG island. *Cancer Res,* **60,** 537-541.

Devereux TR, Horikawa I, Anna CH, Annab LA, Afshari CA and Barrett JC. (1999). DNA methylation analysis of the promoter region of the human telomerase reverse transcriptase (hTERT) gene. *Cancer Res,* **59,** 6087-6090.

Dhaene K, Van ME and Parwaresch R. (2000). Telomeres, telomerase and cancer: an up-date. *Virchows Arch,* **437,** 1-16.

di Fagagna FD, Reaper PM, Clay-Farrace L, Fiegler H, Carr P, von Zglinicki T, *et al*. (2003). A DNA damage checkpoint response in telomere-initiated senescence. *Nature,* **426,** 194-198.

Dikmen ZG, Gellert GC, Jackson S, Gryaznov S, Tressler R, Dogan P, *et al*. (2005). In vivo inhibition of lung cancer by GRN163L: a novel human telomerase inhibitor. *Cancer Res,* **65,** 7866-7873.

Dimri GP, Martinez JL, Jacobs JJ, Keblusek P, Itahana K, van LM, *et al*. (2002). The Bmi-1 oncogene induces telomerase activity and immortalizes human mammary epithelial cells. *Cancer Res,* **62,** 4736-4745.

Dokal I. (2000). Dyskeratosis congenita in all its forms. *Br J Haematol,* **110,** 768-779.

Dokal I, Bungey J, Williamson P, Oscier D, Hows J and Luzzatto L. (1992). Dyskeratosis congenita fibroblasts are abnormal and have unbalanced chromosomal rearrangements. *Blood,* **80,** 3090-3096.

Dong HY, Liu W, Cohen P, Mahle CE and Zhang W. (2005). B-cell specific activation protein encoded by the PAX-5 gene is commonly expressed in merkel cell carcinoma and small cell carcinomas. *Am J Surg Pathol,* **29,** 687-692.

Dorfler P and Busslinger M. (1996). C-terminal activating and inhibitory domains determine the transactivation potential of BSAP (Pax-5), Pax-2 and Pax-8. *EMBO J,* **15,** 1971-1982.

Dragon F, Pogacic V and Filipowicz W. (2000). In vitro assembly of human H/ACA small nucleolar RNPs reveals unique features of U17 and telomerase RNAs. *Molecular and Cellular Biology,* **20,** 3037-3048.

Ducrest AL, Amacker M, Mathieu YD, Cuthbert AP, Trott DA, Newbold RF, *et al*. (2001). Regulation of human telomerase activity: repression by normal chromosome 3 abolishes nuclear telomerase reverse transcriptase transcripts but does not affect c-Myc activity. *Cancer Res,* **61,** 7594-7602.

Ducrest AL, Szutorisz H, Lingner J and Nabholz M. (2002). Regulation of the human telomerase reverse transcriptase gene. *Oncogene,* **21,** 541-552.

Duncan EL and Reddel RR. (1997). Genetic changes associated with immortalization. A review. *Biochemistry (Mosc),* **62,** 1263-1274.

Dunham MA, Neumann AA, Fasching CL and Reddel RR. (2000). Telomere maintenance by recombination in human cells. *Nat Genet,* **26,** 447-450.

Dwyer J, Li H, Xu D and Liu JP. (2007). Transcriptional regulation of telomerase activity: roles of the the Ets transcription factor family. *Ann N Y Acad Sci,* **1114,** 36-47.

Eads CA, Danenberg KD, Kawakami K, Saltz LB, Blake C, Shibata D, *et al*. (2000). MethyLight: a high-throughput assay to measure DNA methylation. *Nucleic Acids Res,* **28,** E32.

Eads CA, Nickel AE and Laird PW. (2002). Complete genetic suppression of polyp formation and reduction of CpG-island hypermethylation in Apc(Min/+) Dnmt1-hypomorphic Mice. *Cancer Res,* **62,** 1296-1299.

Eberhard D and Busslinger M. (1999). The partial homeodomain of the transcription factor Pax-5 (BSAP) is an interaction motif for the retinoblastoma and TATA-binding proteins. *Cancer Res,* **59,** 1716s-1724s.

Ehrlich M. (2002). DNA methylation in cancer: too much, but also too little. *Oncogene,* **21,** 5400-5413.

Ely SA, Chadburn A, Dayton CM, Cesarman E and Knowles DM. (2000). Telomerase activity in B-cell non-Hodgkin lymphoma. *Cancer,* **89,** 445-452.

Emelyanov AV, Kovac CR, Sepulveda MA and Birshtein BK. (2002). The interaction of Pax5 (BSAP) with Daxx can result in transcriptional activation in B cells. *J Biol Chem,* **277,** 11156-11164.

Endoh T, Tsuji N, Asanuma K, Yagihashi A and Watanabe N. (2005). Survivin enhances telomerase activity via up-regulation of specificity protein 1- and c-Myc-mediated human telomerase reverse transcriptase gene transcription. *Exp Cell Res,* **305,** 300-311.

Engelhardt M, Mackenzie K, Drullinsky P, Silver RT and Moore MA. (2000). Telomerase activity and telomere length in acute and chronic leukemia, pre- and post-ex vivo culture. *Cancer Res,* **60,** 610-617.

Espada J, Ballestar E, Fraga MF, Villar-Garea A, Juarranz A, Stockert JC, *et al*. (2004). Human DNA methyltransferase 1 is required for maintenance of the histone H3 modification pattern. *J Biol Chem,* **279,** 37175-37184.

Esteller M. (2005b). Dormant hypermethylated tumour suppressor genes: questions and answers. *J Pathol,* **205,** 172-180.

Esteller M. (2005a). DNA methylation and cancer therapy: new developments and expectations. *Curr Opin Oncol,* **17,** 55-60.

Esteller M. (2007). Cancer epigenomics: DNA methylomes and histone-modification maps. *Nat Rev Genet,* **8,** 286-298.

Esteller M, Catasus L, Matias-Guiu X, Mutter GL, Prat J, Baylin SB, *et al*. (1999a). hMLH1 promoter hypermethylation is an early event in human endometrial tumorigenesis. *Am J Pathol,* **155,** 1767-1772.

Esteller M, Corn PG, Baylin SB and Herman JG. (2001). A gene hypermethylation profile of human cancer. *Cancer Res,* **61,** 3225-3229.

Esteller M, Corn PG, Urena JM, Gabrielson E, Baylin SB and Herman JG. (1998). Inactivation of glutathione S-transferase P1 gene by promoter hypermethylation in human neoplasia. *Cancer Res,* **58,** 4515-4518.

Esteller M, Gaidano G, Goodman SN, Zagonel V, Capello D, Botto B, *et al*. (2002). Hypermethylation of the DNA repair gene O(6)-methylguanine DNA methyltransferase and survival of patients with diffuse large B-cell lymphoma. *J Natl Cancer Inst,* **94,** 26-32.

Esteller M, Garcia-Foncillas J, Andion E, Goodman SN, Hidalgo OF, Vanaclocha V, *et al*. (2000a). Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. *N Engl J Med,* **343,** 1350-1354.

Esteller M, Hamilton SR, Burger PC, Baylin SB and Herman JG. (1999b). Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. *Cancer Res,* **59,** 793-797.

Esteller M, Tortola S, Toyota M, Capella G, Peinado MA, Baylin SB, *et al*. (2000b). Hypermethylation-associated inactivation of p14(ARF) is independent of p16(INK4a) methylation and p53 mutational status. *Cancer Res,* **60,** 129-133.

Eversole A and Maizels N. (2000). In vitro properties of the conserved mammalian protein hnRNP D suggest a role in telomere maintenance. *Mol Cell Biol,* **20,** 5425-5432.

Fan Y, Liu Z, Fang X, Ge Z, Ge N, Jia Y, *et al*. (2005). Differential expression of full-length telomerase reverse transcriptase mRNA and telomerase activity between normal and malignant renal tissues. *Clin Cancer Res,* **11,** 4331-4337.

Farrell CM, West AG and Felsenfeld G. (2002). Conserved CTCF insulator elements flank the mouse and human beta-globin loci. *Mol Cell Biol,* **22,** 3820-3831.

Fasching CL, Bower K and Reddel RR. (2005). Telomerase-independent telomere length maintenance in the absence of alternative lengthening of telomeres-associated promyelocytic leukemia bodies. *Cancer Res,* **65,** 2722-2729.

Fasching CL, Neumann AA, Muntoni A, Yeager TR and Reddel RR. (2007). DNA damage induces alternative lengthening of telomeres (ALT)-associated promyelocytic leukemia bodies that preferentially associate with linear telomeric DNA. *Cancer Research,* **67,** 7072-7077.

Fatemi M and Wade PA. (2006). MBD family proteins: reading the epigenetic code. *J Cell Sci,* **119,** 3033-3037.

Feinberg AP. (2004). The epigenetics of cancer etiology. *Semin Cancer Biol,* **14,** 427-432.

Feinberg AP, Ohlsson R and Henikoff S. (2006). The epigenetic progenitor origin of human cancer. *Nat Rev Genet,* **7,** 21-33.

Feinberg AP and Vogelstein B. (1987). Alterations in DNA methylation in human colon neoplasia. *Semin Surg Oncol,* **3,** 149-151.

Feltus FA, Lee EK, Costello JF, Plass C and Vertino PM. (2003). Predicting aberrant CpG island methylation. *Proc Natl Acad Sci U S A,* **100,** 12253-12258.

Feng J, Funk WD, Wang SS, Weinrich SL, Avilion AA, Chiu CP, *et al*. (1995). The RNA component of human telomerase. *Science,* **269,** 1236-1241.

Feng Q and Zhang Y. (2001). The MeCP1 complex represses transcription through preferential binding, remodeling, and deacetylating methylated nucleosomes. *Genes Dev,* **15,** 827-832.

Filippova GN, Fagerlie S, Klenova EM, Myers C, Dehner Y, Goodwin G, *et al*. (1996). An exceptionally conserved transcriptional repressor, CTCF, employs different combinations of zinc fingers to bind diverged promoter sequences of avian and mammalian c-myc oncogenes. *Mol Cell Biol,* **16,** 2802-2813.

Fitzsimmons D, Hodsdon W, Wheat W, Maira SM, Wasylyk B and Hagman J. (1996). Pax-5 (BSAP) recruits Ets proto-oncogene family proteins to form functional ternary complexes on a B-cell-specific promoter. *Genes Dev,* **10,** 2198-2211.

Fleisher AS, Esteller M, Wang S, Tamura G, Suzuki H, Yin J, *et al*. (1999). Hypermethylation of the hMLH1 gene promoter in human gastric cancers with microsatellite instability. *Cancer Res,* **59,** 1090-1095.

Fletcher TM, Cathers BE, Ravikumar KS, Mamiya BM and Kerwin SM. (2001). Inhibition of human telomerase by 7-deaza-2'-deoxyguanosine nucleoside triphosphate analogs: potent inhibition by 6-thio-7-deaza-2'-deoxyguanosine 5'-triphosphate. *Bioorg Chem,* **29,** 36-55.

Ford LP, Suh JM, Wright WE and Shay JW. (2000). Heterogeneous nuclear ribonucleoproteins C1 and C2 associate with the RNA component of human telomerase. *Mol Cell Biol,* **20,** 9084-9091.

Ford LP, Wright WE and Shay JW. (2002). A model for heterogeneous nuclear ribonucleoproteins in telomere and telomerase regulation. *Oncogene,* **21,** 580-583.

Forsyth NR, Wright WE and Shay JW. (2002). Telomerase and differentiation in multicellular organisms: turn it off, turn it on, and turn it off again. *Differentiation,* **69,** 188-197.

Forsythe HL, Elmore LW, Jensen KO, Landon MR and Holt SE. (2002). Retroviral-mediated expression of telomerase in normal human cells provides a selective growth advantage. *Int J Oncol,* **20,** 1137-1143.

Forsythe HL, Jarvis JL, Turner JW, Elmore LW and Holt SE. (2001). Stable association of hsp90 and p23, but Not hsp70, with active human telomerase. *J Biol Chem,* **276,** 15571-15574.

Fraga MF, Ballestar E, Montoya G, Taysavang P, Wade PA and Esteller M. (2003). The affinity of different MBD proteins for a specific methylated locus depends on their intrinsic binding properties. *Nucleic Acids Res,* **31,** 1765-1774.

Fraga MF, Uriol E, Borja DL, Berdasco M, Esteller M, Canal MJ, *et al*. (2002). High-performance capillary electrophoretic method for the quantification of 5-methyl 2'-deoxycytidine in genomic DNA: application to plant, animal and human cancer tissues. *Electrophoresis,* **23,** 1677-1681.

Frigola J, Ribas M, Risques RA and Peinado MA. (2002). Methylome profiling of cancer cells by amplification of inter-methylated sites (AIMS). *Nucleic Acids Res,* **30,** e28.

Frommer M, McDonald LE, Millar DS, Collis CM, Watt F, Grigg GW, *et al*. (1992). A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc Natl Acad Sci U S A,* **89,** 1827-1831.

Fu C, Turck CW, Kurosaki T and Chan AC. (1998). BLNK: a central linker protein in B cell activation. *Immunity,* **9,** 93-103.

Fu D and Collins K. (2003). Distinct biogenesis pathways for human telomerase RNA and H/ACA small nucleolar RNAs. *Mol Cell,* **11,** 1361-1372.

Fu D and Collins K. (2007). Purification of human telomerase complexes identifies factors involved in telomerase biogenesis and telomere length regulation. *Molecular Cell,* **28,** 773-785.

Fu D and Collins K. (2006). Human telomerase and Cajal body ribonucleoproteins share a unique specificity of Sm protein association. *Genes & Development,* **20,** 531-536.

Fu W, Killen M, Culmsee C, Dhar S, Pandita TK and Mattson MP. (2000). The catalytic subunit of telomerase is expressed in developing brain neurons and serves a cell survival-promoting function. *J Mol Neurosci,* **14,** 3-15.

Fujimoto K, Kyo S, Takakura M, Kanaya T, Kitagawa Y, Itoh H, *et al*. (2000). Identification and characterization of negative regulatory elements of the human telomerase catalytic subunit (hTERT) gene promoter: possible role of MZF-2 in transcriptional repression of hTERT. *Nucleic Acids Res,* **28,** 2557-2562.

Fujita N, Shimotake N, Ohki I, Chiba T, Saya H, Shirakawa M, *et al*. (2000). Mechanism of transcriptional regulation by methyl-CpG binding protein MBD1. *Mol Cell Biol,* **20,** 5107-5118.

Fujiwara T, Urata Y and Tanaka N. (2007). Telomerase-specific oncolytic virotherapy for human cancer with the hTERT promoter. *Curr Cancer Drug Targets,* **7,** 191-201.

Fujiwara-Akita H, Maesawa C, Honda T, Kobayashi S and Masuda T. (2005). Expression of human telomerase reverse transcriptase splice variants is well correlated with low telomerase activity in osteosarcoma cell lines. *Int J Oncol,* **26,** 1009-1016.

Fuks F, Hurd PJ, Wolf D, Nan X, Bird AP and Kouzarides T. (2003). The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation. *J Biol Chem,* **278,** 4035-4040.

Fuxa M, Skok J, Souabni A, Salvagiotto G, Roldan E and Busslinger M. (2004). Pax5 induces V-to-DJ rearrangements and locus contraction of the immunoglobulin heavy-chain gene. *Genes Dev,* **18,** 411-422.

Gallagher WM, Bergin OE, Rafferty M, Kelly ZD, Nolan IM, Fox EJ, *et al*. (2005). Multiple markers for melanoma progression regulated by DNA methylation: insights from transcriptomic studies. *Carcinogenesis,* **26,** 1856-1867.

Gama-Sosa MA, Slagel VA, Trewyn RW, Oxenhandler R, Kuo KC, Gehrke CW, *et al*. (1983). The 5-methylcytosine content of DNA from human tumors. *Nucleic Acids Res,* **11,** 6883-6894.

Garcia-Cao M, O'Sullivan R, Peters AH, Jenuwein T and Blasco MA. (2004). Epigenetic regulation of telomere length in mammalian cells by the Suv39h1 and Suv39h2 histone methyltransferases. *Nat Genet,* **36,** 94-99.

Garvie CW, Hagman J and Wolberger C. (2001). Structural studies of Ets-1/Pax5 complex formation on DNA. *Mol Cell,* **8,** 1267-1276.

Gellert K, Braun KL, Morris R and Starkey V. (2006). The 'Ohana Day Project: a community approach to increasing cancer screening. *Prev Chronic Dis,* **3,** A99.

Geserick C and Blasco MA. (2006). Novel roles for telomerase in aging. *Mechanisms of Ageing and Development,* **127,** 579-583.

Gewin L, Myers H, Kiyono T and Galloway DA. (2004). Identification of a novel telomerase repressor that interacts with the human papillomavirus type-16 E6/E6-AP complex. *Genes Dev,* **18,** 2269-2282.

Ghosh U, Das N and Bhattacharyya NP. (2007). Inhibition of telomerase activity by reduction of poly(ADP-ribosyl)ation of TERT and TEP1/TP1 expression in HeLa cells with knocked down poly(ADP-ribose) polymerase-1 (PARP-1) gene. *Mutat Res,* **615,** 66-74.

Gire W, Roux P, Wynford-Thomas D, Brondello JM and Dulic V. (2004). DNA damage checkpoint kinase Chk2 triggers replicative senescence. *Embo Journal,* **23,** 2554-2563.

Glasspool RM, Teodoridis JM and Brown R. (2006). Epigenetics as a mechanism driving polygenic clinical drug resistance. *Br J Cancer,* **94,** 1087-1092.

Gonda H, Sugai M, Nambu Y, Katakai T, Agata Y, Mori KJ, *et al*. (2003). The balance between Pax5 and Id2 activities is the key to AID gene expression. *J Exp Med,* **198,** 1427-1437.

Gonzalgo ML and Jones PA. (1997). Rapid quantitation of methylation differences at specific sites using methylation-sensitive single nucleotide primer extension (Ms-SNuPE). *Nucleic Acids Res,* **25,** 2529-2531.

Goueli BS and Janknecht R. (2003). Regulation of telomerase reverse transcriptase gene activity by upstream stimulatory factor. *Oncogene,* **22,** 8042-8047.

Goueli BS and Janknecht R. (2004). Upregulation of the Catalytic Telomerase Subunit by the Transcription Factor ER81 and Oncogenic HER2/Neu, Ras, or Raf. *Mol Cell Biol,* **24,** 25-35.

Graziano F, Arduini F, Ruzzo A, Bearzi I, Humar B, More H, *et al*. (2004). Prognostic analysis of E-cadherin gene promoter hypermethylation in patients with surgically resected, node-positive, diffuse gastric cancer. *Clin Cancer Res,* **10,** 2784-2789.

Greider CW. (1996). Telomere length regulation. *Annu Rev Biochem,* **65,** 337-365.

Greider CW and Blackburn EH. (1989). A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis. *Nature,* **337,** 331-337.

Griffith JD, Comeau L, Rosenfield S, Stansel RM, Bianchi A, Moss H, *et al*. (1999). Mammalian telomeres end in a large duplex loop. *Cell,* **97,** 503-514.

Guilleret I and Benhattar J. (2004). Unusual distribution of DNA methylation within the hTERT CpG island in tissues and cell lines. *Biochem Biophys Res Commun,* **325,** 1037-1043.

Guilleret I and Benhattar J. (2003). Demethylation of the human telomerase catalytic subunit (hTERT) gene promoter reduced hTERT expression and telomerase activity and shortened telomeres. *Exp Cell Res,* **289,** 326-334.

Guilleret I, Yan P, Grange F, Braunschweig R, Bosman FT and Benhattar J. (2002a). Hypermethylation of the human telomerase catalytic subunit (hTERT) gene correlates with telomerase activity. *Int J Cancer,* **101,** 335-341.

Guilleret I, Yan P, Guillou L, Braunschweig R, Coindre JM and Benhattar J. (2002b). The human telomerase RNA gene (hTERC) is regulated during carcinogenesis but is not dependent on DNA methylation. *Carcinogenesis,* **23,** 2025-2030.

Gunes C, Lichtsteiner S, Vasserot AP and Englert C. (2000). Expression of the hTERT gene is regulated at the level of transcriptional initiation and repressed by Mad1. *Cancer Res,* **60,** 2116-2121.

Guo X, Deng Y, Lin Y, Cosme-Blanco W, Chan S, He H, *et al*. (2007). Dysfunctional telomeres activate an ATM-ATR-dependent DNA damage response to suppress tumorigenesis. *Embo Journal,* **26,** 4709-4719.

Gupta A, Godwin AK, Vanderveer L, Lu A and Liu J. (2003). Hypomethylation of the synuclein gamma gene CpG island promotes its aberrant expression in breast carcinoma and ovarian carcinoma. *Cancer Res,* **63,** 664-673.

Guy J, Hendrich B, Holmes M, Martin JE and Bird A. (2001). A mouse Mecp2-null mutation causes neurological symptoms that mimic Rett syndrome. *Nat Genet,* **27,** 322-326.

Hahn WC, Stewart SA, Brooks MW, York SG, Eaton E, Kurachi A, *et al*. (1999). Inhibition of telomerase limits the growth of human cancer cells. *Nat Med,* **5,** 1164-1170.

Hakin-Smith V, Jellinek DA, Levy D, Carroll T, Teo M, Timperley WR, *et al*. (2003). Alternative lengthening of telomeres and survival in patients with glioblastoma multiforme. *Lancet,* **361,** 836-838.

Hande MP, Samper E, Lansdorp P and Blasco MA. (1999). Telomere length dynamics and chromosomal instability in cells derived from telomerase null mice. *J Cell Biol,* **144,** 589-601.

Hansen RS, Wijmenga C, Luo P, Stanek AM, Canfield TK, Weemaes CM, *et al*. (1999). The DNMT3B DNA methyltransferase gene is mutated in the ICF immunodeficiency syndrome. *Proc Natl Acad Sci U S A,* **96,** 14412-14417.

Harley CB. (1997). Human ageing and telomeres. *Ciba Found Symp,* **211,** 129-139.

Harley CB. (2008). Telomerase and cancer therapeutics. *Nat Rev Cancer,* **8,** 167-179.

Harley CB, Futcher AB and Greider CW. (1990). Telomeres shorten during ageing of human fibroblasts. *Nature,* **345,** 458-460.

Harrington L, McPhail T, Mar V, Zhou W, Oulton R, Bass MB, *et al*. (1997a). A mammalian telomerase-associated protein. *Science,* **275,** 973-977.

Harrington L, Zhou W, McPhail T, Oulton R, Yeung DS, Mar V, *et al*. (1997b). Human telomerase contains evolutionarily conserved catalytic and structural subunits. *Genes Dev,* **11,** 3109-3115.

Hashimshony T, Zhang J, Keshet I, Bustin M and Cedar H. (2003). The role of DNA methylation in setting up chromatin structure during development. *Nat Genet,* **34,** 187-192.

Hegi ME, Diserens AC, Gorlia T, Hamou MF, de TN, Weller M, *et al*. (2005). MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med,* **352,** 997-1003.

Heine B, Hummel M, Demel G and Stein H. (1998). Demonstration of constant upregulation of the telomerase RNA component in human gastric carcinomas using in situ hybridization. *J Pathol,* **185,** 139-144.

Hemann MT, Strong MA, Hao LY and Greider CW. (2001). The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability. *Cell,* **107,** 67-77.

Hendrich B and Bird A. (1998). Identification and characterization of a family of mammalian methyl-CpG binding proteins. *Mol Cell Biol,* **18,** 6538-6547.

Hendrich B, Guy J, Ramsahoye B, Wilson VA and Bird A. (2001). Closely related proteins MBD2 and MBD3 play distinctive but interacting roles in mouse development. *Genes Dev,* **15,** 710-723.

Hendrich B, Hardeland U, Ng HH, Jiricny J and Bird A. (1999). The thymine glycosylase MBD4 can bind to the product of deamination at methylated CpG sites. *Nature,* **401,** 301-304.

Hendrich B and Tweedie S. (2003). The methyl-CpG binding domain and the evolving role of DNA methylation in animals. *Trends Genet,* **19,** 269-277.

Henson JD, Hannay JA, McCarthy SW, Royds JA, Yeager TR, Robinson RA, *et al*. (2005). A robust assay for alternative lengthening of telomeres in tumors shows the significance of alternative lengthening of telomeres in sarcomas and astrocytomas. *Clinical Cancer Research,* **11,** 217-225.

Henson JD, Neumann AA, Yeager TR and Reddel RR. (2002). Alternative lengthening of telomeres in mammalian cells. *Oncogene,* **21,** 598-610.

Herbert B, Pitts AE, Baker SI, Hamilton SE, Wright WE, Shay JW, *et al*. (1999). Inhibition of human telomerase in immortal human cells leads to progressive telomere shortening and cell death. *Proc Natl Acad Sci U S A,* **96,** 14276-14281.

Herbert BS, Gellert GC, Hochreiter A, Pongracz K, Wright WE, Zielinska D, *et al*. (2005). Lipid modification of GRN163, an N3'-->P5' thio-phosphoramidate oligonucleotide, enhances the potency of telomerase inhibition. *Oncogene,* **24,** 5262-5268.

Herman JG, Graff JR, Myohanen S, Nelkin BD and Baylin SB. (1996). Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci U S A,* **93,** 9821-9826.

Herman JG, Merlo A, Mao L, Lapidus RG, Issa JP, Davidson NE, *et al*. (1995). Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. *Cancer Res,* **55,** 4525-4530.

Herman JG, Umar A, Polyak K, Graff JR, Ahuja N, Issa JP, *et al*. (1998). Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. *Proc Natl Acad Sci U S A,* **95,** 6870-6875.

Heyzer-Williams LJ and Heyzer-Williams MG. (2005). Antigen-specific memory B cell development. *Annu Rev Immunol,* **23,** 487-513.

Hisatomi H, Ohyashiki K, Ohyashiki JH, Nagao K, Kanamaru T, Hirata H, *et al*. (2003). Expression profile of a gamma-deletion variant of the human telomerase reverse transcriptase gene. *Neoplasia,* **5,** 193-197.

Hiyama E, Gollahon L, Kataoka T, Kuroi K, Yokoyama T, Gazdar AF, *et al*. (1996a). Telomerase activity in human breast tumors. *J Natl Cancer Inst,* **88,** 116-122.

Hiyama E, Hiyama K, Tatsumoto N, Kodama T, Shay JW and Yokoyama T. (1996b). Telomerase activity in human intestine. *Int J Oncol,* 453-458.

Hiyama E, Hiyama K, Yokoyama T, Matsuura Y, Piatyszek MA and Shay JW. (1995a). Correlating telomerase activity levels with human neuroblastoma outcomes. *Nat Med,* **1,** 249-255.

Hiyama E, Hiyama K, Yokoyama T and Shay JW. (2001). Immunohistochemical detection of telomerase (hTERT) protein in human cancer tissues and a subset of cells in normal tissues. *Neoplasia,* **3,** 17-26.

Hiyama E, Saeki T, Hiyama K, Takashima S, Shay JW, Matsuura Y, *et al*. (2000). Telomerase activity as a marker of breast carcinoma in fine-needle aspirated samples. *Cancer,* **90,** 235-238.

Hiyama E, Yokoyama T, Tatsumoto N, Hiyama K, Imamura Y, Murakami Y, *et al*. (1995b). Telomerase activity in gastric cancer. *Cancer Res,* **55,** 3258-3262.

Hiyama K, Hirai Y, Kyoizumi S, Akiyama M, Hiyama E, Piatyszek MA, *et al*. (1995c). Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. *J Immunol,* **155,** 3711-3715.

Hiyama K, Ishioka S, Shay JW, Taooka Y, Maeda A, Isobe T, *et al*. (1998). Telomerase activity as a novel marker of lung cancer and immune-associated lung diseases. *Int J Mol Med,* **1,** 545-549.

Hoare SF, Bryce LA, Wisman GB, Burns S, Going JJ, van der Zee AG, *et al*. (2001). Lack of telomerase RNA gene hTERC expression in alternative lengthening of telomeres cells is associated with methylation of the hTERC promoter. *Cancer Res,* **61,** 27-32.

Hoareau-Aveilla C, Bonoli M, Caizergues-Ferrer M and Henry Y. (2006). hNaf1 is required for accumulation of human box H/ACA snoRNPs, scaRNPs, and telomerase. *Rna-A Publication of the Rna Society,* **12,** 832-840.

Holmes ML, Carotta S, Corcoran LM and Nutt SL. (2006). Repression of Flt3 by Pax5 is crucial for B-cell lineage commitment. *Genes Dev,* **20,** 933-938.

Holmes ML, Pridans C and Nutt SL. (2008). The regulation of the B-cell gene expression programme by Pax5. *Immunol Cell Biol,* **86,** 47-53.

Holt SE, Aisner DL, Baur J, Tesmer VM, Dy M, Ouellette M, *et al*. (1999). Functional requirement of p23 and Hsp90 in telomerase complexes. *Genes Dev,* **13,** 817-826.

Holt SE, Aisner DL, Shay JW and Wright WE. (1997). Lack of cell cycle regulation of telomerase activity in human cells. *Proc Natl Acad Sci U S A,* **94,** 10687-10692.

Horcher M, Souabni A and Busslinger M. (2001). Pax5/BSAP maintains the identity of B cells in late B lymphopoiesis. *Immunity,* **14,** 779-790.

Horikawa I, Cable PL, Afshari C and Barrett JC. (1999). Cloning and characterization of the promoter region of human telomerase reverse transcriptase gene. *Cancer Res,* **59,** 826-830.

Hou M, Wang X, Popov N, Zhang A, Zhao X, Zhou R, *et al*. (2002). The histone deacetylase inhibitor trichostatin A derepresses the telomerase reverse transcriptase (hTERT) gene in human cells. *Exp Cell Res,* **274,** 25-34.

Houghtaling BR, Cuttonaro L, Chang W and Smith S. (2004). A dynamic molecular link between the telomere length regulator TRF1 and the chromosome end protector TRF2. *Curr Biol,* **14,** 1621-1631.

Hsu HL, Gilley D, Galande SA, Hande MP, Allen B, Kim SH, *et al*. (2000). Ku acts in a unique way at the mammalian telomere to prevent end joining. *Genes & Development,* **14,** 2807-2812.

Hu B, Tack DC, Liu T, Wu Z, Ullenbruch MR and Phan SH. (2006). Role of Smad3 in the regulation of rat telomerase reverse transcriptase by TGFbeta. *Oncogene,* **25,** 1030-1041.

Hu BT and Insel RA. (1999). Up-regulation of telomerase in human B lymphocytes occurs independently of cellular proliferation and with expression of the telomerase catalytic subunit. *Eur J Immunol,* **29,** 3745-3753.

Hu BT, Lee SC, Marin E, Ryan DH and Insel RA. (1997). Telomerase is up-regulated in human germinal center B cells in vivo and can be re-expressed in memory B cells activated in vitro. *J Immunol,* **159,** 1068-1071.

Huang TH, Perry MR and Laux DE. (1999). Methylation profiling of CpG islands in human breast cancer cells. *Hum Mol Genet,* **8,** 459-470.

Hutchins AS, Mullen AC, Lee HW, Sykes KJ, High FA, Hendrich BD, *et al*. (2002). Gene silencing quantitatively controls the function of a developmental trans-activator. *Mol Cell,* **10,** 81-91.

Igarashi H and Sakaguchi N. (1997). Telomerase activity is induced in human peripheral B lymphocytes by the stimulation to antigen receptor. *Blood,* **89,** 1299-1307.

Illingworth R, Kerr A, Desousa D, Jorgensen H, Ellis P, Stalker J, *et al*. (2008). A novel CpG island set identifies tissue-specific methylation at developmental gene loci. *PLoS Biol,* **6,** e22.

Irizarry RA, Ladd-Acosta C, Carvalho B, Wu H, Brandenburg SA, Jeddeloh JA, *et al*. (2008). Comprehensive high-throughput arrays for relative methylation (CHARM). *Genome Res,* **18,** 780-790.

Irvine RA, Lin IG and Hsieh CL. (2002). DNA methylation has a local effect on transcription and histone acetylation. *Mol Cell Biol,* **22,** 6689-6696.

Isenmann S, Cakouros D, Zannettino A, Shi S and Gronthos S. (2007). hTERT transcription is repressed by Cbfa1 in human mesenchymal stem cell populations. *J Bone Miner Res,* **22,** 897-906.

Ishiai M, Kurosaki M, Pappu R, Okawa K, Ronko I, Fu C, *et al*. (1999). BLNK required for coupling Syk to PLC gamma 2 and Rac1-JNK in B cells. *Immunity,* **10,** 117-125.

Issa JP. (2004). CpG island methylator phenotype in cancer. *Nat Rev Cancer,* **4,** 988-993.

Iwano T, Tachibana M, Reth M and Shinkai Y. (2004). Importance of TRF1 for functional telomere structure. *J Biol Chem,* **279,** 1442-1448.

Iwasaki H and Akashi K. (2007). Myeloid lineage commitment from the hematopoietic stem cell. *Immunity,* **26,** 726-740.

Jackson K, Yu MC, Arakawa K, Fiala E, Youn B, Fiegl H, *et al*. (2004). DNA hypomethylation is prevalent even in low-grade breast cancers. *Cancer Biol Ther,* **3,** 1225-1231.

Jady BE, Bertrand E and Kiss T. (2004). Human telomerase RNA and box H/ACA scaRNAs share a common Cajal body-specific localization signal. *J Cell Biol,* **164,** 647-652.

Jady BE, Richard P, Bertrand E and Kiss T. (2006). Cell cycle-dependent recruitment of telomerase RNA and Cajal bodies to human telomeres. *Mol Biol Cell,* **17,** 944-954.

Jaenisch R and Bird A. (2003). Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet,* **33 Suppl,** 245-254.

Jagadeesh S and Banerjee PP. (2006). Inositol hexaphosphate represses telomerase activity and translocates TERT from the nucleus in mouse and human prostate cancer cells via the deactivation of Akt and PKCalpha. *Biochem Biophys Res Commun,* **349,** 1361-1367.

Jang JS, Lee SJ, Choi JE, Cha SI, Lee EB, Park TI, *et al*. (2005). Methyl-CpG binding domain 1 gene polymorphisms and risk of primary lung cancer. *Cancer Epidemiol Biomarkers Prev,* **14,** 2474-2480.

Jarboe EA, Liaw KL, Thompson LC, Heinz DE, Baker PL, McGregor JA, *et al*. (2002). Analysis of telomerase as a diagnostic biomarker of cervical dysplasia and carcinoma. *Oncogene,* **21,** 664-673.

Jensen KC, Higgins JP, Montgomery K, Kaygusuz G, van de RM and Natkunam Y. (2007). The utility of PAX5 immunohistochemistry in the diagnosis of undifferentiated malignant neoplasms. *Mod Pathol,* **20,** 871-877.

Jia D, Jurkowska RZ, Zhang X, Jeltsch A and Cheng X. (2007). Structure of Dnmt3a bound to Dnmt3L suggests a model for de novo DNA methylation. *Nature,* **449,** 248-251.

Jiang WQ, Zhong ZH, Henson JD and Reddel RR. (2007). Identification of candidate alternative lengthening of telomeres genes by methionine restriction and RNA interference. *Oncogene,* **26,** 4635-4647.

Johnson-Pais T, Degnin C and Thayer MJ. (2001). pRB induces Sp1 activity by relieving inhibition mediated by MDM2. *Proc Natl Acad Sci U S A,* **98,** 2211-2216.

Jones PA. (2002). DNA methylation and cancer. *Oncogene,* **21,** 5358-5360.

Jones PA. (1999). The DNA methylation paradox. *Trends Genet,* **15,** 34-37.

Jones PA and Laird PW. (1999). Cancer epigenetics comes of age. *Nat Genet,* **21,** 163-167.

Jones PL, Veenstra GJ, Wade PA, Vermaak D, Kass SU, Landsberger N, *et al*. (1998). Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nat Genet,* **19,** 187-191.

Jorgensen HF, Ben-Porath I and Bird AP. (2004). Mbd1 is recruited to both methylated and nonmethylated CpGs via distinct DNA binding domains. *Mol Cell Biol,* **24,** 3387-3395.

Judson H, Hayward BE, Sheridan E and Bonthron DT. (2002). A global disorder of imprinting in the human female germ line. *Nature,* **416,** 539-542.

Kafri T, Ariel M, Brandeis M, Shemer R, Urven L, McCarrey J, *et al*. (1992). Developmental pattern of gene-specific DNA methylation in the mouse embryo and germ line. *Genes Dev,* **6,** 705-714.

Kaludov NK and Wolffe AP. (2000). MeCP2 driven transcriptional repression in vitro: selectivity for methylated DNA, action at a distance and contacts with the basal transcription machinery. *Nucleic Acids Res,* **28,** 1921-1928.

Kaminker PG, Kim SH, Taylor RD, Zebarjadian Y, Funk WD, Morin GB, *et al*. (2001). TANK2, a new TRF1-associated poly(ADP-ribose) polymerase, causes rapid induction of cell death upon overexpression. *J Biol Chem,* **276,** 35891-35899.

Kanaya T, Kyo S, Hamada K, Takakura M, Kitagawa Y, Harada H, *et al*. (2000). Adenoviral expression of p53 represses telomerase activity through down-regulation of human telomerase reverse transcriptase transcription. *Clin Cancer Res,* **6,** 1239-1247.

Kanduri C, Pant V, Loukinov D, Pugacheva E, Qi CF, Wolffe A, *et al*. (2000). Functional association of CTCF with the insulator upstream of the H19 gene is parent of origin-specific and methylation-sensitive. *Curr Biol,* **10,** 853-856.

Kane MF, Loda M, Gaida GM, Lipman J, Mishra R, Goldman H, *et al*. (1997). Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. *Cancer Res,* **57,** 808-811.

Kannan K, Nelson AD and Shippen DE. (2008). Dyskerin is a component of the Arabidopsis telomerase RNP required for telomere maintenance. *Mol Cell Biol,* **28,** 2332-2341.

Karlseder J, Hoke K, Mirzoeva OK, Bakkenist C, Kastan MB, Petrini JH, *et al*. (2004). The telomeric protein TRF2 binds the ATM kinase and can inhibit the ATM-dependent DNA damage response. *PLoS Biol,* **2,** E240.

Kass SU, Pruss D and Wolffe AP. (1997). How does DNA methylation repress transcription? *Trends Genet,* **13,** 444-449.

Katzenellenbogen RA, Egelkrout EM, Vliet-Gregg P, Gewin LC, Gafken PR and Galloway DA. (2007). NFX1-123 and poly(A) binding proteins synergistically augment activation of telomerase in human papillomavirus type 16 E6-expressing cells. *J Virol,* **81,** 3786-3796.

Kawasaki H and Taira K. (2004). Induction of DNA methylation and gene silencing by short interfering RNAs in human cells. *Nature,* **431,** 211-217.

Kedde M, Sage CI, Duursma A, Zlotorynski E, van Leeuwen B, Nijkamp W, *et al*. (2006). Telomerase-independent regulation of ATR by human telomerase RNA. *Journal of Biological Chemistry,* **281,** 40503-40514.

Keen JC, Yan L, Mack KM, Pettit C, Smith D, Sharma D, *et al*. (2003). A novel histone deacetylase inhibitor, scriptaid, enhances expression of functional estrogen receptor alpha (ER) in ER negative human breast cancer cells in combination with 5-aza 2'-deoxycytidine. *Breast Cancer Res Treat,* **81,** 177-186.

Keith WN, Bilsland A, Hardie M and Evans TR. (2004). Drug insight: Cancer cell immortality-telomerase as a target for novel cancer gene therapies. *Nat Clin Pract Oncol,* **1,** 88-96.

Keith WN, Thomson CM, Howcroft J, Maitland NJ and Shay JW. (2007). Seeding drug discovery: integrating telomerase cancer biology and cellular senescence to uncover new therapeutic opportunities in targeting cancer stem cells. *Drug Discov Today,* **12,** 611-621.

Kelleher C, Kurth I and Lingner J. (2005). Human protection of telomeres 1 (POT1) is a negative regulator of telomerase activity in vitro. *Mol Cell Biol,* **25,** 808-818.

Kelsoe G. (1996). The germinal center: a crucible for lymphocyte selection. *Semin Immunol,* **8,** 179-184.

Keppler BR, Grady AT and Jarstfer MB. (2006). The biochemical role of the heat shock protein 90 chaperone complex in establishing human telomerase activity. *J Biol Chem,* **281,** 19840-19848.

Keshet I, Schlesinger Y, Farkash S, Rand E, Hecht M, Segal E, *et al*. (2006). Evidence for an instructive mechanism of de novo methylation in cancer cells. *Nat Genet,* **38,** 149-153.

Kharbanda S, Kumar V, Dhar S, Pandey P, Chen C, Majumder P, *et al*. (2000). Regulation of the hTERT telomerase catalytic subunit by the c-Abl tyrosine kinase. *Curr Biol,* **10,** 568-575.

Khulan B, Thompson RF, Ye K, Fazzari MJ, Suzuki M, Stasiek E, *et al*. (2006). Comparative isoschizomer profiling of cytosine methylation: the HELP assay. *Genome Res,* **16,** 1046-1055.

Kilian A, Bowtell DD, Abud HE, Hime GR, Venter DJ, Keese PK, *et al*. (1997). Isolation of a candidate human telomerase catalytic subunit gene, which reveals complex splicing patterns in different cell types. *Hum Mol Genet,* **6,** 2011-2019.

Kim Sh SH, Kaminker P and Campisi J. (2002). Telomeres, aging and cancer: in search of a happy ending. *Oncogene,* **21,** 503-511.

Kim H, Kim YH, Kim SE, Kim NG, Noh SH and Kim H. (2003). Concerted promoter hypermethylation of hMLH1, p16INK4A, and E-cadherin in gastric carcinomas with microsatellite instability. *J Pathol,* **200,** 23-31.

Kim JH, Park SM, Kang MR, Oh SY, Lee TH, Muller MT, *et al*. (2005). Ubiquitin ligase MKRN1 modulates telomere length homeostasis through a proteolysis of hTERT. *Genes Dev,* **19,** 776-781.

Kim NW. (1997). Clinical implications of telomerase in cancer. *Eur J Cancer,* **33,** 781-786.

Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, *et al*. (1994a). Specific association of human telomerase activity with immortal cells and cancer. *Science,* **266,** 2011-2015.

Kim S, Beausejour C, Davalos AR, Kaminker P, Heo SJ and Campisi J. (2004). TIN2 mediates functions of TRF2 at human telomeres. *Journal of Biological Chemistry,* **279,** 43799-43804.

Kim SH, Kaminker P and Campisi J. (1999). TIN2, a new regulator of telomere length in human cells. *Nat Genet,* **23,** 405-412.

Kim YI, Giuliano A, Hatch KD, Schneider A, Nour MA, Dallal GE, *et al*. (1994b). Global DNA hypomethylation increases progressively in cervical dysplasia and carcinoma. *Cancer,* **74,** 893-899.

Kirk KE, Harmon BP, Reichardt IK, Sedat JW and Blackburn EH. (1997). Block in anaphase chromosome separation caused by a telomerase template mutation. *Science,* **275,** 1478-1481.

Kishi H, Jin ZX, Wei XC, Nagata T, Matsuda T, Saito S, *et al*. (2002). Cooperative binding of c-Myb and Pax-5 activates the RAG-2 promoter in immature B cells. *Blood,* **99,** 576-583.

Klapper W, Parwaresch R and Krupp G. (2001). Telomere biology in human aging and aging syndromes. *Mech Ageing Dev,* **122,** 695-712.

Knudson CM, Johnson GM, Lin Y and Korsmeyer SJ. (2001). Bax accelerates tumorigenesis in p53-deficient mice. *Cancer Res,* **61,** 659-665.

Koering CE, Pollice A, Zibella MP, Bauwens S, Puisieux A, Brunori M, *et al*. (2002). Human telomeric position effect is determined by chromosomal context and telomeric chromatin integrity. *EMBO Rep,* **3,** 1055-1061.

Kondo E, Gu Z, Horii A and Fukushige S. (2005). The thymine DNA glycosylase MBD4 represses transcription and is associated with methylated p16(INK4a) and hMLH1 genes. *Mol Cell Biol,* **25,** 4388-4396.

Kondo M, Weissman IL and Akashi K. (1997). Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell,* **91,** 661-672.

Kouzarides T. (2007). Chromatin modifications and their function. *Cell,* **128,** 693-705.

Kozmik Z, Sure U, Ruedi D, Busslinger M and Aguzzi A. (1995). Deregulated expression of PAX5 in medulloblastoma. *Proc Natl Acad Sci U S A,* **92,** 5709-5713.

Kozmik Z, Wang S, Dorfler P, Adams B and Busslinger M. (1992). The promoter of the CD19 gene is a target for the B-cell-specific transcription factor BSAP. *Mol Cell Biol,* **12,** 2662-2672.

Kraemer K, Fuessel S, Schmidt U, Kotzsch M, Schwenzer B, Wirth MP, *et al*. (2003). Antisense-mediated hTERT inhibition specifically reduces the growth of human bladder cancer cells. *Clin Cancer Res,* **9,** 3794-3800.

Krajewski WA. (2002). Histone acetylation status and DNA sequence modulate ATP-dependent nucleosome repositioning. *J Biol Chem,* **277,** 14509-14513.

Krenacs L, Himmelmann AW, Quintanilla-Martinez L, Fest T, Riva A, Wellmann A, *et al*. (1998). Transcription factor B-cell-specific activator protein (BSAP) is differentially expressed in B cells and in subsets of B-cell lymphomas. *Blood,* **92,** 1308-1316.

Kress C, Thomassin H and Grange T. (2006). Active cytosine demethylation triggered by a nuclear receptor involves DNA strand breaks. *Proc Natl Acad Sci U S A,* **103,** 11112-11117.

Kristensen LS, Mikeska T, Krypuy M and Dobrovic A. (2008). Sensitive Melting Analysis after Real Time-Methylation Specific PCR (SMART-MSP): high-throughput and probe-free quantitative DNA methylation detection. *Nucleic Acids Research,* **36**.

Kubuki Y, Suzuki M, Sasaki H, Toyama T, Yamashita K, Maeda K, *et al*. (2005). Telomerase activity and telomere length as prognostic factors of adult T-cell leukemia. *Leuk Lymphoma,* **46,** 393-399.

Kumaki F, Kawai T, Hiroi S, Shinomiya N, Ozeki Y, Ferrans VJ, *et al*. (2001). Telomerase activity and expression of human telomerase RNA component and human telomerase reverse transcriptase in lung carcinomas. *Hum Pathol,* **32,** 188-195.

Kumaki F, Takeda K, Yu ZX, Moss J and Ferrans VJ. (2002). Expression of human telomerase reverse transcriptase in lymphangioleiomyomatosis. *Am J Respir Crit Care Med,* **166,** 187-191.

Kumakura S, Tsutsui TW, Yagisawa J, Barrett JC and Tsutsui T. (2005). Reversible conversion of immortal human cells from telomerase-positive to telomerase-negative cells. *Cancer Res,* **65,** 2778-2786.

Kyo S, Takakura M, Fujiwara T and Inoue M. (2008). Understanding and exploiting hTERT promoter regulation for diagnosis and treatment of human cancers. *Cancer Sci,* **99,** 1528-1538.

Kyo S, Takakura M, Kanaya T, Zhuo W, Fujimoto K, Nishio Y, *et al*. (1999). Estrogen activates telomerase. *Cancer Res,* **59,** 5917-5921.

Kyo S, Takakura M, Taira T, Kanaya T, Itoh H, Yutsudo M, *et al*. (2000). Sp1 cooperates with c-Myc to activate transcription of the human telomerase reverse transcriptase gene (hTERT). *Nucleic Acids Res,* **28,** 669-677.

LaBranche H, Dupuis S, Ben-David Y, Bani MR, Wellinger RJ and Chabot B. (1998). Telomere elongation by hnRNP A1 and a derivative that interacts with telomeric repeats and telomerase. *Nat Genet,* **19,** 199-202.

Lacerte A, Korah J, Roy M, Yang XJ, Lemay S and Lebrun JJ. (2008). Transforming growth factor-beta inhibits telomerase through SMAD3 and E2F transcription factors. *Cell Signal,* **20,** 50-59.

Lachner M and Jenuwein T. (2002). The many faces of histone lysine methylation. *Curr Opin Cell Biol,* **14,** 286-298.

Ladetto M, Compagno M, Ricca I, Pagano M, Rocci A, Astolfi M, *et al*. (2004). Telomere length correlates with histopathogenesis according to the germinal center in mature B-cell lymphoproliferative disorders. *Blood,* **103,** 4644-4649.

Lai CK, Mitchell JR and Collins K. (2001). RNA binding domain of telomerase reverse transcriptase. *Mol Cell Biol,* **21,** 990-1000.

Laird PW. (2003). The power and the promise of DNA methylation markers. *Nat Rev Cancer,* **3,** 253-266.

Laird PW. (2005). Cancer epigenetics. *Hum Mol Genet,* **14 Spec No 1,** R65-R76.

Le S, Sternglanz R and Greider CW. (2000). Identification of two RNA-binding proteins associated with human telomerase RNA. *Mol Biol Cell,* **11,** 999-1010.

Lee HW, Blasco MA, Gottlieb GJ, Horner JW, Greider CW and DePinho RA. (1998). Essential role of mouse telomerase in highly proliferative organs. *Nature,* **392,** 569-574.

Lei M, Zaug AJ, Podell ER and Cech TR. (2005). Switching human telomerase on and off with hPOT1 protein in vitro. *J Biol Chem,* **280,** 20449-20456.

Lewis JD, Meehan RR, Henzel WJ, Maurer-Fogy I, Jeppesen P, Klein F, *et al*. (1992). Purification, sequence, and cellular localization of a novel chromosomal protein that binds to methylated DNA. *Cell,* **69,** 905-914.

Li B and de Lange T. (2003). Rap1 affects the length and heterogeneity of human telomeres. *Mol Biol Cell,* **14,** 5060-5068.

Li B, Oestreich S and de Lange T. (2000). Identification of human Rap1: implications for telomere evolution. *Cell,* **101,** 471-483.

Li B, Zhou J, Liu P, Hu J, Jin H, Shimono Y, *et al*. (2007). Polycomb protein Cbx4 promotes SUMO modification of de novo DNA methyltransferase Dnmt3a. *Biochem J,* **405,** 369-378.

Li H, Cao Y, Berndt MC, Funder JW and Liu JP. (1999). Molecular interactions between telomerase and the tumor suppressor protein p53 in vitro. *Oncogene,* **18,** 6785-6794.

Li H, Xu D, Li J, Berndt MC and Liu JP. (2006a). Transforming growth factor beta suppresses human telomerase reverse transcriptase (hTERT) by Smad3 interactions with c-Myc and the hTERT gene. *J Biol Chem,* **281,** 25588-25600.

Li H, Xu D, Toh BH and Liu JP. (2006b). TGF-beta and cancer: is Smad3 a repressor of hTERT gene? *Cell Res,* **16,** 169-173.

Li H, Zhao L, Yang Z, Funder JW and Liu JP. (1998). Telomerase is controlled by protein kinase Calpha in human breast cancer cells. *J Biol Chem,* **273,** 33436-33442.

Liang XZ, Lucy AP, Ding SW and Wong SM. (2002). The p23 protein of hibiscus chlorotic ringspot virus is indispensable for host-specific replication. *J Virol,* **76,** 12312-12319.

Lida S, Rao PH, Nallasivam P, Hibshoosh H, Butler M, Louie DC, *et al*. (1996). The t(9;14)(p13;q32) chromosomal translocation associated with lymphoplasmacytoid lymphoma involves the PAX-5 gene. *Blood,* **88,** 4110-4117.

Lillard-Wetherell K, Machwe A, Langland GT, Combs KA, Behbehani GK, Schonberg SA, *et al*. (2004). Association and regulation of the BLM helicase by the telomere proteins TRF1 and TRF2. *Human Molecular Genetics,* **13,** 1919-1932.

Lin CH, Hsieh SY, Sheen IS, Lee WC, Chen TC, Shyu WC, *et al*. (2001). Genome-wide hypomethylation in hepatocellular carcinogenesis. *Cancer Res,* **61,** 4238-4243.

Lin H, Yamada Y, Nguyen S, Linhart H, Jackson-Grusby L, Meissner A, *et al*. (2006). Suppression of intestinal neoplasia by deletion of Dnmt3b. *Mol Cell Biol,* **26,** 2976-2983.

Lin J and Blackburn EH. (2004). Nucleolar protein PinX1p regulates telomerase by sequestering its protein catalytic subunit in an inactive complex lacking telomerase RNA. *Genes Dev,* **18,** 387-396.

Lin SY and Elledge SJ. (2003). Multiple tumor suppressor pathways negatively regulate telomerase. *Cell,* **113,** 881-889.

Lin X and Nelson WG. (2003). Methyl-CpG-binding domain protein-2 mediates transcriptional repression associated with hypermethylated GSTP1 CpG islands in MCF-7 breast cancer cells. *Cancer Res,* **63,** 498-504.

Linderson Y, Eberhard D, Malin S, Johansson A, Busslinger M and Pettersson S. (2004). Corecruitment of the Grg4 repressor by PU.1 is critical for Pax5-mediated repression of B-cell-specific genes. *EMBO Rep,* **5,** 291-296.

Ling Y, Sankpal UT, Robertson AK, McNally JG, Karpova T and Robertson KD. (2004). Modification of de novo DNA methyltransferase 3a (Dnmt3a) by SUMO-1 modulates its interaction with histone deacetylases (HDACs) and its capacity to repress transcription. *Nucleic Acids Res,* **32,** 598-610.

Lingner J, Hughes TR, Shevchenko A, Mann M, Lundblad V and Cech TR. (1997). Reverse transcriptase motifs in the catalytic subunit of telomerase. *Science,* **276,** 561-567.

Liou JM, Wu MS, Lin JT, Wang HP, Huang SP, Chiu HM, *et al*. (2007). Loss of imprinting of insulin-like growth factor II is associated with increased risk of proximal colon cancer. *Eur J Cancer,* **43,** 1276-1282.

Lippman Z, Gendrel AV, Colot V and Martienssen R. (2005). Profiling DNA methylation patterns using genomic tiling microarrays. *Nat Methods,* **2,** 219-224.

Liu C, Fang X, Ge Z, Jalink M, Kyo S, Bjorkholm M, *et al*. (2007). The telomerase reverse transcriptase (hTERT) gene is a direct target of the histone methyltransferase SMYD3. *Cancer Res,* **67,** 2626-2631.

Liu D, O'Connor MS, Qin J and Songyang Z. (2004). Telosome, a mammalian telomere-associated complex formed by multiple telomeric proteins. *J Biol Chem,* **279,** 51338-51342.

Liu X, Yuan H, Fu B, Disbrow GL, Apolinario T, Tomaic V, *et al*. (2005). The E6AP ubiquitin ligase is required for transactivation of the hTERT promoter by the human papillomavirus E6 oncoprotein. *J Biol Chem,* **280,** 10807-10816.

Liu Y, Snow BE, Hande MP, Yeung D, Erdmann NJ, Wakeham A, *et al*. (2000). The telomerase reverse transcriptase is limiting and necessary for telomerase function in vivo. *Curr Biol,* **10,** 1459-1462.

Loayza D and De Lange T. (2003). POT1 as a terminal transducer of TRF1 telomere length control. *Nature,* **423,** 1013-1018.

Loayza D, Parsons H, Donigian J, Hoke K and De Lange T. (2004). DNA binding features of human POT1: a nonamer 5'-TAGGGTTAG-3' minimal binding site, sequence specificity, and internal binding to multimeric sites. *J Biol Chem,* **279,** 13241-13248.

Lopatina NG, Poole JC, Saldanha SN, Hansen NJ, Key JS, Pita MA, *et al*. (2003). Control mechanisms in the regulation of telomerase reverse transcriptase expression in differentiating human teratocarcinoma cells. *Biochem Biophys Res Commun,* **306,** 650-659.

Lopez-Serra L, Ballestar E, Fraga MF, Alaminos M, Setien F and Esteller M. (2006). A profile of methyl-CpG binding domain protein occupancy of hypermethylated promoter CpG islands of tumor suppressor genes in human cancer. *Cancer Res,* **66,** 8342-8346.

Lopez-Serra L, Ballestar E, Ropero S, Setien F, Billard LM, Fraga MF, *et al*. (2008). Unmasking of epigenetically silenced candidate tumor suppressor genes by removal of methyl-CpG-binding domain proteins. *Oncogene,* **27,** 3556-3566.

Lopez-Serra L and Esteller M. (2008). Proteins that bind methylated DNA and human cancer: reading the wrong words. *Br J Cancer,* **98,** 1881-1885.

Ludwig A, Saretzki G, Holm PS, Tiemann F, Lorenz M, Emrich T, *et al*. (2001). Ribozyme cleavage of telomerase mRNA sensitizes breast epithelial cells to inhibitors of topoisomerase. *Cancer Res,* **61,** 3053-3061.

Lundblad V. (2000). DNA ends: maintenance of chromosome termini versus repair of double strand breaks. *Mutat Res,* **451,** 227-240.

Luscher B. (2001). Function and regulation of the transcription factors of the Myc/Max/Mad network. *Gene,* **277,** 1-14.

Lv J, Liu H, Wang Q, Tang Z, Hou L and Zhang B. (2003). Molecular cloning of a novel human gene encoding histone acetyltransferase-like protein involved in transcriptional activation of hTERT. *Biochem Biophys Res Commun,* **311,** 506-513.

Mack GS. (2006). Epigenetic cancer therapy makes headway. *J Natl Cancer Inst,* **98,** 1443-1444.

MacNamara B, Wang W, Chen Z, Hou M, Mazur J, Gruber A, *et al*. (2001). Telomerase activity in relation to pro- and anti-apoptotic protein expression in high grade non-Hodgkin's lymphomas. *Haematologica,* **86,** 386-393.

Maeda K, Kawakami K, Ishida Y, Ishiguro K, Omura K and Watanabe G. (2003). Hypermethylation of the CDKN2A gene in colorectal cancer is associated with shorter survival. *Oncol Rep,* **10,** 935-938.

Magdinier F and Wolffe AP. (2001). Selective association of the methyl-CpG binding protein MBD2 with the silent p14/p16 locus in human neoplasia. *Proc Natl Acad Sci U S A,* **98,** 4990-4995.

Maida Y, Kyo S, Kanaya T, Wang Z, Yatabe N, Tanaka M, *et al*. (2002). Direct activation of telomerase by EGF through Ets-mediated transactivation of TERT via MAP kinase signaling pathway. *Oncogene,* **21,** 4071-4079.

Maier H, Ostraat R, Parenti S, Fitzsimmons D, Abraham LJ, Garvie CW, *et al*. (2003). Requirements for selective recruitment of Ets proteins and activation of mb-1/Ig-alpha gene transcription by Pax-5 (BSAP). *Nucleic Acids Res,* **31,** 5483-5489.

Maitra S and Atchison M. (2000). BSAP can repress enhancer activity by targeting PU.1 function. *Mol Cell Biol,* **20,** 1911-1922.

Marchetti A, Bertacca G, Buttitta F, Chella A, Quattrocolo G, Angeletti CA, *et al*. (1999). Telomerase activity as a prognostic indicator in stage I non-small cell lung cancer. *Clin Cancer Res,* **5,** 2077-2081.

Marciniak RA, Cavazos D, Montellano R, Chen Q, Guarente L and Johnson FB. (2005). A novel telomere structure in a human alternative lengthening of telomeres cell line. *Cancer Res,* **65,** 2730-2737.

Martens UM, Brass V, Sedlacek L, Pantic M, Exner C, Guo Y, *et al*. (2002). Telomere maintenance in human B lymphocytes. *Br J Haematol,* **119,** 810-818.

Martin C and Zhang Y. (2005). The diverse functions of histone lysine methylation. *Nat Rev Mol Cell Biol,* **6,** 838-849.

Martinez-Agosto JA, Mikkola HK, Hartenstein V and Banerjee U. (2007). The hematopoietic stem cell and its niche: a comparative view. *Genes Dev,* **21,** 3044-3060.

Maruyama R, Toyooka S, Toyooka KO, Virmani AK, Zochbauer-Muller S, Farinas AJ, *et al*. (2002). Aberrant promoter methylation profile of prostate cancers and its relationship to clinicopathological features. *Clin Cancer Res,* **8,** 514-519.

Maser RS, Choudhury B, Campbell PJ, Feng B, Wong KK, Protopopov A, *et al*. (2007). Chromosomally unstable mouse tumours have genomic alterations similar to diverse human cancers. *Nature,* **447,** 966-971.

Mason PJ. (2003). Stem cells, telomerase and dyskeratosis congenita. *Bioessays,* **25,** 126-133.

Masutomi K, Possemato R, Wong JMY, Currier JL, Tothova Z, Manola JB, *et al*. (2005). The telomerase reverse transcriptase regulates chromatin state and DNA damage responses. *Proceedings of the National Academy of Sciences of the United States of America,* **102,** 8222-8227.

Matera AG, Terns RM and Terns MP. (2007). Non-coding RNAs: lessons from the small nuclear and small nucleolar RNAs. *Nature Reviews Molecular Cell Biology,* **8,** 209-220.

Matthews P, Jones CJ, Skinner J, Haughton M, de MC and Wynford-Thomas D. (2001). Telomerase activity and telomere length in thyroid neoplasia: biological and clinical implications. *J Pathol,* **194,** 183-193.

Mattick JS and Makunin IV. (2005). Small regulatory RNAs in mammals. *Hum Mol Genet,* **14 Spec No 1,** R121-R132.

Mauro LJ and Foster DN. (2002). Regulators of telomerase activity. *Am J Respir Cell Mol Biol,* **26,** 521-524.

Meehan RR, Lewis JD, McKay S, Kleiner EL and Bird AP. (1989). Identification of a mammalian protein that binds specifically to DNA containing methylated CpGs. *Cell,* **58,** 499-507.

Meeker AK and Argani P. (2004). Telomere shortening occurs early during breast tumorigenesis: A cause of chromosome destabilization underlying malignant transformation? *Journal of Mammary Gland Biology and Neoplasia,* **9,** 285-296.

Meffre E, Davis E, Schiff C, Cunningham-Rundles C, Ivashkiv LB, Staudt LM, *et al*. (2000). Circulating human B cells that express surrogate light chains and edited receptors. *Nat Immunol,* **1,** 207-213.

Merlo A, Herman JG, Mao L, Lee DJ, Gabrielson E, Burger PC, *et al*. (1995). 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. *Nat Med,* **1,** 686-692.

Meyerson M, Counter CM, Eaton EN, Ellisen LW, Steiner P, Caddle SD, *et al*. (1997). hEST2, the putative human telomerase catalytic subunit gene, is up- regulated in tumor cells and during immortalization. *Cell,* **90,** 785-795.

Mhawech-Fauceglia P, Saxena R, Zhang S, Terracciano L, Sauter G, Chadhuri A, *et al*. (2007). Pax-5 immunoexpression in various types of benign and malignant tumours: a high-throughput tissue microarray analysis. *J Clin Pathol,* **60,** 709-714.

Mikkola I, Heavey B, Horcher M and Busslinger M. (2002). Reversion of B cell commitment upon loss of Pax5 expression. *Science,* **297,** 110-113.

Minegishi Y, Rohrer J, Coustan-Smith E, Lederman HM, Pappu R, Campana D, *et al*. (1999). An essential role for BLNK in human B cell development. *Science,* **286,** 1954-1957.

Misiti S, Nanni S, Fontemaggi G, Cong YS, Wen J, Hirte HW, *et al*. (2000). Induction of hTERT expression and telomerase activity by estrogens in human ovary epithelium cells. *Mol Cell Biol,* **20,** 3764-3771.

Mitchell JR, Cheng J and Collins K. (1999a). A box H/ACA small nucleolar RNA-like domain at the human telomerase RNA 3' end. *Mol Cell Biol,* **19,** 567-576.

Mitchell JR and Collins K. (2000). Human telomerase activation requires two independent interactions between telomerase RNA and telomerase reverse transcriptase. *Mol Cell,* **6,** 361-371.

Mitchell JR, Wood E and Collins K. (1999b). A telomerase component is defective in the human disease dyskeratosis congenita. *Nature,* **402,** 551-555.

Montgomery E, Argani P, Hicks JL, DeMarzo AM and Meeker AK. (2004). Telomere lengths of translocation-associated and nontranslocation-associated sarcomas differ dramatically. *Am J Pathol,* **164,** 1523-1529.

Moriarty TJ, Marie-Egyptienne DT and Autexier C. (2004). Functional organization of repeat addition processivity and DNA synthesis determinants in the human telomerase multimer. *Mol Cell Biol,* **24,** 3720-3733.

Morris KV, Chan SW, Jacobsen SE and Looney DJ. (2004). Small interfering RNA-induced transcriptional gene silencing in human cells. *Science,* **305,** 1289-1292.

Morrison AM, Jager U, Chott A, Schebesta M, Haas OA and Busslinger M. (1998). Deregulated PAX-5 transcription from a translocated IgH promoter in marginal zone lymphoma. *Blood,* **92,** 3865-3878.

Muller HM, Fiegl H, Goebel G, Hubalek MM, Widschwendter A, Muller-Holzner E, *et al*. (2003). MeCP2 and MBD2 expression in human neoplastic and non-neoplastic breast tissue and its association with oestrogen receptor status. *Br J Cancer,* **89,** 1934-1939.

Munoz-Jordan JL and Cross GA. (2001). Telomere shortening and cell cycle arrest in Trypanosoma brucei expressing human telomeric repeat factor TRF1. *Mol Biochem Parasitol,* **114,** 169-181.

Murnane JP, Sabatier L, Marder BA and Morgan WF. (1994). Telomere dynamics in an immortal human cell line. *EMBO J,* **13,** 4953-4962.

Nakamura M, Saito H, Ebinuma H, Wakabayashi K, Saito Y, Takagi T, *et al*. (2001a). Reduction of telomerase activity in human liver cancer cells by a histone deacetylase inhibitor. *J Cell Physiol,* **187,** 392-401.

Nakamura M, Watanabe T, Yonekawa Y, Kleihues P and Ohgaki H. (2001b). Promoter methylation of the DNA repair gene MGMT in astrocytomas is frequently associated with G:C --> A:T mutations of the TP53 tumor suppressor gene. *Carcinogenesis,* **22,** 1715-1719.

Nakamura TM, Morin GB, Chapman KB, Weinrich SL, Andrews WH, Lingner J, *et al*. (1997). Telomerase catalytic subunit homologs from fission yeast and human. *Science,* **277,** 955-959.

Nakayama J, Tahara H, Tahara E, Saito M, Ito K, Nakamura H, *et al*. (1998). Telomerase activation by hTRT in human normal fibroblasts and hepatocellular carcinomas. *Nat Genet,* **18,** 65-68.

Nan X, Meehan RR and Bird A. (1993). Dissection of the methyl-CpG binding domain from the chromosomal protein MeCP2. *Nucleic Acids Res,* **21,** 4886-4892.

Nan X, Ng HH, Johnson CA, Laherty CD, Turner BM, Eisenman RN, *et al*. (1998). Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature,* **393,** 386-389.

Nanni S, Narducci M, Della PL, Moretti F, Grasselli A, De CP, *et al*. (2002). Signaling through estrogen receptors modulates telomerase activity in human prostate cancer. *J Clin Invest,* **110,** 219-227.

Narayan A, Ji W, Zhang XY, Marrogi A, Graff JR, Baylin SB, *et al*. (1998). Hypomethylation of pericentromeric DNA in breast adenocarcinomas. *Int J Cancer,* **77,** 833-838.

Neuberger MS and Milstein C. (1995). Somatic hypermutation. *Curr Opin Immunol,* **7,** 248-254.

Ng HH, Jeppesen P and Bird A. (2000). Active repression of methylated genes by the chromosomal protein MBD1. *Mol Cell Biol,* **20,** 1394-1406.

Ng HH, Zhang Y, Hendrich B, Johnson CA, Turner BM, Erdjument-Bromage H, *et al*. (1999). MBD2 is a transcriptional repressor belonging to the MeCP1 histone deacetylase complex. *Nat Genet,* **23,** 58-61.

Nguyen C, Liang G, Nguyen TT, Tsao-Wei D, Groshen S, Lubbert M, *et al*. (2001). Susceptibility of nonpromoter CpG islands to de novo methylation in normal and neoplastic cells. *J Natl Cancer Inst,* **93,** 1465-1472.

Nikitina T and Woodcock CL. (2004). Closed chromatin loops at the ends of chromosomes. *J Cell Biol,* **166,** 161-165.

Nishi H, Nakada T, Kyo S, Inoue M, Shay JW and Isaka K. (2004). Hypoxia-inducible factor 1 mediates upregulation of telomerase (hTERT). *Mol Cell Biol,* **24,** 6076-6083.

Nittis T, Guittat L and Stewart SA. (2008). Alternative lengthening of telomeres (ALT) and chromatin: Is there a connection? *Biochimie,* **90,** 5-12.

Nomoto K, Maekawa M, Sugano K, Ushiama M, Fukayama N, Fujita S, *et al*. (2002). Methylation status and expression of human telomerase reverse transcriptase mRNA in relation to hypermethylation of the p16 gene in colorectal cancers as analyzed by bisulfite PCR-SSCP. *Jpn J Clin Oncol,* **32,** 3-8.

Norrback KF, Dahlenborg K, Carlsson R and Roos G. (1996). Telomerase activation in normal B lymphocytes and non-Hodgkin's lymphomas. *Blood,* **88,** 222-229.

Norrback KF, Hultdin M, Dahlenborg K, Osterman P, Carlsson R and Roos G. (2001). Telomerase regulation and telomere dynamics in germinal centers. *Eur J Haematol,* **67,** 309-317.

Nutt SL, Heavey B, Rolink AG and Busslinger M. (1999). Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. *Nature,* **401,** 556-562.

Nutt SL and Kee BL. (2007). The transcriptional regulation of B cell lineage commitment. *Immunity,* **26,** 715-725.

Nutt SL, Morrison AM, Dorfler P, Rolink A and Busslinger M. (1998). Identification of BSAP (Pax-5) target genes in early B-cell development by loss- and gain-of-function experiments. *EMBO J,* **17,** 2319-2333.

Nutt SL, Urbanek P, Rolink A and Busslinger M. (1997). Essential functions of Pax5 (BSAP) in pro-B cell development: difference between fetal and adult B lymphopoiesis and reduced V-to-DJ recombination at the IgH locus. *Genes Dev,* **11,** 476-491.

O'Connor CM and Collins K. (2006). A novel RNA binding domain in tetrahymena telomerase p65 initiates hierarchical assembly of telomerase holoenzyme. *Mol Cell Biol,* **26,** 2029-2036.

O'Connor MS, Safari A, Liu D, Qin J and Songyang Z. (2004). The human Rap1 protein complex and modulation of telomere length. *J Biol Chem,* **279,** 28585-28591.

Oh S, Song Y, Yim J and Kim TK. (1999). The Wilms' tumor 1 tumor suppressor gene represses transcription of the human telomerase reverse transcriptase gene. *J Biol Chem,* **274,** 37473-37478.

Oh S, Song YH, Yim J and Kim TK. (2000). Identification of Mad as a repressor of the human telomerase (hTERT) gene. *Oncogene,* **19,** 1485-1490.

Ohlsson R, Renkawitz R and Lobanenkov V. (2001). CTCF is a uniquely versatile transcription regulator linked to epigenetics and disease. *Trends Genet,* **17,** 520-527.

Ohyashiki JH, Hayashi S, Yahata N, Iwama H, Ando K, Tauchi T, *et al*. (2001). Impaired telomere regulation mechanism by TRF1 (telomere-binding protein), but not TRF2 expression, in acute leukemia cells. *Int J Oncol,* **18,** 593-598.

Ohyashiki JH, Hisatomi H, Nagao K, Honda S, Takaku T, Zhang Y, *et al*. (2005). Quantitative relationship between functionally active telomerase and major telomerase components (hTERT and hTR) in acute leukaemia cells. *Br J Cancer,* **92,** 1942-1947.

Ohyashiki JH, Ohyashiki K, Iwama H, Hayashi S, Toyama K and Shay JW. (1997). Clinical implications of telomerase activity levels in acute leukemia. *Clin Cancer Res,* **3,** 619-625.

Okano M, Bell DW, Haber DA and Li E. (1999). DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell,* **99,** 247-257.

Okitsu CY and Hsieh CL. (2007). DNA methylation dictates histone H3K4 methylation. *Mol Cell Biol,* **27,** 2746-2757.

Opresko PL, von KC, Laine JP, Harrigan J, Hickson ID and Bohr VA. (2002). Telomere-binding protein TRF2 binds to and stimulates the Werner and Bloom syndrome helicases. *J Biol Chem,* **277,** 41110-41119.

Orlando C, Gelmini S, Selli C and Pazzagli M. (2001). Telomerase in urological malignancy. *J Urol,* **166,** 666-673.

Ouellette MM, Liao M, Herbert BS, Johnson M, Holt SE, Liss HS, *et al*. (2000). Subsenescent telomere lengths in fibroblasts immortalized by limiting amounts of telomerase. *J Biol Chem,* **275,** 10072-10076.

Pandita TK. (2002). ATM function and telomere stability. *Oncogene,* **21,** 611-618.

Pardal R, Clarke MF and Morrison SJ. (2003). Applying the principles of stem-cell biology to cancer. *Nat Rev Cancer,* **3,** 895-902.

Park TJ, Han SU, Cho YK, Paik WK, Kim YB and Lim IK. (2001). Methylation of O(6)-methylguanine-DNA methyltransferase gene is associated significantly with K-ras mutation, lymph node invasion, tumor staging, and disease free survival in patients with gastric carcinoma. *Cancer,* **92,** 2760-2768.

Pascual V, Liu YJ, Magalski A, de BO, Banchereau J and Capra JD. (1994). Analysis of somatic mutation in five B cell subsets of human tonsil. *J Exp Med,* **180,** 329-339.

Patra SK and Bettuzzi S. (2007). Epigenetic DNA-methylation regulation of genes coding for lipid raft-associated components: a role for raft proteins in cell transformation and cancer progression (review). *Oncol Rep,* **17,** 1279-1290.

Patra SK, Patra A and Dahiya R. (2001). Histone deacetylase and DNA methyltransferase in human prostate cancer. *Biochem Biophys Res Commun,* **287,** 705-713.

Patra SK, Patra A, Rizzi F, Ghosh TC and Bettuzzi S. (2008). Demethylation of (Cytosine-5-C-methyl) DNA and regulation of transcription in the epigenetic pathways of cancer development. *Cancer Metastasis Rev,* **27,** 315-334.

Patra SK, Patra A, Zhao H and Dahiya R. (2002). DNA methyltransferase and demethylase in human prostate cancer. *Mol Carcinog,* **33,** 163-171.

Paz MF, Fraga MF, Avila S, Guo M, Pollan M, Herman JG, *et al*. (2003). A systematic profile of DNA methylation in human cancer cell lines. *Cancer Res,* **63,** 1114-1121.

Pedram M, Sprung CN, Gao Q, Lo AWI, Reynolds GE and Murnane JP. (2006). Telomere position effect and silencing of transgenes near telomeres in the mouse. *Mol Cell Biol,* **26,** 1865-1878.

Pendino F, Tarkanyi I, Dudognon C, Hillion J, Lanotte M, Aradi J, *et al*. (2006). Telomeres and telomerase: Pharmacological targets for new anticancer strategies? *Curr Cancer Drug Targets,* **6,** 147-180.

Peschon JJ, Morrissey PJ, Grabstein KH, Ramsdell FJ, Maraskovsky E, Gliniak BC, *et al*. (1994). Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J Exp Med,* **180,** 1955-1960.

Peterson CL and Laniel MA. (2004). Histones and histone modifications. *Curr Biol,* **14,** R546-R551.

Plumb JA, Bilsland A, Kakani R, Zhao J, Glasspool RM, Knox RJ, *et al*. (2001). Telomerase-specific suicide gene therapy vectors expressing bacterial nitroreductase sensitize human cancer cells to the pro-drug CB1954. *Oncogene,* **20,** 7797-7803.

Poole JC, Andrews LG and Tollefsbol TO. (2001). Activity, function, and gene regulation of the catalytic subunit of telomerase (hTERT). *Gene,* **269,** 1-12.

Poremba C, Willenbring H, Hero B, Christiansen H, Schafer KL, Brinkschmidt C, *et al*. (1999). Telomerase activity distinguishes between neuroblastomas with good and poor prognosis. *Ann Oncol,* **10,** 715-721.

Prathapam R, Witkin KL, O'Connor CM and Collins K. (2005). A telomerase holoenzyme protein enhances telomerase RNA assembly with telomerase reverse transcriptase. *Nat Struct Mol Biol,* **12,** 252-257.

Pridans C, Holmes ML, Polli M, Wettenhall JM, Dakic A, Corcoran LM, *et al*. (2008). Identification of Pax5 target genes in early B cell differentiation. *J Immunol,* **180,** 1719-1728.

Primeau M, Gagnon J and Momparler RL. (2003). Synergistic antineoplastic action of DNA methylation inhibitor 5-AZA-2'-deoxycytidine and histone deacetylase inhibitor depsipeptide on human breast carcinoma cells. *Int J Cancer,* **103,** 177-184.

Prokhortchouk E and Hendrich B. (2002). Methyl-CpG binding proteins and cancer: are MeCpGs more important than MBDs? *Oncogene,* **21,** 5394-5399.

Qu GZ, Grundy PE, Narayan A and Ehrlich M. (1999). Frequent hypomethylation in Wilms tumors of pericentromeric DNA in chromosomes 1 and 16. *Cancer Genet Cytogenet,* **109,** 34-39.

Rahman R, Latonen L and Wiman KG. (2005). hTERT antagonizes p53-induced apoptosis independently of telomerase activity. *Oncogene,* **24,** 1320-1327.

Ramakrishnan S, Eppenberger U, Mueller H, Shinkai Y and Narayanan R. (1998). Expression profile of the putative catalytic subunit of the telomerase gene. *Cancer Res,* **58,** 622-625.

Ramirez RD, Morales CP, Herbert BS, Rohde JM, Passons C, Shay JW, *et al*. (2001). Putative telomere-independent mechanisms of replicative aging reflect inadequate growth conditions. *Genes Dev,* **15,** 398-403.

Rand K, Qu W, Ho T, Clark SJ and Molloy P. (2002). Conversion-specific detection of DNA methylation using real-time polymerase chain reaction (ConLight-MSP) to avoid false positives. *Methods,* **27,** 114-120.

Razin A and Cedar H. (1991). DNA methylation and gene expression. *Microbiol Rev,* **55,** 451-458.

Reddel RR. (2003). Alternative lengthening of telomeres, telomerase, and cancer. *Cancer Lett,* **194,** 155-162.

Reichenbach P, Hoss M, Azzalin CM, Nabholz M, Bucher P and Lingner J. (2003). A human homolog of yeast Est1 associates with telomerase and uncaps chromosome ends when overexpressed. *Curr Biol,* **13,** 568-574.

Remes K, Norrback KF, Rosenquist R, Mehle C, Lindh J and Roos G. (2000). Telomere length and telomerase activity in malignant lymphomas at diagnosis and relapse. *Br J Cancer,* **82,** 601-607.

Renaud S, Loukinov D, Abdullaev Z, Guilleret I, Bosman FT, Lobanenkov V, *et al*. (2007). Dual role of DNA methylation inside and outside of CTCF-binding regions in the transcriptional regulation of the telomerase hTERT gene. *Nucleic Acids Res,* **35,** 1245-1256.

Renaud S, Loukinov D, Bosman FT, Lobanenkov V and Benhattar J. (2005). CTCF binds the proximal exonic region of hTERT and inhibits its transcription. *Nucleic Acids Res,* **33,** 6850-6860.

Richmond TJ and Davey CA. (2003). The structure of DNA in the nucleosome core. *Nature,* **423,** 145-150.

Riek R, Pervushin K, Fernandez C, Kainosho M and Wuthrich K. (2001). [(13)C,(13)C]- and [(13)C,(1)H]-TROSY in a triple resonance experiment for ribose-base and intrabase correlations in nucleic acids. *J Am Chem Soc,* **123,** 658-664.

Roberts EC, Deed RW, Inoue T, Norton JD and Sharrocks AD. (2001). Id helix-loop-helix proteins antagonize pax transcription factor activity by inhibiting DNA binding. *Mol Cell Biol,* **21,** 524-533.

Robertson KD. (2002). DNA methylation and chromatin - unraveling the tangled web. *Oncogene,* **21,** 5361-5379.

Robertson KD, it-Si-Ali S, Yokochi T, Wade PA, Jones PL and Wolffe AP. (2000). DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. *Nat Genet,* **25,** 338-342.

Robertson KD, Uzvolgyi E, Liang G, Talmadge C, Sumegi J, Gonzales FA, *et al*. (1999). The human DNA methyltransferases (DNMTs) 1, 3a and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors. *Nucleic Acids Res,* **27,** 2291-2298.

Roessler S, Gyory I, Imhof S, Spivakov M, Williams RR, Busslinger M, *et al*. (2007). Distinct promoters mediate the regulation of Ebf1 gene expression by interleukin-7 and Pax5. *Mol Cell Biol,* **27,** 579-594.

Roh TY, Cuddapah S, Cui K and Zhao K. (2006). The genomic landscape of histone modifications in human T cells. *Proc Natl Acad Sci U S A,* **103,** 15782-15787.

Roh TY, Cuddapah S and Zhao K. (2005). Active chromatin domains are defined by acetylation islands revealed by genome-wide mapping. *Genes Dev,* **19,** 542-552.

Rothenberg EV. (2007). Negotiation of the T lineage fate decision by transcription-factor interplay and microenvironmental signals. *Immunity,* **26,** 690-702.

Rountree MR, Bachman KE and Baylin SB. (2000). DNMT1 binds HDAC2 and a new co-repressor, DMAP1, to form a complex at replication foci. *Nat Genet,* **25,** 269-277.

Rubio MA, Davalos AR and Campisi J. (2004). Telomere length mediates the effects of telomerase on the cellular response to genotoxic stress. *Exp Cell Res,* **298,** 17-27.

Rudolph KL, Chang S, Lee HW, Blasco M, Gottlieb GJ, Greider C, *et al*. (1999). Longevity, stress response, and cancer in aging telomerase-deficient mice. *Cell,* **96,** 701-712.

Rufer N, Brummendorf TH, Kolvraa S, Bischoff C, Christensen K, Wadsworth L, *et al*. (1999). Telomere fluorescence measurements in granulocytes and T lymphocyte subsets point to a high turnover of hematopoietic stem cells and memory T cells in early childhood. *J Exp Med,* **190,** 157-167.

Saeboe-Larssen S, Fossberg E and Gaudernack G. (2006). Characterization of novel alternative splicing sites in human telomerase reverse transcriptase (hTERT): analysis of expression and mutual correlation in mRNA isoforms from normal and tumour tissues. *BMC Mol Biol,* **7,** 26.

Saito M, Nakagawa K, Hamada K, Hirose S, Harada H, Kohno S, *et al*. (2004). Introduction of p16INK4a inhibits telomerase activity through transcriptional suppression of human telomerase reverse transcriptase expression in human gliomas. *Int J Oncol,* **24,** 1213-1220.

Saldanha SN, Andrews LG and Tollefsbol TO. (2003). Assessment of telomere length and factors that contribute to its stability. *Eur J Biochem,* **270,** 389-403.

Sansom OJ, Berger J, Bishop SM, Hendrich B, Bird A and Clarke AR. (2003a). Deficiency of Mbd2 suppresses intestinal tumorigenesis. *Nat Genet,* **34,** 145-147.

Sansom OJ, Bishop SM, Court H, Dudley S, Liskay RM and Clarke AR. (2003b). Apoptosis and mutation in the murine small intestine: loss of Mlh1- and Pms2-dependent apoptosis leads to increased mutation in vivo. *DNA Repair (Amst),* **2,** 1029-1039.

Santini V, Kantarjian HM and Issa JP. (2001). Changes in DNA methylation in neoplasia: pathophysiology and therapeutic implications. *Ann Intern Med,* **134,** 573-586.

Santos JH, Meyer JN and Van HB. (2006). Mitochondrial localization of telomerase as a determinant for hydrogen peroxide-induced mitochondrial DNA damage and apoptosis. *Hum Mol Genet,* **15,** 1757-1768.

Saretzki G, Ludwig A, von ZT and Runnebaum IB. (2001). Ribozyme-mediated telomerase inhibition induces immediate cell loss but not telomere shortening in ovarian cancer cells. *Cancer Gene Ther,* **8,** 827-834.

Sarin KY, Cheung P, Gilison D, Lee E, Tennen RI, Wang E, *et al*. (2005). Conditional telomerase induction causes proliferation of hair follicle stem cells. *Nature,* **436,** 1048-1052.

Sarraf SA and Stancheva I. (2004). Methyl-CpG binding protein MBD1 couples histone H3 methylation at lysine 9 by SETDB1 to DNA replication and chromatin assembly. *Mol Cell,* **15,** 595-605.

Sato M, Horio Y, Sekido Y, Minna JD, Shimokata K and Hasegawa Y. (2002). The expression of DNA methyltransferases and methyl-CpG-binding proteins is not associated with the methylation status of p14(ARF), p16(INK4a) and RASSF1A in human lung cancer cell lines. *Oncogene,* **21,** 4822-4829.

Sawan C, Vaissiere T, Murr R and Herceg Z. (2008). Epigenetic drivers and genetic passengers on the road to cancer. *Mutat Res,* **642,** 1-13.

Schaniel C, Bruno L, Melchers F and Rolink AG. (2002a). Multiple hematopoietic cell lineages develop in vivo from transplanted Pax5-deficient pre-B I-cell clones. *Blood,* **99,** 472-478.

Schaniel C, Gottar M, Roosnek E, Melchers F and Rolink AG. (2002b). Extensive in vivo self-renewal, long-term reconstitution capacity, and hematopoietic multipotency of Pax5-deficient precursor B-cell clones. *Blood,* **99,** 2760-2766.

Schebesta A, McManus S, Salvagiotto G, Delogu A, Busslinger GA and Busslinger M. (2007). Transcription factor Pax5 activates the chromatin of key genes involved in B cell signaling, adhesion, migration, and immune function. *Immunity,* **27,** 49-63.

Schebesta M, Pfeffer PL and Busslinger M. (2002). Control of pre-BCR signaling by Pax5-dependent activation of the BLNK gene. *Immunity,* **17,** 473-485.

Schnapp G, Rodi HP, Rettig WJ, Schnapp A and Damm K. (1998). One-step affinity purification protocol for human telomerase. *Nucleic Acids Res,* **26,** 3311-3313.

Schoeftner S and Blasco MA. (2008). Developmentally regulated transcription of mammalian telomeres by DNA-dependent RNA polymerase II. *Nat Cell Biol,* **10,** 228-236.

Sedivy JM. (1998). Can ends justify the means?: telomeres and the mechanisms of replicative senescence and immortalization in mammalian cells. *Proc Natl Acad Sci U S A,* **95,** 9078-9081.

Shaffer AL, Lin KI, Kuo TC, Yu X, Hurt EM, Rosenwald A, *et al*. (2002). Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. *Immunity,* **17,** 51-62.

Shay JW and Gazdar AF. (1997). Telomerase in the early detection of cancer. *J Clin Pathol,* **50,** 106-109.

Shay JW and Keith WN. (2008). Targeting telomerase for cancer therapeutics. *Br J Cancer,* **98,** 677-683.

Shay JW, Pereira-Smith OM and Wright WE. (1991). A role for both RB and p53 in the regulation of human cellular senescence. *Exp Cell Res,* **196,** 33-39.

Shay JW and Roninson IB. (2004). Hallmarks of senescence in carcinogenesis and cancer therapy. *Oncogene,* **23,** 2919-2933.

Shay JW and Wright WE. (2001). Ageing and cancer: the telomere and telomerase connection. *Novartis Found Symp,* **235,** 116-125.

Shi B, Hsu HL, Evens AM, Gordon LI and Gartenhaus RB. (2003). Expression of the candidate MCT-1 oncogene in B- and T-cell lymphoid malignancies. *Blood,* **102,** 297-302.

Shi Y, Lan F, Matson C, Mulligan P, Whetstine JR, Cole PA, *et al*. (2004). Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell,* **119,** 941-953.

Shia WJ, Li B and Workman JL. (2006). SAS-mediated acetylation of histone H4 Lys 16 is required for H2A.Z incorporation at subtelomeric regions in Saccharomyces cerevisiae. *Genes Dev,* **20,** 2507-2512.

Shin JH, Chung J, Kim HO, Kim YH, Hur YM, Rhim JH, *et al*. (2002). Detection of cancer cells in peripheral blood of stomach cancer patients using RT-PCR amplification of tumour-specific mRNAs. *Aliment Pharmacol Ther,* **16 Suppl 2,** 137-144.

Shin KH, Kang MK, Dicterow E and Park NH. (2003). Hypermethylation of the hTERT promoter inhibits the expression of telomerase activity in normal oral fibroblasts and senescent normal oral keratinocytes. *Br J Cancer,* **89,** 1473-1478.

Shogren-Knaak M and Peterson CL. (2006). Switching on chromatin: mechanistic role of histone H4-K16 acetylation. *Cell Cycle,* **5,** 1361-1365.

Singal R and Ginder GD. (1999). DNA methylation. *Blood,* **93,** 4059-4070.

Singal R, vanWert JM and Ferdinand L, Jr. (2002). Methylation of alpha-type embryonic globin gene alpha pi represses transcription in primary erythroid cells. *Blood,* **100,** 4217-4222.

Sitnicka E, Bryder D, Theilgaard-Monch K, Buza-Vidas N, Adolfsson J and Jacobsen SE. (2002). Key role of flt3 ligand in regulation of the common lymphoid progenitor but not in maintenance of the hematopoietic stem cell pool. *Immunity,* **17,** 463-472.

Smilenov LB, Dhar S and Pandita TK. (1999). Altered telomere nuclear matrix interactions and nucleosomal periodicity in ataxia telangiectasia cells before and after ionizing radiation treatment. *Mol Cell Biol,* **19,** 6963-6971.

Smith LL, Coller HA and Roberts JM. (2003a). Telomerase modulates expression of growth-controlling genes and enhances cell proliferation. *Nat Cell Biol,* **5,** 474-479.

Smith ND, Rubenstein JN, Eggener SE and Kozlowski JM. (2003b). The p53 tumor suppressor gene and nuclear protein: Basic science review and relevance in the management of bladder cancer. *Journal of Urology,* **169,** 1219-1228.

Smith S and De Lange T. (1997). TRF1, a mammalian telomeric protein. *Trends Genet,* **13,** 21-26.

Smith S and De Lange T. (2000). Tankyrase promotes telomere elongation in human cells. *Curr Biol,* **10,** 1299-1302.

Smith S, Giriat I, Schmitt A and de Lange T. (1998). Tankyrase, a poly(ADP-ribose) polymerase at human telomeres. *Science,* **282,** 1484-1487.

Smogorzewska A and De Lange T. (2004). Regulation of telomerase by telomeric proteins. *Annu Rev Biochem,* **73,** 177-208.

Smogorzewska A, van Steensel B, Bianchi A, Oelmann S, Schaefer MR, Schnapp G, *et al*. (2000). Control of human telomere length by TRF1 and TRF2. *Mol Cell Biol,* **20,** 1659-1668.

Snow BE, Erdmann N, Cruickshank J, Goldman H, Gill RM, Robinson MO, *et al*. (2003). Functional conservation of the telomerase protein Est1p in humans. *Curr Biol,* **13,** 698-704.

Soder AI, Going JJ, Kaye SB and Keith WN. (1998). Tumour specific regulation of telomerase RNA gene expression visualized by in situ hybridization. *Oncogene,* **16,** 979-983.

Soder AI, Hoare SF, Muir S, Going JJ, Parkinson EK and Keith WN. (1997). Amplification, increased dosage and in situ expression of the telomerase RNA gene in human cancer. *Oncogene,* **14,** 1013-1021.

Son NH, Joyce B, Hieatt A, Chrest FJ, Yanovski J and Weng NP. (2003). Stable telomere length and telomerase expression from naive to memory B-lymphocyte differentiation. *Mech Ageing Dev,* **124,** 427-432.

Son NH, Murray S, Yanovski J, Hodes RJ and Weng N. (2000). Lineage-specific telomere shortening and unaltered capacity for telomerase expression in human T and B lymphocytes with age. *J Immunol,* **165,** 1191-1196.

Song K, Jung D, Jung Y, Lee SG and Lee I. (2000). Interaction of human Ku70 with TRF2. *Febs Letters,* **481,** 81-85.

Souabni A, Cobaleda C, Schebesta M and Busslinger M. (2002). Pax5 promotes B lymphopoiesis and blocks T cell development by repressing Notch1. *Immunity,* **17,** 781-793.

Spits H. (2002). Development of alphabeta T cells in the human thymus. *Nat Rev Immunol,* **2,** 760-772.

Spotswood HT and Turner BM. (2002). An increasingly complex code. *J Clin Invest,* **110,** 577-582.

Stamenkovic I and Seed B. (1988). CD19, the earliest differentiation antigen of the B cell lineage, bears three extracellular immunoglobulin-like domains and an Epstein-Barr virus-related cytoplasmic tail. *J Exp Med,* **168,** 1205-1210.

Stansel RM, De Lange T and Griffith JD. (2001). T-loop assembly in vitro involves binding of TRF2 near the 3' telomeric overhang. *EMBO J,* **20,** 5532-5540.

Steinert S, Shay JW and Wright WE. (2000). Transient expression of human telomerase extends the life span of normal human fibroblasts. *Biochem Biophys Res Commun,* **273,** 1095-1098.

Stewart SA. (2005). Telomere maintenance and tumorigenesis: an "ALT"ernative road. *Curr Mol Med,* **5,** 253-257.

Stewart SA, Ben-Porath I, Carey VJ, O'Connor BF, Hahn WC and Weinberg RA. (2003). Erosion of the telomeric single-strand overhang at replicative senescence. *Nat Genet,* **33,** 492-496.

Stone MD, Mihalusova M, O'Connor CM, Prathapam R, Collins K and Zhuang X. (2007). Stepwise protein-mediated RNA folding directs assembly of telomerase ribonucleoprotein. *Nature,* **446,** 458-461.

Strahl BD and Allis CD. (2000). The language of covalent histone modifications. *Nature,* **403,** 41-45.

Streutker CJ, Thorner P, Fabricius N, Weitzman S and Zielenska M. (2001). Telomerase activity as a prognostic factor in neuroblastomas. *Pediatr Dev Pathol,* **4,** 62-67.

Stuart ET, Kioussi C, Aguzzi A and Gruss P. (1995). PAX5 expression correlates with increasing malignancy in human astrocytomas. *Clin Cancer Res,* **1,** 207-214.

Su Z, Dannull J, Yang BK, Dahm P, Coleman D, Yancey D, *et al*. (2005). Telomerase mRNA-transfected dendritic cells stimulate antigen-specific CD8+ and CD4+ T cell responses in patients with metastatic prostate cancer. *J Immunol,* **174,** 3798-3807.

Suetake I, Shinozaki F, Miyagawa J, Takeshima H and Tajima S. (2004). DNMT3L stimulates the DNA methylation activity of Dnmt3a and Dnmt3b through a direct interaction. *J Biol Chem,* **279,** 27816-27823.

Szyf M. (2003). DNA methylation and cancer therapy. *Drug Resist Updat,* **6,** 341-353.

Tagoh H, Ingram R, Wilson N, Salvagiotto G, Warren AJ, Clarke D, *et al*. (2006). The mechanism of repression of the myeloid-specific c-fms gene by Pax5 during B lineage restriction. *EMBO J,* **25,** 1070-1080.

Tahara H, Yasui W, Tahara E, Fujimoto J, Ito K, Tamai K, *et al*. (1999). Immuno-histochemical detection of human telomerase catalytic component, hTERT, in human colorectal tumor and non-tumor tissue sections. *Oncogene,* **18,** 1561-1567.

Takakura M, Kyo S, Inoue M, Wright WE and Shay JW. (2005). Function of AP-1 in transcription of the telomerase reverse transcriptase gene (TERT) in human and mouse cells. *Mol Cell Biol,* **25,** 8037-8043.

Takakura M, Kyo S, Kanaya T, Hirano H, Takeda J, Yutsudo M, *et al*. (1999). Cloning of human telomerase catalytic subunit (hTERT) gene promoter and identification of proximal core promoter sequences essential for transcriptional activation in immortalized and cancer cells. *Cancer Res,* **59,** 551-557.

Takakura M, Kyo S, Sowa Y, Wang Z, Yatabe N, Maida Y, *et al*. (2001). Telomerase activation by histone deacetylase inhibitor in normal cells. *Nucleic Acids Res,* **29,** 3006-3011.

Tang Z, Zhao Y, Mei F, Yang S, Li X, Lv J, *et al*. (2004). Molecular cloning and characterization of a human gene involved in transcriptional regulation of hTERT. *Biochem Biophys Res Commun,* **324,** 1324-1332.

Tate PH and Bird AP. (1993). Effects of DNA methylation on DNA-binding proteins and gene expression. *Curr Opin Genet Dev,* **3,** 226-231.

Tatsumoto N, Hiyama E, Murakami Y, Imamura Y, Shay JW, Matsuura Y, *et al*. (2000). High telomerase activity is an independent prognostic indicator of poor outcome in colorectal cancer. *Clin Cancer Res,* **6,** 2696-2701.

Tedder TF and Isaacs CM. (1989). Isolation of cDNAs encoding the CD19 antigen of human and mouse B lymphocytes. A new member of the immunoglobulin superfamily. *J Immunol,* **143,** 712-717.

Teodoridis JM, Strathdee G and Brown R. (2004). Epigenetic silencing mediated by CpG island methylation: potential as a therapeutic target and as a biomarker. *Drug Resist Updat,* **7,** 267-278.

Terrin L, Dal CJ, Rampazzo E, Zancai P, Pedrotti M, Ammirabile G, *et al*. (2008). Latent membrane protein 1 of Epstein-Barr virus activates the hTERT promoter and enhances telomerase activity in B lymphocytes. *J Virol,* **82,** 10175-10187.

Tesmer VM, Ford LP, Holt SE, Frank BC, Yi XM, Aisner DL, *et al*. (1999). Two inactive fragments of the integral RNA cooperate to assemble active telomerase with the human protein catalytic subunit (hTERT) in vitro. *Molecular and Cellular Biology,* **19,** 6207-6216.

Thompson CA. (2006). Vorinostat approved for rare lymphoma. *Am J Health Syst Pharm,* **63,** 2168.

Tiacci E, Pileri S, Orleth A, Pacini R, Tabarrini A, Frenguelli F, *et al*. (2004). PAX5 expression in acute leukemias: higher B-lineage specificity than CD79a and selective association with t(8;21)-acute myelogenous leukemia. *Cancer Res,* **64,** 7399-7404.

Tollefsbol TO and Andrews LG. (2001). Mechanisms for telomerase gene control in aging cells and tumorigenesis. *Med Hypotheses,* **56,** 630-637.

Tomlinson RL, Ziegler TD, Supakorndej T, Terns RM and Terns MP. (2006). Cell cycle-regulated trafficking of human telomerase to telomeres. *Mol Biol Cell,* **17,** 955-965.

Toogun OA, Dezwaan DC and Freeman BC. (2008). The hsp90 molecular chaperone modulates multiple telomerase activities. *Mol Cell Biol,* **28,** 457-467.

Torlakovic E, Slipicevic A, Robinson C, DeCoteau JF, Alfsen GC, Vyberg M, *et al*. (2006). Pax-5 expression in nonhematopoietic tissues. *Am J Clin Pathol,* **126,** 798-804.

Torlakovic E, Torlakovic G, Nguyen PL, Brunning RD and Delabie J. (2002). The value of anti-pax-5 immunostaining in routinely fixed and paraffin-embedded sections: a novel pan pre-B and B-cell marker. *Am J Surg Pathol,* **26,** 1343-1350.

Toyooka S, Suzuki M, Maruyama R, Toyooka KO, Tsukuda K, Fukuyama Y, *et al*. (2004). The relationship between aberrant methylation and survival in non-small-cell lung cancers. *Br J Cancer,* **91,** 771-774.

Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB and Issa JP. (1999a). CpG island methylator phenotype in colorectal cancer. *Proc Natl Acad Sci U S A,* **96,** 8681-8686.

Toyota M, Ho C, Ahuja N, Jair KW, Li Q, Ohe-Toyota M, *et al*. (1999b). Identification of differentially methylated sequences in colorectal cancer by methylated CpG island amplification. *Cancer Res,* **59,** 2307-2312.

Traver D, Akashi K, Manz M, Merad M, Miyamoto T, Engleman EG, *et al*. (2000). Development of CD8alpha-positive dendritic cells from a common myeloid progenitor. *Science,* **290,** 2152-2154.

Trentin L, Ballon G, Ometto L, Perin A, Basso U, Chieco-Bianchi L, *et al*. (1999). Telomerase activity in chronic lymphoproliferative disorders of B-cell lineage. *Br J Haematol,* **106,** 662-668.

Tycko B. (2000). Epigenetic gene silencing in cancer. *J Clin Invest,* **105,** 401-407.

Uhlmann K, Rohde K, Zeller C, Szymas J, Vogel S, Marczinek K, *et al*. (2003). Distinct methylation profiles of glioma subtypes. *Int J Cancer,* **106,** 52-59.

Ulaner GA, Hu JF, Vu TH, Giudice LC and Hoffman AR. (1998). Telomerase activity in human development is regulated by human telomerase reverse transcriptase (hTERT) transcription and by alternate splicing of hTERT transcripts. *Cancer Res,* **58,** 4168-4172.

Ulaner GA, Hu JF, Vu TH, Oruganti H, Giudice LC and Hoffman AR. (2000). Regulation of telomerase by alternate splicing of human telomerase reverse transcriptase (hTERT) in normal and neoplastic ovary, endometrium and myometrium. *Int J Cancer,* **85,** 330-335.

Ulaner GA, Huang HY, Otero J, Zhao Z, Ben-Porat L, Satagopan JM, *et al*. (2003). Absence of a telomere maintenance mechanism as a favorable prognostic factor in patients with osteosarcoma. *Cancer Res,* **63,** 1759-1763.

Underhill DA. (2000). Genetic and biochemical diversity in the Pax gene family. *Biochem Cell Biol,* **78,** 629-638.

Urbanek P, Wang ZQ, Fetka I, Wagner EF and Busslinger M. (1994). Complete block of early B cell differentiation and altered patterning of the posterior midbrain in mice lacking Pax5/BSAP. *Cell,* **79,** 901-912.

Valk-Lingbeek ME, Bruggeman SW and van LM. (2004). Stem cells and cancer; the polycomb connection. *Cell,* **118,** 409-418.

Van Steensel B. and De Lange T. (1997). Control of telomere length by the human telomeric protein TRF1. *Nature,* **385,** 740-743.

Vaziri H and Benchimol S. (1998). Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. *Curr Biol,* **8,** 279-282.

Veldman T, Etheridge KT and Counter CM. (2004). Loss of hPot1 function leads to telomere instability and a cut-like phenotype. *Curr Biol,* **14,** 2264-2270.

Veldman T, Liu X, Yuan H and Schlegel R. (2003). Human papillomavirus E6 and Myc proteins associate in vivo and bind to and cooperatively activate the telomerase reverse transcriptase promoter. *Proc Natl Acad Sci U S A,* **100,** 8211-8216.

Verdun RE and Karlseder J. (2007). Replication and protection of telomeres. *Nature,* **447,** 924-931.

Villar-Garea A and Esteller M. (2003). DNA demethylating agents and chromatin-remodelling drugs: which, how and why? *Curr Drug Metab,* **4,** 11-31.

Vostrov AA and Quitschke WW. (1997). The zinc finger protein CTCF binds to the APBbeta domain of the amyloid beta-protein precursor promoter. Evidence for a role in transcriptional activation. *J Biol Chem,* **272,** 33353-33359.

Vulliamy T, Marrone A, Goldman F, Dearlove A, Bessler M, Mason PJ, *et al*. (2001). The RNA component of telomerase is mutated in autosomal dominant dyskeratosis congenita. *Nature,* **413,** 432-435.

Wade PA. (2001). Methyl CpG binding proteins: coupling chromatin architecture to gene regulation. *Oncogene,* **20,** 3166-3173.

Walne AJ, Vulliamy T, Marrone A, Beswick R, Kirwan M, Masunari Y, *et al*. (2007). Genetic heterogeneity in autosomal recessive dyskeratosis congenita with one subtype due to mutations in the telomerase-associated protein NOP10. *Human Molecular Genetics,* **16,** 1619-1629.

Wang F, Podell ER, Zaug AJ, Yang Y, Baciu P, Cech TR, *et al*. (2007). The POT1-TPP1 telomere complex is a telomerase processivity factor. *Nature,* **445,** 506-510.

Wang J, Xie LY, Allan S, Beach D and Hannon GJ. (1998). Myc activates telomerase. *Genes Dev,* **12,** 1769-1774.

Wang Q, Bai Z, Li X, Hou L and Zhang B. (2004). The evidences of human orphan receptor COUP-TFII inhibiting telomerase activity through decreasing hTERT transcription. *Cancer Lett,* **214,** 81-90.

Wang S and Zhu J. (2003). Evidence for a relief of repression mechanism for activation of the human telomerase reverse transcriptase promoter. *J Biol Chem,* **278,** 18842-18850.

Wang Y, Kowalski J, Tsai HL, Marik R, Prasad N, Somervell H, *et al*. (2008). Differentiating alternative splice variant patterns of human telomerase reverse transcriptase in thyroid neoplasms. *Thyroid,* **18,** 1055-1063.

Wang Z, Kyo S, Takakura M, Tanaka M, Yatabe N, Maida Y, *et al*. (2000). Progesterone regulates human telomerase reverse transcriptase gene expression via activation of mitogen-activated protein kinase signaling pathway. *Cancer Res,* **60,** 5376-5381.

Weber M, Davies JJ, Wittig D, Oakeley EJ, Haase M, Lam WL, *et al*. (2005). Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. *Nat Genet,* **37,** 853-862.

Wei SH, Balch C, Paik HH, Kim YS, Baldwin RL, Liyanarachchi S, *et al*. (2006). Prognostic DNA methylation biomarkers in ovarian cancer. *Clin Cancer Res,* **12,** 2788-2794.

Weinrich SL, Pruzan R, Ma L, Ouellette M, Tesmer VM, Holt SE, *et al*. (1997). Reconstitution of human telomerase with the template RNA component hTR and the catalytic protein subunit hTRT. *Nat Genet,* **17,** 498-502.

Weng NP. (2002). Regulation of telomerase expression in human lymphocytes. *Springer Semin Immunopathol,* **24,** 23-33.

Weng NP, Granger L and Hodes RJ. (1997a). Telomere lengthening and telomerase activation during human B cell differentiation. *Proc Natl Acad Sci U S A,* **94,** 10827-10832.

Weng NP, Hathcock KS and Hodes RJ. (1998). Regulation of telomere length and telomerase in T and B cells: a mechanism for maintaining replicative potential. *Immunity,* **9,** 151-157.

Weng NP, Levine BL, June CH and Hodes RJ. (1996). Regulated expression of telomerase activity in human T lymphocyte development and activation. *J Exp Med,* **183,** 2471-2479.

Weng NP, Palmer LD, Levine BL, Lane HC, June CH and Hodes RJ. (1997b). Tales of tails: regulation of telomere length and telomerase activity during lymphocyte development, differentiation, activation, and aging. *Immunol Rev,* **160,** 43-54.

Wenz C, Enenkel B, Amacker M, Kelleher C, Damm K and Lingner J. (2001). Human telomerase contains two cooperating telomerase RNA molecules. *EMBO J,* **20,** 3526-3534.

Wick M, Zubov D and Hagen G. (1999). Genomic organization and promoter characterization of the gene encoding the human telomerase reverse transcriptase (hTERT). *Gene,* **232,** 97-106.

Wilson AS, Power BE and Molloy PL. (2007). DNA hypomethylation and human diseases. *Biochim Biophys Acta,* **1775,** 138-162.

Wojdacz TK and Dobrovic A. (2007). Methylation-sensitive high resolution melting (MS-HRM): a new approach for sensitive and high-throughput assessment of methylation. *Nucleic Acids Research,* **35**.

Wolf P, Hu YC, Doffek K, Sidransky D and Ahrendt SA. (2001). O(6)-Methylguanine-DNA methyltransferase promoter hypermethylation shifts the p53 mutational spectrum in non-small cell lung cancer. *Cancer Res,* **61,** 8113-8117.

Wolffe AP. (1996). Histone deacetylase: a regulator of transcription. *Science,* **272,** 371-372.

Won J, Yim J and Kim TK. (2002a). Opposing regulatory roles of E2F in human telomerase reverse transcriptase (hTERT) gene expression in human tumor and normal somatic cells. *FASEB J,* **16,** 1943-1945.

Won J, Yim J and Kim TK. (2002b). Sp1 and Sp3 recruit histone deacetylase to repress transcription of human telomerase reverse transcriptase (hTERT) promoter in normal human somatic cells. *J Biol Chem,* **277,** 38230-38238.

Wong JM and Collins K. (2006). Telomerase RNA level limits telomere maintenance in X-linked dyskeratosis congenita. *Genes Dev,* **20,** 2848-2858.

Wright WE, Brasiskyte D, Piatyszek MA and Shay JW. (1996a). Experimental elongation of telomeres extends the lifespan of immortal x normal cell hybrids. *EMBO J,* **15,** 1734-1741.

Wright WE, Pereira-Smith OM and Shay JW. (1989). Reversible cellular senescence: implications for immortalization of normal human diploid fibroblasts. *Mol Cell Biol,* **9,** 3088-3092.

Wright WE, Piatyszek MA, Rainey WE, Byrd W and Shay JW. (1996b). Telomerase activity in human germline and embryonic tissues and cells. *Dev Genet,* **18,** 173-179.

Wright WE and Shay JW. (1992). Telomere positional effects and the regulation of cellular senescence. *Trends Genet,* **8,** 193-197.

Wu GK, Lee WH and Chen PL. (2000). NBS1 and TRF1 colocalize at promyelocytic leukemia bodies during late S/G(2) phases in immortalized telomerase-negative cells - Implication of NBS1 in alternative lengthening of telomeres. *Journal of Biological Chemistry,* **275,** 30618-30622.

Wu KJ, Grandori C, Amacker M, Simon-Vermot N, Polack A, Lingner J, *et al*. (1999). Direct activation of TERT transcription by c-MYC. *Nat Genet,* **21,** 220-224.

Wu YL, Dudognon C, Nguyen E, Hillion J, Pendino F, Tarkanyi I, *et al*. (2006). Immunodetection of human telomerase reverse-transcriptase (hTERT) re-appraised: nucleolin and telomerase cross paths. *J Cell Sci,* **119,** 2797-2806.

Xin H, Liu D, Wan M, Safari A, Kim H, Sun W, *et al*. (2007). TPP1 is a homologue of ciliate TEBP-beta and interacts with POT1 to recruit telomerase. *Nature,* **445,** 559-562.

Xiong Z and Laird PW. (1997). COBRA: a sensitive and quantitative DNA methylation assay. *Nucleic Acids Res,* **25,** 2532-2534.

Xu D, Gruber A, Peterson C and Pisa P. (1998). Telomerase activity and the expression of telomerase components in acute myelogenous leukaemia. *Br J Haematol,* **102,** 1367-1375.

Xu D, Popov N, Hou M, Wang Q, Bjorkholm M, Gruber A, *et al*. (2001). Switch from Myc/Max to Mad1/Max binding and decrease in histone acetylation at the telomerase reverse transcriptase promoter during differentiation of HL60 cells. *Proc Natl Acad Sci U S A,* **98,** 3826-3831.

Xu D, Wang Q, Gruber A, Bjorkholm M, Chen Z, Zaid A, *et al*. (2000). Downregulation of telomerase reverse transcriptase mRNA expression by wild type p53 in human tumor cells. *Oncogene,* **19,** 5123-5133.

Xu HE, Rould MA, Xu W, Epstein JA, Maas RL and Pabo CO. (1999). Crystal structure of the human Pax6 paired domain-DNA complex reveals specific roles for the linker region and carboxy-terminal subdomain in DNA binding. *Genes Dev,* **13,** 1263-1275.

Xu M, Luo W, Elzi DJ, Grandori C and Galloway DA. (2008). NFX1 interacts with mSin3A/histone deacetylase to repress hTERT transcription in keratinocytes. *Mol Cell Biol,* **28,** 4819-4828.

Xu W, Rould MA, Jun S, Desplan C and Pabo CO. (1995). Crystal structure of a paired domain-DNA complex at 2.5 A resolution reveals structural basis for Pax developmental mutations. *Cell,* **80,** 639-650.

Yamada Y, Jackson-Grusby L, Linhart H, Meissner A, Eden A, Lin H, *et al*. (2005). Opposing effects of DNA hypomethylation on intestinal and liver carcinogenesis. *Proc Natl Acad Sci U S A,* **102,** 13580-13585.

Yan P, Saraga EP, Bouzourene H, Bosman FT and Benhattar J. (2001). Expression of telomerase genes correlates with telomerase activity in human colorectal carcinogenesis. *J Pathol,* **193,** 21-26.

Yang H, Kyo S, Takatura M and Sun L. (2001). Autocrine transforming growth factor beta suppresses telomerase activity and transcription of human telomerase reverse transcriptase in human cancer cells. *Cell Growth Differ,* **12,** 119-127.

Yaswen P and Stampfer MR. (2002). Molecular changes accompanying senescence and immortalization of cultured human mammary epithelial cells. *Int J Biochem Cell Biol,* **34,** 1382-1394.

Yatabe N, Kyo S, Maida Y, Nishi H, Nakamura M, Kanaya T, *et al*. (2004). HIF-1-mediated activation of telomerase in cervical cancer cells. *Oncogene,* **23,** 3708-3715.

Ye JZ and de Lange T. (2004). TIN2 is a tankyrase 1 PARP modulator in the TRF1 telomere length control complex. *Nat Genet,* **36,** 618-623.

Ye JZ, Hockemeyer D, Krutchinsky AN, Loayza D, Hooper SM, Chait BT, *et al*. (2004a). POT1-interacting protein PIP1: a telomere length regulator that recruits POT1 to the TIN2/TRF1 complex. *Genes Dev,* **18,** 1649-1654.

Ye JZS, Donigian JR, van Overbeek M, Loayza D, Luo Y, Krutchinsky AN, *et al*. (2004b). TIN2 binds TRF1 and TRF2 simultaneously and stabilizes the TRF2 complex on telomeres. *J Biol Chem,* **279,** 47264-47271.

Yeager TR, Neumann AA, Englezou A, Huschtscha LI, Noble JR and Reddel RR. (1999). Telomerase-negative immortalized human cells contain a novel type of promyelocytic leukemia (PML) body. *Cancer Research,* **59,** 4175-4179.

Yi X, Shay JW and Wright WE. (2001). Quantitation of telomerase components and hTERT mRNA splicing patterns in immortal human cells. *Nucleic Acids Res,* **29,** 4818-4825.

Yi X, White DM, Aisner DL, Baur JA, Wright WE and Shay JW. (2000). An alternate splicing variant of the human telomerase catalytic subunit inhibits telomerase activity. *Neoplasia,* **2,** 433-440.

Yokoyama Y, Wan X, Takahashi Y, Shinohara A and Tamaya T. (2001). Alternatively spliced variant deleting exons 7 and 8 of the human telomerase reverse transcriptase gene is dominantly expressed in the uterus. *Mol Hum Reprod,* **7,** 853-857.

Yu CC, Lo SC and Wang TC. (2001). Telomerase is regulated by protein kinase C-zeta in human nasopharyngeal cancer cells. *Biochem J,* **355,** 459-464.

Zaffaroni N, Della PC, Villa R, Botti C, Buglioni S, Mottolese M, *et al*. (2002). Transcription and alternative splicing of telomerase reverse transcriptase in benign and malignant breast tumours and in adjacent mammary glandular tissues: implications for telomerase activity. *J Pathol,* **198,** 37-46.

Zaffaroni N, Villa R, Pastorino U, Cirincione R, Incarbone M, Alloisio M, *et al*. (2005). Lack of telomerase activity in lung carcinoids is dependent on human telomerase reverse transcriptase transcription and alternative splicing and is associated with long telomeres. *Clin Cancer Res,* **11,** 2832-2839.

Zardo G and Caiafa P. (1998). The unmethylated state of CpG islands in mouse fibroblasts depends on the poly(ADP-ribosyl)ation process. *J Biol Chem,* **273,** 16517-16520.

Zeschnigk M, Bohringer S, Price EA, Onadim Z, Masshofer L and Lohmann DR. (2004). A novel real-time PCR assay for quantitative analysis of methylated alleles (QAMA): analysis of the retinoblastoma locus. *Nucleic Acids Res,* **32,** e125.

Zhang W, Piatyszek MA, Kobayashi T, Estey E, Andreeff M, Deisseroth AB, *et al*. (1996). Telomerase activity in human acute myelogenous leukemia: inhibition of telomerase activity by differentiation-inducing agents. *Clin Cancer Res,* **2,** 799-803.

Zhang X, Mar V, Zhou W, Harrington L and Robinson MO. (1999a). Telomere shortening and apoptosis in telomerase-inhibited human tumor cells. *Genes Dev,* **13,** 2388-2399.

Zhang Y, Ng HH, Erdjument-Bromage H, Tempst P, Bird A and Reinberg D. (1999b). Analysis of the NuRD subunits reveals a histone deacetylase core complex and a connection with DNA methylation. *Genes Dev,* **13,** 1924-1935.

Zhao J, Bilsland A, Hoare SF and Keith WN. (2003). Involvement of NF-Y and Sp1 binding sequences in basal transcription of the human telomerase RNA gene. *FEBS Lett,* **536,** 111-119.

Zhao J, Bilsland A, Jackson K and Keith WN. (2005). MDM2 negatively regulates the human telomerase RNA gene promoter. *BMC Cancer,* **5,** 6.

Zhao JQ, Glasspool RM, Hoare SF, Bilsland A, Szatmari I and Keith WN. (2000). Activation of telomerase rna gene promoter activity by NF-Y, Sp1, and the retinoblastoma protein and repression by Sp3. *Neoplasia,* **2,** 531-539.

Zhao JQ, Hoare SF, McFarlane R, Muir S, Parkinson EK, Black DM, *et al*. (1998). Cloning and characterization of human and mouse telomerase RNA gene promoter sequences. *Oncogene,* **16,** 1345-1350.

Zhou XZ and Lu KP. (2001). The Pin2/TRF1-interacting protein PinX1 is a potent telomerase inhibitor. *Cell,* **107,** 347-359.

Zhu J, Wang H, Bishop JM and Blackburn EH. (1999). Telomerase extends the lifespan of virus-transformed human cells without net telomere lengthening. *Proc Natl Acad Sci U S A,* **96,** 3723-3728.

Zhu Q, Liu C, Ge Z, Fang X, Zhang X, Straat K, *et al*. (2008). Lysine-specific demethylase 1 (LSD1) Is required for the transcriptional repression of the telomerase reverse transcriptase (hTERT) gene. *PLoS ONE,* **3,** e1446.

Zhu XD, Kuster B, Mann M, Petrini JHJ and de Lange T. (2000). Cell-cycle-regulated association of RAD50/MRE11/NBS1 with TRF2 and human telomeres. *Nature Genetics,* **25,** 347-352.

Zhu XD, Niedernhofer L, Kuster B, Mann M, Hoeijmakers JHJ and de Lange T. (2003). ERCC1/XPF removes the 3' overhang from uncapped telomeres and represses formation of telomeric DNA-containing double minute chromosomes. *Molecular Cell,* **12,** 1489-1498.

Zinn RL, Pruitt K, Eguchi S, Baylin SB and Herman JG. (2007). hTERT is expressed in cancer cell lines despite promoter DNA methylation by preservation of unmethylated DNA and active chromatin around the transcription start site. *Cancer Research,* **67,** 194-201.

Zou L, Zhang PH, Luo CL and Tu ZG. (2005). Transcript regulation of human telomerase reverse transcriptase by c-myc and mad1. *Acta Biochim Biophys Sin (Shanghai),* **37,** 32-38.

CHAPTER 2

Specific association between the Methyl-CpG binding domain protein 2 and the hypermethylated region of the human telomerase reverse transcriptase promoter in cancer cells.

Stéphanie Bougel[1]*, Amandine Chatagnon[2]*, Laury Perriaud[2], Joël Lachuer[3], Robert Dante [2] and Jean Benhattar[1]

[1] Institut de Pathologie, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Lausanne, Switzerland. [2] INSERM, U590, Lyon, F-69008, France. [3] ProfileXpert/Neurobiotec Service, INSERM U842, Bron, F-69676, France.

*AC and SB equally participated to this work.

Carcinogenesis (2009) 1:28-34.

ABSTRACT

Human telomerase reverse transcriptase (*hTERT*) is expressed in most cancer cells. Paradoxically, its promoter is embedded in a hypermethylated CpG island. A short region escapes to this alteration, allowing a basal level of transcription. However, the methylation of adjacent regions may play a role in the maintenance of low *hTERT* expression. It is now well established that Methyl-CpG Binding Domain proteins mediate the transcriptional silencing of hypermethylated genes. The potential involvement of these proteins in the control of *hTERT* expression was firstly investigated in HeLa cells. Chromatin immunoprecipitation assays showed that only MBD2 associated the hypermethylated *hTERT* promoter. In *MBD2* knockdown HeLa cells, constitutively depleted in MBD2, neither MeCP2 nor MBD1 acted as substitutes for MBD2. MBD2 depletion by transient or constitutive RNA interference led to an upregulation of *hTERT* transcription that can be down-regulated by expressing mouse Mbd2 protein. Our results indicate that MBD2 is specifically and directly involved in the transcriptional repression of *hTERT* in HeLa cells. This specific transcriptional repression was also observed in breast, liver and neuroblastoma cancer cell lines. Thus, MBD2 seems to be a general repressor of *hTERT* in *hTERT*-methylated telomerase-positive cells.

Key words: DNA methylation/ MBD2/ *hTERT*/ transcriptional regulation/ chromatin immunoprecipitation/ ChIP on chip

INTRODUCTION

An increasing body of evidences indicates that the alterations of DNA methylation patterns are a characteristic of cancer cells (1). Generally, global reduction of DNA methylation level is associated with localized hypermethylation (1). Specifically, an aberrant hypermethylation of CpG islands at the 5' end of tumor-suppressor genes, leading to transcriptional repression, has been described both in cancer cell lines and tumor tissues (2,3).

In human epithelial and fibroblast cells, telomere shortening is a key event in replicative senescence. In more than 85% of cancer cells, telomere length is maintained through telomerase holoenzyme activity (4,5). Although germ cells and stem cells also exhibit high telomerase activity (4), in normal somatic cells, the catalytic subunit of the telomerase (*hTERT)* is silenced, leading to a limited life span (6). The regulation of *hTERT* transcription has been extensively investigated and several inductors and repressors have been identified, including c-Myc, Sp1, hALP, Hif-1, Mbi-1, USF1/2, estrogen response element, p53, Mad1, myeloid-specific zinc finger protein 2 (MZF-2), TGF-β, Wilms' Tumor 1 (WT1) and CTCF (7-9). In addition, the *hTERT* promoter region is embedded in a large CpG island spanning nucleotides –1100 to +1500 from the transcription start site, suggesting that transcription of the gene might be regulated by DNA methylation.

The first studies examining the methylation status of the *hTERT* CpG island had led to a paradox. In normal somatic cells, this CpG island was found unmethylated while the gene was transcriptionnally silent. However, in most of cancer cells this region was aberrantly methylated whereas telomerase activities and *hTERT* mRNAs were unambiguously detected (10-13). This paradox was recently solved. *hTERT* methylation prevents the binding of negatively-acting transcription factors such as CTCF inhibitor (9), and a partial hypomethylation of the *hTERT* promoter region can result in some level of transcriptional activity (14). In several cancer cell lines and tumor tissues, careful analysis of *hTERT* methylation patterns has shown that a short region of the CpG island (positions nucleotide (nt) -165 to nt -80) is unmethylated or slightly methylated despite highly methylated border regions (14). This unmethylated region is located in the *hTERT* core promoter (positions nt -279 to nt +5) (15), and chromatin immunoprecipitation (ChIP) assays have shown that active chromatin marks are associated with this unmethylated region (16). Moreover, studies using plasmid expression vectors and patch methylation techniques indicate that the *hTERT* core promoter does not show any activity when all CpG sites are methylated (17). In contrast, the selective demethylation of a small region upstream the transcription start site significantly

activates the *hTERT* promoter in a reporter plasmid. Nevertheless, the activity of the promoter under these conditions is significantly lower than when using the unmethylated core promoter. In this condition, *hTERT* does not provide a real exception to the general model of gene silencing by promoter methylation and the hypermethylation around the unmethylated region seems to play a major role in the reduction of *hTERT* transcriptional activity. Indeed, in telomerase-positive cancer cells only 0.2 to 6 mRNA molecules per cell can be detected (18,19), suggesting that the transcriptional activity of the *hTERT* promoter is limited by cellular factor(s), since high transcription rates are induced when the unmethylated promoter is used in plasmid expression vectors (14).

Transcriptional repression mediated by CpG methylation often involves methyl-CpG binding domain (MDB) proteins. The five MBD proteins identified to date, MeCP2, MBD1, MBD2, MBD3 and MBD4 share a highly conserved MBD. With the exception of MBD4, which is primarily a thymine glycosylase involved in DNA repair (20), all MBD proteins are involved in the transcriptional repression mediated by DNA methylation. It has now been well established that MeCP2, MBD1 and MBD2 bind to methylated DNA and recruit different histone deacetylase complexes (HDAC) and histone methyl transferases (HMT), belonging to the chromatin remodelling complexes that control chromatin compaction and induce gene silencing (21-23). MBD3 lacks a functional MBD but is an integral subunit of the Mi2/NuRD complex which is recruited through MBD2 (24,25).

These data have prompted us to investigate whether MBD proteins are involved in the repression of *hTERT* expression when hypermethylated in telomerase-positive cells.

MATERIALS AND METHODS

Cell culture

Five human tumor cell lines, HeLa (cervical adenocarcinoma), MCF7 (breast adenocarcinoma), HepG2 (hepatocellular carcinoma), LAN-1 (neuroblastoma) and NCCIT (teratocarcinoma), and a normal human embryonic lung fibroblast cell line, MRC5, were used in this study. Cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD), except for LAN-1, and grown in the medium recommended at 37°C in a humidified 5% CO_2 atmosphere.

Chromatin immunoprecipitation (ChIP) assays

ChIP was done as previously described (26). Cross-linked chromatin was immunoprecipitated using 15 µl of two different polyclonal anti-MBD2 antibodies (kindly provided by Dr. P. Wade and Dr. E. Ballestar) or 20 µl of polyclonal anti-MeCP2 (Upstate Biotechnology, Lake Placid, NY), anti-MBD1 (Abcam) antibodies or anti-mouse IgG (Dakocytomation, Trappes, France).

Purified DNA obtained from the input, unbound and bound fractions were quantified by densitometry using the VersaFluor™ Fluorometer (Biorad, Ivry, France) and RiboGreen reagent (Molecular Probes, Interchim, Montluçon, France).

PCR analysis were then performed to asses the binding of MBD proteins to the *hTERT* core promoter. An equal quantity (0.4 ng) of each DNA fraction was amplified by dose-dependent PCR (Supplementary Fig. S1) using HotStar Taq polymerase Kit (Qiagen, Courtaboeuf, France), 5 % of DMSO and 0.5 µM of primers spanning a region from nt -296 to nt -84 of *hTERT* (*hTERT* ChIP, see Supplementary Table S1). The thermal cycler program was 37 cycles of 94°C for 30 s, 65°C for 60 s and 72°C for 75 s. PCR products were analyzed on a 2% agarose gel containing 1 µg/ml ethidium bromide and were quantified by densitometry using a Fluor's fluorimeter and Quantity One software (Biorad).

Primer name	Forward primer	Reverse primer	Tm (°C)
hTERT ChIP	5'-CAG GCC GGG CTC CCA GTG GAT-3'	5'-GGG GCC GCG GAA AGG AAG G-3'	65
hTERT RT-PCR	5'-TGT TTC TGG ATT TGC AGG TG-3'	5'-GTT CTT GGC TTT CAG GAT GG-3'	55
PBGD RT-PCR	5'-GAG TGA TTC GCG TGG GTA CC-3'	5'-GGC TCC GAT GGT GAA GCC-3'	55
FAM-labeled probe	5'-ACC CTG GTC CGA GGT GTC CCT GAG-3'		60
hTERT RT-PCR Q	5'-TGA CAC CTC ACC TCA CCC AC-3'	5'-CAC TGT CTT CCG CAA GTT CAC-3'	60

Table S1. Primers used in the PCR and RT-PCR reactions

ChIP-on-chip

For ChIP-on-Chip analysis, the specific protein-DNA complexes were obtained from independent immunoprecipitations using two different polyclonal anti-MBD2 antibodies (kindly provided by Dr. P. Wade and Dr. E. Ballestar). The ChIP DNAs from the input and bound fractions were amplified, labelled and hybridized on microarrays by ProfileXpert service according to Affymetrix™ protocols. Briefly, the ChIP DNA was amplified by ligation-mediated (LM)-PCR. To test for enrichment of MBD2-bound sites, PCR amplification of *NBR2* (26) and *pS2* promoters (27) was performed on each ChIP samples before and after amplification. The amplified DNAs were then labelled using the GeneChip® WT Double - Stranded DNA Terminal Labelling Kit and hybridized to the human tiling arrays (Human Promoter 1.0R Arrays), which were then washed and scanned. Raw data from the scans were analyzed using Affymetrix® Tiling Analysis Software (TAS) and the results were viewed in Affymetrix' Integrated Genome Browser (IGB) Software.

DNA methylation analysis

Bisulfite sequencing used to determined the CpG methylation pattern of *hTERT* promoter and proximal exonic region was performed as described previously (12,14). Briefly bisulfite-modified genomic DNA was amplified by two primer sets (P1, positions nt -442 to nt -219 (12); and P2, positions nt -206 to nt +108 (14)) to analyse a region from nt -442 to nt +108 from the transcription start site of *hTERT*. PCR amplifications were accomplished using the master mix (Promega, Madison, WI), with the following conditions: 40 cycles of 94°C for 30 s, 54°C (P1) or 57°C (P2) for 45 s and 72°C for 50 s. DNA methylation status was then established by a direct sequencing of PCR products or by a sequencing of cloned PCR products. For the last procedure PCR products were cloned into the pGEM-T vector using the pGEM-T vector system II (Promega). After transformation of JM109 E. Coli competent cells (Promega), plasmid DNA was extracted from clones with the QIAprep Spin Miniprep Kit (Qiagen). Each clone was sequenced with the M13 forward primer (5'-GTAAAACGACGGCCAG-3'), using a Big Dye Terminator Cycle Sequencing Kit, and an ABI Prism 3100 sequencer (Applied Biosystems, Foster City, CA).

Transient transfection

siRNA duplexes for *MBD2* (sense: 5'-GGAGGAAGUGUACCGAAAATT-3', antisense: 5'-UUUUCGGAUCACUUCCUCCTT-3'; Eurogentec, Seraing, Belgium); non-specific siRNA control (Eurogenetec); pRev-Mbd2 (kindly provided by Dr A. Bird) (Auriol *et al.*, 2005), and

pGL3 basic (Promega) were transfected in cell lines using lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Briefly, cells were seeded at 2 x 10^5 cells per well in six-well plates, and grown to 50-60% confluence on the day of transfection. All transfections were done in Opti-MEM medium (Invitrogen) with 625nM of MBD2 siRNA or 1µg of Mbd2 expression plasmid. Lipofectamine 2000 complexes were incubated for 4-5 hours. The medium was then removed and replaced with fresh medium. Cells were grown and harvested at various time after the transfection.

RNA extraction and reverse transcription (RT)–PCR analysis

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen). After extraction, the integrity of total RNA was examined on a 1.2% agarose gel containing 1 µg/ml ethidium bromide and quantified by densitometry using a Fluor's fluorimeter and Quantity One software (Biorad) by comparison with serial dilutions of a standard RNA (Roche, Molecular Biochemicals, Maylan, France).

MBD2 mRNA was quantified by competitive quantitative RT-PCR as previously described (28). *hTERT* mRNA levels were monitored by relative RT-PCR using One Step RT-PCR kit (Qiagen), and 0.1 µg of total RNA. Cycling parameters were 50°C for 30 min followed by 95°C for 15 min, and then 32 cycles of 94°C for 30 s, 55°C for 60 s, and 72°C for 90 s. *hTERT* transcripts were co-amplified with *PBGD* transcripts used as internal controls (for primers *hTERT* RT-PCR and *PBGD* RT-PCR, see Supplementary Table S1). PCR products were analyzed on a 2% agarose gel containing 1 µg/ml ethidium bromide and quantified by densitometry. The ratio between *hTERT* and *PBGD* signals was determined. To quantify more precisely *hTERT* mRNA, real-time RT-PCR were also carried out on a Rotorgene 6000 cycler (Corbett Research, Sydney, Australia) using *C. therm.* Polymerase One-Step RT-PCR System (Roche). Each reaction mixture included 50 ng of template RNA, 250 nM of FAM-labeled probe (see Supplementary Table S1), and 500 nM of primers specific for *hTERT* (*hTERT* RT-PCR Q, see Supplementary Table S1). Cycling parameters were 60°C for 30 min followed by 95°C for 5 min, and then 45 cycles at 95°C for 15 s and 60°C for 60 s. *β-actin* mRNA levels were used to normalize *hTERT* expression. The relative level of each mRNA was calculated on the basis of two standard curves using the relative quantification method.

RESULTS

hTERT hypermethylated CpG island is selectively associated with MBD2 in HeLa cells

To explore the potential involvement of MBD proteins in *hTERT* regulation, we have chosen cervical cancer cell line HeLa, as a first model. In these telomerase-positive tumor cells, as was shown in our earlier studies, the *hTERT* core promoter is also regionally hypermethylated (17) and might be a target of MBD proteins.

To address this issue, ChIP assays were performed using antibodies directed against MeCP2, MBD1 and MBD2. As a control, the fractions immunoprecipitated with a non-MBD protein-specific antibody (anti-mouse IgG) were also analyzed. The relative amounts of *hTERT* core promoter were determined by a dose-dependent PCR assay (Supplementary Figure 1S) using a constant amount of DNA from each fractionation process: input, unbound and bound fractions.

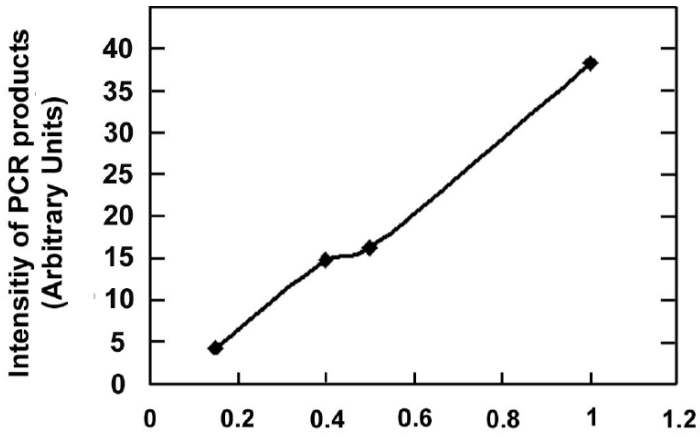


Figure S1. **Dose dependent PCR assay of the *hTERT* promoter DNA in ChIP fractions.** Increasing amounts of DNA from the "input fraction" of ChIP assays were amplified. Data are obtained from 3 independent ChIP assays. The intensity of the PCR products were analyzed on a 2% agarose gel containing 1 µg/ml ethidium bromide and were quantified by densitometry (see materials and methods). A linear relationship between the amount of DNA amplified and the intensity of the signals corresponding to the PCR products was observed over a wide range of "input DNA" (0.1 ng to 1 ng).

Representative data from at least three independent experiments are shown in Figure 1A. A strong enrichment in *hTERT* promoter was observed in the fraction immunoprecipitated by anti-MBD2 antibodies when compared with input or non-retained fractions (Figure 1A "input", "unbound" and "IgG"). Since dose-dependent PCR assays were performed with a constant amount of DNA, these data strongly suggest that MBD2 is associated with the methylated region of the *hTERT* promoter. In contrast, analysis of the fractions immunoprecipitated by anti-MeCP2 or anti-MBD1 antibodies showed that these fractions were depleted in *hTERT* DNA (Figure 1A), indicating that these two proteins are not

bound to *hTERT* promoter in HeLa cells. Furthermore, the depletion in *hTERT* DNA observed in the fractions bound by anti-MeCP2 and anti-MBD1 antibodies suggests that these two proteins are probably linked to other chromatin domains in HeLa cells. Taken together these data strongly suggest that the methylated regions adjacent to the unmethylated region of the *hTERT* core promoter are selectively associated with MBD2.

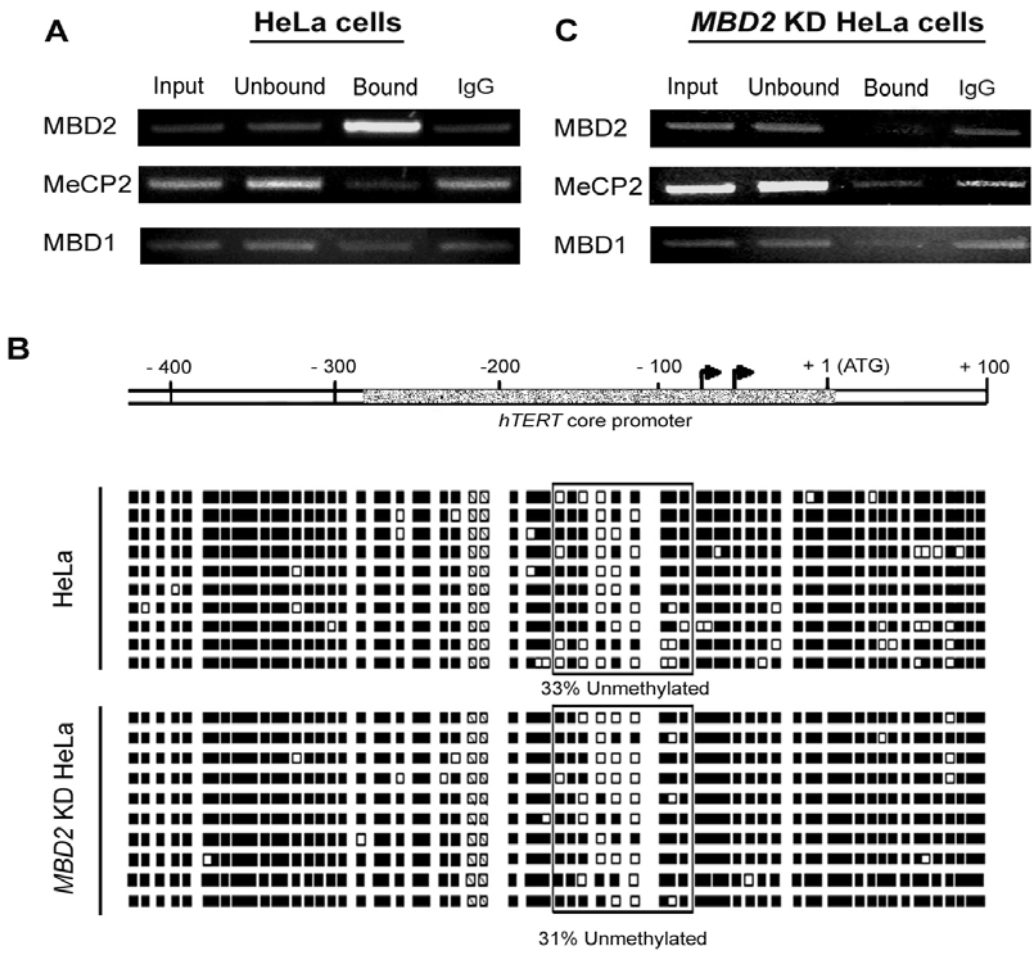


Figure 1. MBD2 specifically binds the hypermethylated region of the *hTERT* promoter in HeLa cells. *(***A**) ChIP analysis of MBD proteins binding to the hypermethylated region of the *hTERT* promoter (positions nt -296 to nt -84 from the ATG translational start site) in HeLa cells. Cross-linked chromatin was immunoprecipitated using anti-MeCP2, anti-MBD1, and anti-MBD2 antibodies or anti-mouse IgG. The relative amounts of *hTERT* core promoter were determined by a dose-dependent PCR assay using a constant amount of DNA from each step of the fractionation process: input, unbound and bound fractions. The intensities of the bands corresponding to representative PCR products amplified from the input, unbound and bound fractions are shown. (**B**) Genomic bisulfite sequencing profiles of *hTERT* promoter and proximal exonic region in wild-type HeLa and *MBD2* KD HeLa cells. A 550 bp region (positions nt -442 to nt +108) of the *hTERT* CpG island is presented on a schematic map. White dotted box, core promoter; black arrows, transcription start sites. Bisulfite-sequencing status of *hTERT* promoter and proximal exonic region are shown (number of analyzed clones for each cell line, n=10). Each line represents a single DNA template molecule. Black and open squares respectively represent methylated and unmethylated CpGs. Sequencing was performed from two different regions which do not overlap (see materials and methods section), leading to unanalyzed CpGs, barred squares. The slightly methylated region (positions nt -165 to nt -80) is framed. (**C**) MeCP2 and MBD1 do not compensate for MBD2 depletion at the *hTERT* promoter in *MBD2* knockdown (KD) HeLa cells. Representative examples of ChIP assays performed in *MBD2* KD HeLa cells are presented.

Recently, we have developed high-throughput analyses of MBD2 binding pattern using a ChIP on chip approach (Chatagnon *et al.*, manuscript in preparation). DNAs obtained from the chromatin immunoprecipitated by anti-MBD2 antibodies were hybridized to Affymetrix Human Promoter 1.0R Array. On this chip, 25,500 human promoter regions tiled at 35bp resolution are representing. Each promoter region covers approximately 7.5 kb upstream through 2.45 kb downstream of 5' transcription start site and for 1,300 cancer-associated genes, additional 2.45 kb are also represented. Data obtained from two independent experiments performed with two different anti-MBD2 antibodies indicated that MBD2 not only bound the hypermethylated region of the *hTERT* core promoter but also covered all the hypermethylated *hTERT* CpG island (Figure 2A). As a control, results obtained for a previously identified MBD2 free hypermethylated promoter (29), *PARVG*, are also shown on Figure 2B. As expected, no MBD2 positive signal was observed along this hypermethylated promoter (Figure 2B).

The hypermethylated hTERT promoter remains free of MBD proteins in MBD2 depleted cells

In HeLa cells, among the *MBD* transcripts, *MBD2* mRNAs are the most abundant (28), suggesting that the selective binding of MBD2 to *hTERT* promoter might be due to its prominent expression. Thus, we investigated *hTERT* promoter occupancy in a HeLa clone cell line (*MBD2* knockdown (KD) HeLa cells) constitutively depleted in MBD2 by a transgene expressing a siRNA targeting the mRNA coding for this protein (26).

Quantitative competitive RT-PCR assays indicated that 89% to 96% *MBD2* mRNA depletion was obtained and maintained over many passages in *MBD2* KD HeLa cells. This low level of *MBD2* transcripts was correlated with a very low level of MBD2 protein, which was almost undetectable in western blot experiments (26). In addition, neither the amounts of MeCP2 and MBD1 transcripts nor the amounts of the corresponding proteins were altered by MBD2 depletion (data not shown).

As MBD2 belongs to the DNA methylation machinery, a prolonged MBD2 depletion might alter DNA methylation patterns. Therefore, the DNA methylation patterns of the *hTERT* promoter were determined in *MBD2* KD HeLa cells. DNA extracted from the cell lines was modified and amplified by PCR with primers specifically designed to amplify bisulfite-modified DNA sequence of the *hTERT* promoter and proximal exonic region. PCR fragments were cloned and sequenced. The analysis of 10 clones from wild-type HeLa cells and *MBD2* KD HeLa cells indicated that over the hypermethylated *hTERT* promoter and proximal exonic region, a small sequence (positions nt –165 to nt –80), corresponding to a

part of the *hTERT* core promoter, was hypomethylated in the two cell lines analyzed (Figure 1B). Indeed, this region exhibit a low level of methylation (about 30%) but no significant difference was observed between wild-type HeLa cells and HeLa cells depleted in MBD2. Thus, the methylation patterns of the *hTERT* promoter in *MBD2* KD HeLa cells were not altered by the absence of MBD2.

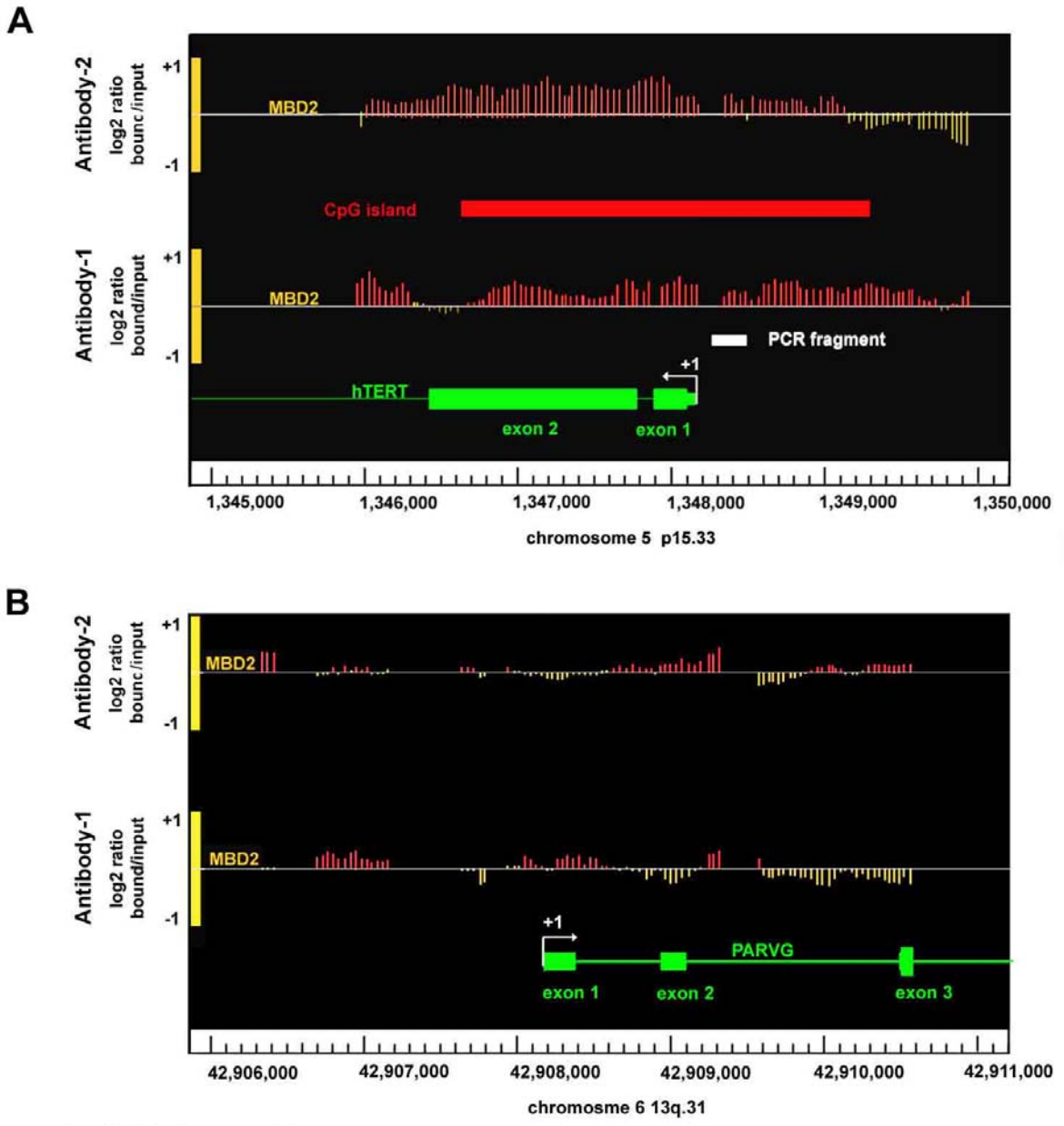


Figure 2. ChIP on chip analysis of MBD2-binding sites on *hTERT* promoter. (**A**) Array peaks on *hTERT* promoter of MBD2 log2 signal ratio (MBD2 / Input) values are shown below the Affymetrix' Integrated Genome Browser (IGB) window. Gene is transcribed from right to left. *hTERT* CpG island is shown by a red box. DNA fragment analyzed by PCR following MBD2 ChIP is represented by a white box. (**B**) *PARVG* 5' end viewed as a MBD2 free control. Gene is transcribed from left to right.

As expected, in HeLa cells depleted in MBD2 proteins, ChIP assays indicated that MBD2 was no longer detected at the hypermethylated region of the *hTERT* core promoter (Figure 1C). Furthermore, this region seemed to remain free of MBD proteins since the immunoprecipitated fractions are depleted in the methylated *hTERT* promoter when anti-MeCP2 or anti-MBD1 antibodies were used in ChIP experiments (Figure 1C).

Thus, the hypermethylated region of the *hTERT* promoter is specifically targeted by MBD2 in HeLa cells and no redundancy between MBD2 and MeCP2 or MBD1 was observed at this hypermethylated region.

MBD2 depletion enhances hTERT gene transcription in HeLa cells

MBD2 is a member of the MBD protein family and their methylation-dependent repressive activities are now well established (30). Therefore, we investigated the potential involvement of MBD2 in the repression of the endogenous *hTERT* promoter in HeLa cells.

First, we determined the consequence of a transient MBD2 depletion on the expression of *hTERT* in HeLa cells. At 24 hours intervals after MBD2-specific siRNA transfection, RNA was extracted and the levels of *hTERT* and *MBD2* transcripts determined by RT-PCR assays were compared with their levels in HeLa cells transfected with a non-specific siRNA. Quantitative competitive RT-PCR assays (28) indicated that a reduction of ~ 90 % in *MBD2* mRNA level was reached 24 hours after transfection with MBD2 siRNA and maintained over 96 hours after transfection (Figure 3A). Western blot analysis also showed a dramatic decrease in MBD2 proteins in these cells (data not shown). In HeLa cells, 48h after the MBD2 siRNA transfection, an elevation of 1.2-fold of *hTERT* mRNA level was observed when compared with HeLa cells transfected with a non-specific siRNA. This stimulation increased to a maximum of 1.9 at 96h after MBD2 siRNA treatment. The expression level of *hTERT* was determined using relative RT-PCR (Figure 3A) and identical results were obtained with real-time RT-PCR for critical points (Figure 3B). It should be noted that neither the level of *MBD2* transcripts nor the level of *hTERT* transcripts were altered by non-specific siRNA transfection when compared with untreated wild-type HeLa cells (data not shown). These data suggest that MBD2 actually represses *hTERT* expression in HeLa cells.

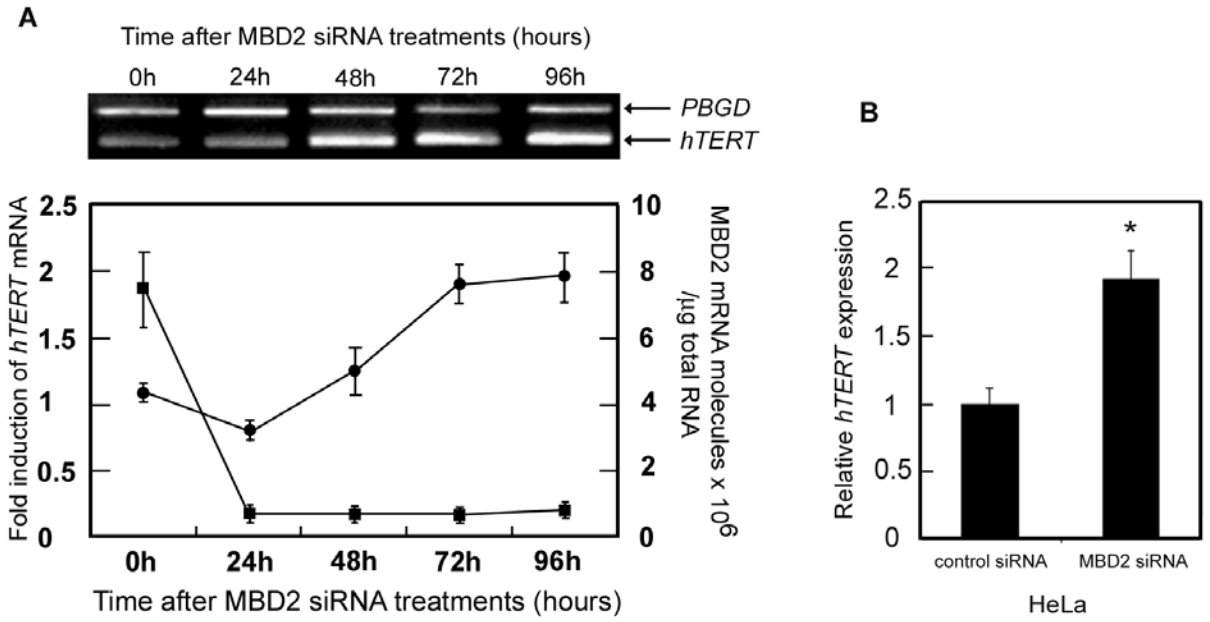


Figure 3. Transient depletion of MBD2 proteins by MBD2-specific siRNA induces time-dependent stimulation of *hTERT* expression in HeLa cells. (**A**) HeLa cells were transiently transfected, with either MBD2 siRNA or a negative control scrambled siRNA. After 24, 48, 72 or 96h following the transfection, RNA was extracted and the efficiency of the MBD2 siRNA treatment was determined by quantification of *MBD2* mRNA as previously described (28). *hTERT* mRNA levels were monitored by relative RT-PCR. The fold induction of *hTERT* expression was calculated from the ratio *hTERT* mRNA / *PBGD* mRNA in MBD2 siRNA transfected cells, versus scrambled siRNA transfected cells. Mean values (± standard deviation) obtained from at least three independent transfection experiments are shown. Black squares, *MBD2* mRNA level; black circles, fold induction of *hTERT* mRNA. (**B**) Quantitative RT-PCR expression analysis of *hTERT* in HeLa cells 96h after transfection with either a negative control siRNA or MBD2 siRNA. Real time RT-PCR was done on cells lines, and *hTERT* expression was normalized to *β-actin*. The relative level of each mRNA was calculated on the basis of the two standard curves using the relative quantification method. At least three independent determinations of fold differences were used to calculate the average fold difference values and associated standard deviation (∗P = 0.0027; t-test).

Stimulation of hTERT transcription in MBD2 KD HeLa cells is reversed by ectopic expression of Mbd2

The *MBD2* KD HeLa cell line offers the opportunity to investigate the functional control of the specific repression of *hTERT* by MBD2. In this cell line, MBD2 expression can be rescued using pRev-Mbd2, a vector coding for a mouse *Mbd2* RNA containing five silent point mutations and, therefore, refractory to siRNA-mediated decay (26). We also observed a 2-fold stimulation of *hTERT* expression in the *MBD2* KD HeLa cells as compared to wild-type HeLa cells (Figure 4). In these cells, the ectopic expression of Mbd2 reduced *hTERT* transcription to a level similar to the level observed in wild-type HeLa cells (Figure 4), suggesting that MBD2 directly represses *hTERT* expression in HeLa cells. In wild-type HeLa cells, the abundance of MBD2 does not seem to be a limiting factor since the overexpression

of MBD2 mediated by pRev-Mbd2 transfection did not modify *hTERT* expression level in cells containing normal amounts of MBD2 (Figure 4).

Our results demonstrate that MBD2 specifically binds to *hTERT* and represses its expression in HeLa cells.

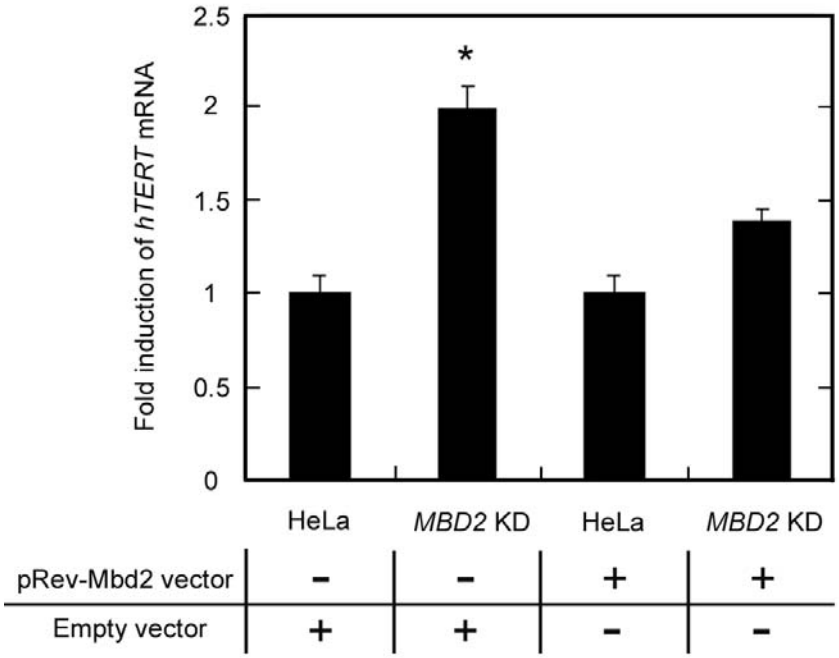


Figure 4. Mbd2 expression rescues the reduction of *hTERT* transcript in *MBD2* knockdown HeLa cells. The transcriptional expression of *hTERT* was analyzed by quantitative RT-PCR 48h after lipofectamine transfection of HeLa and *MBD2* knockdown HeLa cells using pRev-Mbd2, an Mbd2 vector expressing a transcript resistant to RNAi, or an empty pGL3 basic vector. The relative *hTERT* mRNA in pRev-Mbd2 transfected cells or in pGL3 transfected cells or in KD HeLa cells was calculated using the same reference: the *hTERT* mRNA level in untransfected HeLa cells. Mean values obtained from at least three independent transfection experiments are shown (∗P = 0.0035, *t*-test).

MBD2 hTERT repression is specifically observed in hTERT-methylated telomerase-positive cancer cell lines and independent of the cellular types

Data obtained suggest that MBD2 might be general repressor of *hTERT* transcription in *hTERT*-methylated telomerase-positive cells. In order to address this point, we tested whether the above-mentioned *MBD2* knockdown-mediated *hTERT* stimulation in HeLa cells could be recapitulated in other *hTERT*-methylated cell lines. A functional study was undertaken in different cell lines exhibiting characteristic *hTERT* DNA methylation patterns: (i) MCF-7, LAN-1 and HepG2 cells, three telomerase-positive cancer cell lines showing an *hTERT* core promoter unmethylated or slightly methylated despite an hypermethylated CpG island (ii) NCCIT cells, an *hTERT*-unmethylated telomerase-positive teratocarcinoma cell line, and (iii)

MRC5 cells, an *hTERT*-unmethylated telomerase-negative normal embryonic cell line. The *hTERT* core promoter methylation status of these cell lines and the corresponding transcript levels are shown in Figure 5. Control experiments indicated that these cell lines exhibited approximatively the same level of MBD2 transcripts ($4.8 \pm 2,7 \times 10^6$ mRNA molecules/µg of total RNA), with the exception of NCCIT (7×10^4 mRNA molecules/µg of total RNA).

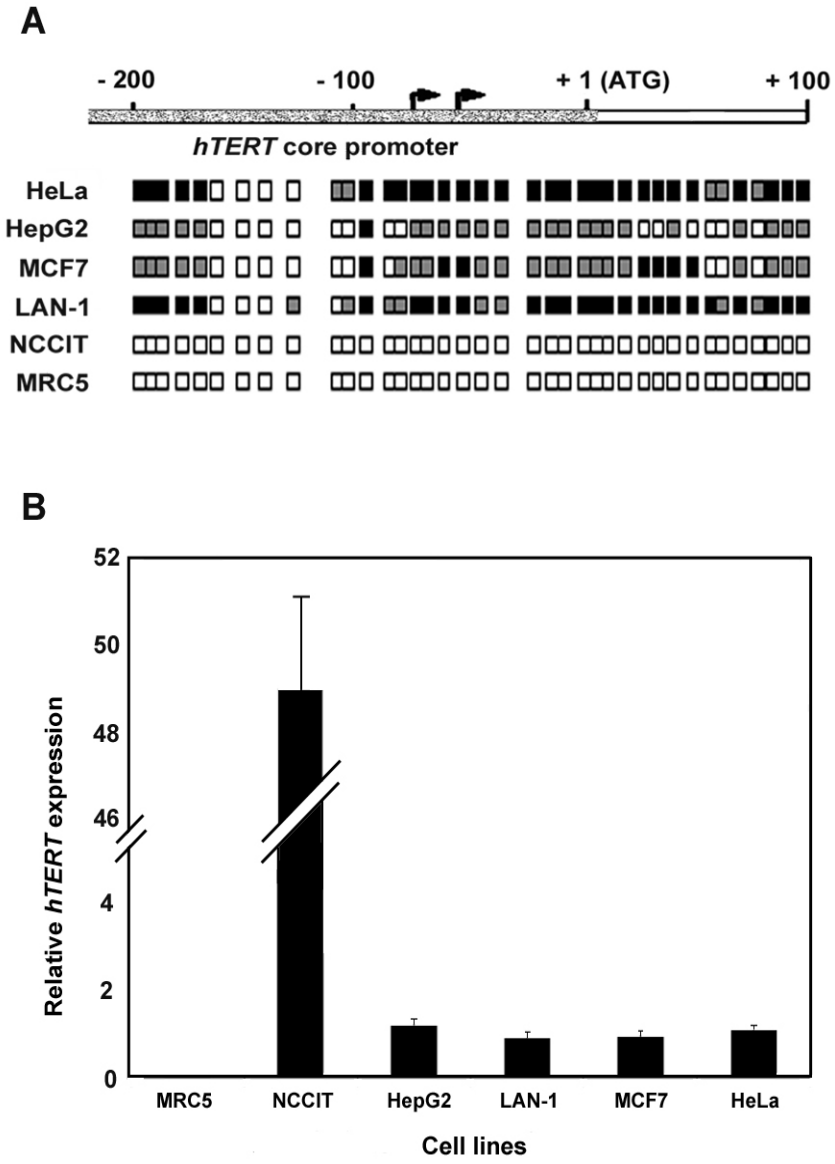
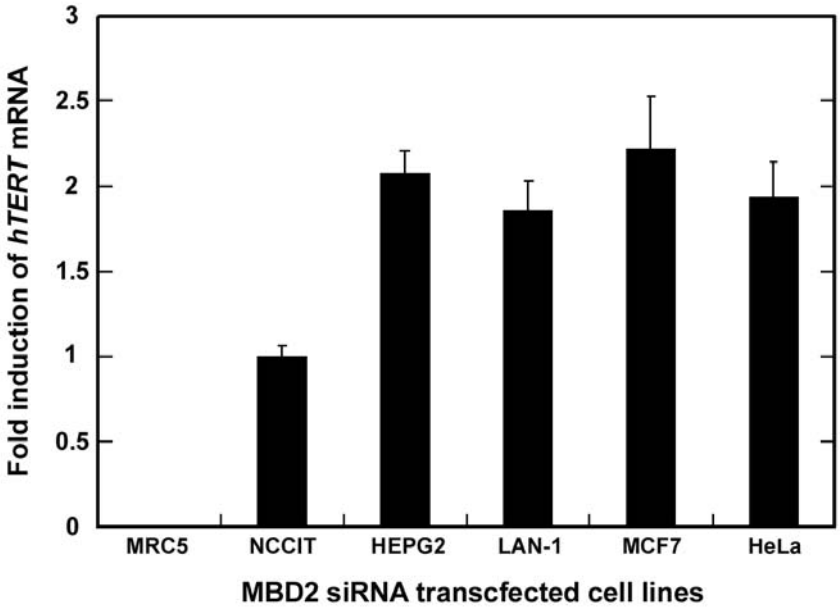


Figure 5. DNA methylation patterns of *hTERT* and the corresponding transcript levels in different cell lines. (**A**) Bisulfite sequencing of five telomerase-positive cancer cell lines (HeLa, MCF7, HepG2, LAN-1 and NCCIT) and one normal telomerase-negative fibroblast cell line (MRC5). Bisulfite-treated DNA was PCR amplified using a primer set spanning the end of the promoter and the proximal exonic region of *hTERT*. PCR products were then directly sequenced. The analyzed 314 bp region (-206 to +108) is presented on a schematic map and results are shown for each cell lines. Black and open squares respectively represent complete methylated and unmethylated CpG sites, whereas grey squares correspond to partial methylated CpG sites. (**B**) Quantitative expression of *hTERT* in MRC5, NCCIT, HepG2, LAN-1, MCF7 and HeLa cells. Real time RT-PCR was done on cell lines, and *β-actin* was used as a reference.

As observed in HeLa cells, a significant decrease of *MBD2* transcripts (60-79%) was observed in the different cell lines 96h after MBD2 siRNA transfection. Relative to the transfection with a non-specific siRNA, a ~2-fold increased in *hTERT* mRNA was observed in all *hTERT*-methylated cell lines, LAN-1, MCF7, and HepG2 cells. In contrast, MBD2 depletion did not affect *hTERT* expression in *hTERT*-unmethylated cell lines, NCCIT, and MRC5 cells (Figure 6). Since MBD2 does no bind to unmethylated DNA (24,25), these data indicate that *hTERT* induction due to MBD2 depletion is not mediated by an indirect effect.



Efficiency of MBD2 siRNA	91% ± 9%	63% ± 5%	63% ± 7%	73% ± 6%	72% ± 3%	87% ± 7%

Figure 6. Depletion of MBD2 by siRNA results in transcriptional activation of *hTERT* in *hTERT*-methylated telomerase-positive cancer cell lines. Fold change of *hTERT* expression between MBD2 depleted cells (MBD2 siRNA transfected cells) and control cells (scrambled siRNA transfected cells). *hTERT* expression was monitored by quantitative RT-PCR 96h after transfection. Mean values (± standard deviation) obtained from at least three independent transfection experiments are shown.

Taken together, the specific transcriptional repression of *hTERT* by MBD2 does not seem to be restricted to a particular cancer cell line since this effect was observed in cervix, breast, liver and neuroblastoma cancer cell lines. Thus, MBD2 seems to be a general repressor of *hTERT* in *hTERT*-methylated telomerase-positive cells.

DISCUSSION

Most (about 96%) of the CpG islands are unmethylated in normal cells (31), while hypermethylation of these sequences is a characteristic of cancer cells (1). These aberrant DNA methylation patterns have been correlated with the transcriptional silencing of genes undergoing such alterations at their 5' end CpG islands. The *hTERT* gene has provided an interesting exception, since a bimodal alteration of the DNA methylation status of its 5' end CpG island is associated with its expression in about 85% of cancer cells and tumors tissues (4,5). The large CpG island, 2.6 kb in length, lying from nucleotide nt –1102 to nt +1519 from the *hTERT* transcription, is hypermethylated at the exception of a short region (positions nt -165 to nt -80) which is unmethylated or slightly methylated despite highly methylated border regions (16,17). This particular pattern of methylation seems crucial for establishing *hTERT* expression at a basal level. Indeed, hypermethylation of CpG islands seem to be a crucial event in carcinogenesis. Thus, the methylation free region in *hTERT* promoter may result from antagonistic pressure between the mechanisms leading to aberrant methylation and the need to keep *hTERT* expressed for unlimited life-span of cancer cells.

A body of evidence has been accumulated concerning association between hypermethylation of CpG islands, transcriptional silencing and MBD proteins binding (21). ChIP experiments and ChIP on chip analysis indicate that MBD2 associated the hypermethylated CpG island of *hTERT*. In this context, MBD2 seems to be a limiting factor rather than a transcriptionnal silencer.

Recently, a large screening of tumor suppressor gene promoters in ten cell lines showed that human cancer cell lines tend to use a particular MBD protein (32). Furthermore, in MRC5 cells, ChIP experiments have indicated that MeCP2 and MBD2 proteins have non-overlapping binding specificities *in vivo* (23). These data, as well as our data are in favour of the "one gene-one MBD" hypothesis, at least for some genes. Indeed, MBD2 was specifically associated with the methylated region of the *hTERT* CpG island, while MeCP2 and MBD1 were not detected at this locus. Moreover, MBD2 depletion did not induce MeCP2 or MBD1 binding at this methylated area in HeLa cells. Thus, the specificity of MBD proteins does not seem to be driven by their relative concentrations in a cell line, as it was suggested in an other study (32): a strong expression of a particular MBD is not necessarily associated with its preferential use in promoters.

Several studies have shown that transcriptional activation could be realized upon depletion of MBD proteins by RNAi (26,29). A large scale microarray analysis indicated that 15% of 6386 genes analyzed exhibit an increased expression change between untreated and triple MBD-depleted cells (29). It should be noted that for single MBD interference, MBD2 depletion was the protein most commonly involved in the observed release of gene silencing by far (29). These data suggest that MBD2 plays an important role in methylation dependent gene silencing. MBD2 depletion mediated by RNAi stimulates *hTERT* expression, either in stable knockdown HeLa clones or in transiently transfected HeLa cells. A 2-fold stimulation of *hTERT* expression upon *MBD2* depletion was observed, suggesting that MBD2 plays an important role in the regulation of this gene. Indeed, in human cell lines fold changes induced by MBD2 depletion is also of the same range, 2-fold for NBR2 induced by MBD2 siRNA (26) and microarray analysis of MBD2 depleted cells exhibited a mean fold change of 7 (29). Furthermore, the fold change observed in Mbd2-deficient mouse cells is not very different from our own data. For example, it has been shown (33) that $Mbd2^{-/-}$ fibroblasts had 3-fold higher levels of Xist than wild type cells. Moreover, elevated *hTERT* transcription upon MBD2 depletion is not limited to cervix (HeLa cells), since this effect was observed in breast, liver and neuroblastoma cancer cell lines. MBD2 RNAi experiments in cell lines exhibiting unmethylated *hTERT* CpG island (MRC5, NCCIT) did not affect *hTERT* transcription indicating that MBD2 specifically and directly represses *hTERT* expression in methylation dependent manner. Collectively these data strongly suggest that MBD2 is a general repressor of *hTERT* expression in cancer cells.

A considerable number of transcription factors have been proposed as negative regulators of *hTERT* transcription. Nevertheless direct evidence indicating that *hTERT* gene transcription is down-regulated remains to be firmly established. Thus MBD2 may represent a new factor directly involved in the negative regulation of *hTERT* expression. In cancer cells MBD2 seems to play a specific role since its binding to the hypermethylated part of the *hTERT* promoter establishes a direct link between a common DNA alteration of tumor cells, CpG hypermethylation, and a reduced level of *hTERT* transcription. The binding of MBD2 represents an additional layer for the control of *hTERT* expression, thereby underlying the importance of *hTERT* regulation in immortalized and cancers cells.

ACKNOLEDGEMENTS

The present work was supported by the Ligue Nationale contre le Cancer (Comité du Rhône and Comité de la Loire), the Institut National contre le Cancer (EpiPro, CircBio and grant 419807-37/NG-LC) and the Swiss National Science Foundation (Grants 3100A0-101732 and 3100A0-113505).

REFERENCES

1. Ehrlich, M. (2002) DNA methylation in cancer: too much, but also too little. *Oncogene*, **21**, 5400-5413.

2. Esteller, M. (2007) Cancer epigenomics: DNA methylomes and histone-modification maps. *Nat Rev Genet*, **8**, 286-298.

3. Herman, J.G. and Baylin, S.B. (2003) Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med*, **349**, 2042-2054.

4. Kim, N.W., Piatyszek, M.A., Prowse, K.R., Harley, C.B., West, M.D., Ho, P.L., Coviello, G.M., Wright, W.E., Weinrich, S.L. and Shay, J.W. (1994) Specific association of human telomerase activity with immortal cells and cancer. *Science*, **266**, 2011-2015.

5. Stampfer, M.R. and Yaswen, P. (2003) Human epithelial cell immortalization as a step in carcinogenesis. *Cancer Lett*, **194**, 199-208.

6. Yasumoto, S., Kunimura, C., Kikuchi, K., Tahara, H., Ohji, H., Yamamoto, H., Ide, T. and Utakoji, T. (1996) Telomerase activity in normal human epithelial cells. *Oncogene*, **13**, 433-439.

7. Goueli, B.S. and Janknecht, R. (2003) Regulation of telomerase reverse transcriptase gene activity by upstream stimulatory factor. *Oncogene*, **22**, 8042-8047.

8. Poole, J.C., Andrews, L.G. and Tollefsbol, T.O. (2001) Activity, function, and gene regulation of the catalytic subunit of telomerase (hTERT). *Gene*, **269**, 1-12.

9. Renaud, S., Loukinov, D., Bosman, F.T., Lobanenkov, V. and Benhattar, J. (2005) CTCF binds the proximal exonic region of hTERT and inhibits its transcription. *Nucleic Acids Res*, **33**, 6850-6860.

10. Dessain, S.K., Yu, H., Reddel, R.R., Beijersbergen, R.L. and Weinberg, R.A. (2000) Methylation of the human telomerase gene CpG island. *Cancer Res*, **60**, 537-541.

11. Devereux, T.R., Horikawa, I., Anna, C.H., Annab, L.A., Afshari, C.A. and Barrett, J.C. (1999) DNA methylation analysis of the promoter region of the human telomerase reverse transcriptase (hTERT) gene. *Cancer Res*, **59**, 6087-6090.

12. Guilleret, I., Yan, P., Grange, F., Braunschweig, R., Bosman, F.T. and Benhattar, J. (2002) Hypermethylation of the human telomerase catalytic subunit (hTERT) gene correlates with telomerase activity. *Int J Cancer*, **101**, 335-341.

13. Nomoto, K., Maekawa, M., Sugano, K., Ushiama, M., Fukayama, N., Fujita, S. and Kakizoe, T. (2002) Methylation status and expression of human telomerase reverse transcriptase mRNA in relation to hypermethylation of the p16 gene in colorectal cancers as analyzed by bisulfite PCR-SSCP. *Jpn J Clin Oncol*, **32**, 3-8.

14. Renaud, S., Loukinov, D., Abdullaev, Z., Guilleret, I., Bosman, F.T., Lobanenkov, V. and Benhattar, J. (2007) Dual role of DNA methylation inside and outside of CTCF-binding regions in the transcriptional regulation of the telomerase hTERT gene. *Nucleic Acids Res*, **35**, 1245-1256.

15. Horikawa, I., Cable, P.L., Afshari, C. and Barrett, J.C. (1999) Cloning and characterization of the promoter region of human telomerase reverse transcriptase gene. *Cancer Res*, **59**, 826-830.

16. Zinn, R.L., Pruitt, K., Eguchi, S., Baylin, S.B. and Herman, J.G. (2007) hTERT is expressed in cancer cell lines despite promoter DNA methylation by preservation of unmethylated DNA and active chromatin around the transcription start site. *Cancer Res*, **67**, 194-201.

17. Guilleret, I. and Benhattar, J. (2004) Unusual distribution of DNA methylation within the hTERT CpG island in tissues and cell lines. *Biochem Biophys Res Commun*, **325**, 1037-1043.

18. Yi, X., Shay, J.W. and Wright, W.E. (2001) Quantitation of telomerase components and hTERT mRNA splicing patterns in immortal human cells. *Nucleic Acids Res*, **29**, 4818-4825.

19. Ducrest, A.L., Amacker, M., Mathieu, Y.D., Cuthbert, A.P., Trott, D.A., Newbold, R.F., Nabholz, M. and Lingner, J. (2001) Regulation of human telomerase activity: repression by normal chromosome 3 abolishes nuclear telomerase reverse transcriptase transcripts but does not affect c-Myc activity. *Cancer Res*, **61**, 7594-7602.

20. Hendrich, B., Hardeland, U., Ng, H.H., Jiricny, J. and Bird, A. (1999) The thymine glycosylase MBD4 can bind to the product of deamination at methylated CpG sites. *Nature*, **401**, 301-304.

21. Bird, A.P. and Wolffe, A.P. (1999) Methylation-induced repression--belts, braces, and chromatin. *Cell*, **99**, 451-454.

22. Fatemi, M. and Wade, P.A. (2006) MBD family proteins: reading the epigenetic code. *J Cell Sci*, **119**, 3033-3037.

23. Klose, R.J., Sarraf, S.A., Schmiedeberg, L., McDermott, S.M., Stancheva, I. and Bird, A.P. (2005) DNA binding selectivity of MeCP2 due to a requirement for A/T sequences adjacent to methyl-CpG. *Mol Cell*, **19**, 667-678.

24. Ng, H.H., Zhang, Y., Hendrich, B., Johnson, C.A., Turner, B.M., Erdjument-Bromage, H., Tempst, P., Reinberg, D. and Bird, A. (1999) MBD2 is a transcriptional repressor belonging to the MeCP1 histone deacetylase complex. *Nat Genet*, **23**, 58-61.

25. Wade, P.A., Gegonne, A., Jones, P.L., Ballestar, E., Aubry, F. and Wolffe, A.P. (1999) Mi-2 complex couples DNA methylation to chromatin remodelling and histone deacetylation. *Nat Genet*, **23**, 62-66.

26. Auriol, E., Billard, L.M., Magdinier, F. and Dante, R. (2005) Specific binding of the methyl binding domain protein 2 at the BRCA1-NBR2 locus. *Nucleic Acids Res*, **33**, 4243-4254.

27. Martin, V., Ribieras, S., Song-Wang, X.G., Lasne, Y., Frappart, L., Rio, M.C. and Dante, R. (1997) Involvement of DNA methylation in the control of the expression of an estrogen-induced breast-cancer-associated protein (pS2) in human breast cancers. *J Cell Biochem*, **65**, 95-106.

28. Billard, L.M., Magdinier, F., Lenoir, G.M., Frappart, L. and Dante, R. (2002) MeCP2 and MBD2 expression during normal and pathological growth of the human mammary gland. *Oncogene*, **21**, 2704-2712.

29. Lopez-Serra, L., Ballestar, E., Ropero, S., Setien, F., Billard, L.M., Fraga, M.F., Lopez-Nieva, P., Alaminos, M., Guerrero, D., Dante, R. and Esteller, M. (2008) Unmasking of epigenetically silenced candidate tumor suppressor genes by removal of methyl-CpG-binding domain proteins. *Oncogene*, **27**, 3556–3566.

30. Klose, R.J. and Bird, A.P. (2006) Genomic DNA methylation: the mark and its mediators. *Trends Biochem Sci*, **31**, 89-97.

31. Weber, M., Hellmann, I., Stadler, M.B., Ramos, L., Paabo, S., Rebhan, M. and Schubeler, D. (2007) Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. *Nat Genet*, **39**, 457-466.

32. Lopez-Serra, L., Ballestar, E., Fraga, M.F., Alaminos, M., Setien, F. and Esteller, M. (2006) A profile of methyl-CpG binding domain protein occupancy of hypermethylated promoter CpG islands of tumor suppressor genes in human cancer. *Cancer Res*, **66**, 8342-8346.

33. Barr, H., Hermann, A., Berger, J., Tsai, H.H., Adie, K., Prokhortchouk, A., Hendrich, B. and Bird, A. (2007) Mbd2 contributes to DNA methylation-directed repression of the Xist gene. *Mol Cell Biol*, **27**, 3750-3757.

34. Lin, X. and Nelson, W.G. (2003) Methyl-CpG-binding domain protein-2 mediates transcriptional repression associated with hypermethylated GSTP1 CpG islands in MCF-7 breast cancer cells. *Cancer Res*, **63**, 498-504.

35. Curradi, M., Izzo, A., Badaracco, G. and Landsberger, N. (2002) Molecular mechanisms of gene silencing mediated by DNA methylation. *Mol Cell Biol*, **22**, 3157-3173.

CHAPTER 3

PAX5 plays a key role in the transcriptional activation of the human telomerase reverse transcriptase gene in B cells

Stéphanie Bougel,[1] Stéphanie Renaud,[2] Richard Braunschweig,[1] Dmitri Loukinov,[2] Herbert C Morse III, [2] Fred T. Bosman,[1] Victor Lobanenkov[2] and Jean Benhattar[1]

[1]Institute of Pathology, Centre Hospitalier Universitaire Vaudois and University of Lausanne, CH-1011 Lausanne, Switzerland and [2]Laboratory of Immunopathology, NIAID, NIH, Rockville, MD 20815

Manuscript submitted

ABSTRACT

Telomerase is an RNA-dependent DNA polymerase that synthesizes telomeric DNA. Its activity is not detectable in most somatic cells, but it is reactivated during tumorigenesis. In most cancers, the combination of hTERT hypermethylation and hypomethylation of a short promoter region is permissive for low-level hTERT transcription. Activated and malignant lymphocytes express high telomerase activity, through a mechanism that seems methylation independent. The aim of this study was to determine which mechanism is involved in the enhanced expression of hTERT in lymphoid cells. Our data confirm that in B cells, some T cell lymphomas, and in non-neoplastic lymph nodes, the hTERT promoter is unmethylated. Binding sites for the B cell-specific transcription factor PAX5 were identified downstream the ATG translational start site through EMSA and ChIP experiments. ChIP assays indicated that the transcriptional activation of hTERT by PAX5 does not involve repression of CTCF binding. In a B-cell lymphoma cell line, siRNA-induced knockdown of PAX5 expression repressed hTERT transcription. Moreover, ectopic expression of PAX5 in a telomerase-negative normal fibroblast cell line was found to be sufficient to activate hTERT expression. These data show that activation of hTERT in telomerase-positive B cells is due to a methylation-independent mechanism in which PAX5 plays a crucial role.

Key words: hTERT, PAX5, CTCF, B cells, telomerase, DNA methylation

INTRODUCTION

The telomerase enzyme allows germ cells, stems cells and cancer cells to divide indefinitely (1). Human telomerase possesses a highly regulated subunit called hTERT, for telomerase reverse transcriptase, which is the limiting factor for its activity (2,3). The hTERT expression is nearly imperceptible in the majority of differentiated somatic cells, which lead to inevitable telomeric attrition and subsequently cellular senescence. High levels of hTERT are detected in proliferative somatic cells like endometrial tissues or activated lymphocytes, but also in most immortalized and cancer cells.

The hTERT transcription has been shown to be influenced by numerous activators and inhibitors, such as c-Myc, Sp1, Hif-1, Mbi-1, USF1/2, estrogen response element, p53, Mad1, myeloid-specific zinc finger protein 2 (MZF-2), TGF-β, Wilms' Tumor 1 (WT1) and CTCF (4-6). In addition, a possible role of DNA methylation in hTERT transcription regulation can be expected, as the hTERT promoter is situated within a dense CG-rich CpG island. In normal somatic cells, the hTERT promoter is unmethylated although the transcription of the gene is repressed. However, in most cancer cells, hypermethylation of this region correlates with expression of the gene and with perceptible telomerase activity (7-10). This apparent contradiction with the classical mechanism of transcriptional repression by DNA methylation was recently clarified. DNA methylation exhibits a dual role in hTERT transcriptional regulation by interfering with the binding of inhibitors, such as the CTCF transcription factor, and by partial hypomethylation of the core promoter, which allows the hTERT gene to be permissive for transcription (11).

In a small subset of telomerase-positive tumors, hTERT expression appears to be regulated by a methylation-independent mechanism (12-14). For example, the hTERT promoter is methylated in only 30% of ovarian cancers, almost all of which are telomerase positive (13). Cells of the lymphoid system also seem to escape methylation-dependant mechanism of hTERT regulation. Lymphocytes express telomerase during development, and turn off its activity after maturation in response to a specific antigen (15). Leukemias and lymphomas, including B-cell chronic lymphocytic leukemia (CLL), express high levels of telomerase but exhibit low levels of hTERT promoter methylation (12). More recently, the acute myeloblastic leukemic cell line (HL-60) and Burkitt lymphoma (Raji) cell lines as well as normal lymphocytes were found to have hypomethylated hTERT promoters (14).

Paired box (PAX) proteins include nine members that are important regulators in early development for tissue specificity (16). Once bound to DNA, PAX proteins can play the role of transcriptional activators or repressors (17-19). Deregulation of PAX genes has been associated with a variety of cancers, including astrocytoma, medulloblastoma, lymphoma, and Wilm's tumor (20,21). Moreover, PAX expression has been suggested to be essential for survival of cancer cells. Recently, PAX8 has been implicated in the activation of hTERT and hTR promoters, which in turn activate telomerase in glioma (22). PAX2, PAX5, and PAX8 belong to the same subgroup, and thus could impact on hTERT regulation in a tissue-specific manner.

During B-cell development, the PAX5 gene is expressed in early B cell precursors (pro-B cells) and continues to be expressed up to mature B cells, but not in terminally differentiated plasma cells (23,24). As a consequence, PAX5 expression is used as a lineage-specific marker in B-cells neoplasms (25,26). Accordingly, a large study revealed that PAX5 is expressed in 91.5% B-cell non-Hodgkin lymphomas (B-NHL) and in 85.7% Hodgkin lymphomas (HL) (27). PAX5 has been shown to promote the expression of target genes encoding crucial components of the (pre)BCR signaling cascade, such as the receptor signaling chain Igα, also called CD79a and mb-1 (24,28), the costimulatory receptor CD19 (29,30), and the central adaptor protein BLNK (31). PAX5 also facilitates the VH-DJH recombination step and can activate other transcription factor genes. Overall, the pleiotropic role of PAX5 is involved in control of the B-lineage commitment and simultaneously suppression of other lineage-specific genes (29,32,33).

Our working hypothesis for the experiments reported in this paper was that hTERT regulation in B-cells is methylation independent and involves an activating role of the PAX5 transcription factor. Our data confirm that hTERT expression in telomerase-positive B cells is methylation independent. We found that PAX5 activates the hTERT promoter, supporting the hypothesis that PAX5 is a critical determinant of hTERT expression in telomerase-positive B cells.

EXPERIMENTAL PROCEDURES

Cell culture

The Burkitt lymphoma cell lines, Daudi and Ramos, and the pre-B cell leukemia line, Nalm6, were kindly provided by Dr. Benedicte Baisse (CHUV, Lausanne, Switzerland). The Burkitt line, Raji, was kindly provided by Apoxis (Lausanne, Switzerland). The cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated Fetal Bovine Serum (HI-FBS; Invitrogen, Basel, Switzerland). Normal BJ fibroblasts were obtained from and grown in the medium recommended by ATCC (Manassas, VA).

Tissue Samples

The lymphomas and normal tissues came from the files of the Institute of Pathology of Lausanne. The samples included: 4 histologically non-neoplastic lymphoid tissues (2 lymph node biopsies and 2 tonsil biopsies); 6 B-cell non-Hodgkin lymphomas (NHL), comprising 3 high-grade NHL and 3 low-grade NHL; and 6 T-cell NHL. All diagnosis were confirmed by a pathologist (R.B.). The lymphoma cases were selected to ensure that sufficient populations of T or B cells were present. The use of human tissues for this study was done according to the guidelines of the local ethics committee.

RT-PCR

Total RNA of frozen tissues and cultured cells was extracted using Trizol-LS (Invitrogen, Basel, Switzerland) according to the manufacturer's protocol. The extraction protocol for fixed tissues was described previously (34). Both cDNA synthesis and PCR were performed in a single tube using SuperScript One-Step RT-PCR or Quantitative RT-PCR ThermoScript™ One-Step System (Invitrogen). Primers and RT-PCR for each individual gene are described in the supplemental Table S1. The amplification products were analyzed by electrophoresis on 2% agarose gels.

Table S1 - Primers sequences for RT-PCR

Gene	Primer sequences (sense and antisense)	PCR product	Program
hTERT	5' CGG AAG AGT GTC TGG AGC AA 3' 5' GGA TGA AGC GGA GTC TGG A 3'	145 bp	RT: 60°C (THERMOSCRIPT™) PCR: Tm = 60°C - 40 cycles
PAX5	5'-GAGCGGGTGTGTGACAATGA -3' 5'-GCACCGGAGACTCCTGAATAC -3'	265 bp	RT: 50 °C (SUPERSCRIPT™) PCR: Tm = 64°C - 30 cycles
β-actin	5' AGG CCA ACC GCG AGA AGA TGA 3' 5' GCC GTG GTG GTG AAG CTG TAG 3'	273 bp	RT: 50 °C (SUPERSCRIPT™) PCR: Tm = 60°C - 30 cycles
CD19	5'-GTGGCAACCTGACCATGTCATT -3' 5'-GACCAGGGCTCTTTGAAGATGA -3'	167 bp	RT: 50 °C (SUPERSCRIPT™) PCR: Tm = 58°C - 40 cycles

DNA methylation analysis

DNA was extracted from frozen and fixed tissues, and cultured cells using the DNeasy tissue kit (Qiagen). Two μg of DNA were modified with sodium bisulfite and used to amplify a 224 bp fragment of the hTERT promoter as previously described (35). PCR products were analyzed by a methylation-sensitive dot blot assay (MS-DBA) (35) and confirmed by direct sequencing (9) and methylation-sensitive single-strand conformation analysis (MS-SSCA) (36,37).

Analysis of the hTERT promoter

Potential binding sites for transcription factors in the hTERT promoter were identified using MatInspector software (http://www.genomatix.de/matinspector.html).

Electrophoretic mobility shift assay (EMSA)

Double strand oligonucleotides of the hTERT exonic region and the CD79A promoter region (hTERT sense: 5'-GCTGGTGCAGCGCGGGGACCCGGCGGCTTT-3'; CD79A: sense 5'-AGCGAGGGCCACTGGAGCCCATCTCCGGGG-3') were labeled with the DIG-Oligonucleotide 3'-End Labeling Kit (Roche). Cellular extracts were obtained by incubating the Nalm6 and Raji cell lines with a Triton lysis buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 2.5 mM β-

glycerophosphate, 1 mM NaF, 1 mM DTT, Complete protease inhibitor). Gel shift reactions were performed using the DIG Gel Shift Kit (Roche) with 0.5 pmol of DIG-labeled oligonucleotide and 5 µg of cell extract. A supershift assay was performed with a PAX5 rabbit antibody (Active Motif, Carlsbad) on Raji cell extracts according to the manufacturer's protocol. One quarter of the reaction solution was loaded on a 6% polyacrylamide gel. The transfer was done by electroblotting on a nylon membrane using a transblot semi-dry system (Bio-Rad, Hercules, CA), for 90 min at 9 V. After crosslinking, the generated chemiluminescence signals were recorded on X-ray film. A negative control was performed without cell extract. Competition assays with unlabeled oligonucleotides were performed with the same double strand oligonucleotides for hTERT and CD79A.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed using EZ ChIP (Upstate Biotechnology, Lake Placid, NY) following the manufacturer's instructions with some modifications. A 1% formaldehyde solution was added to the cultured cells to crosslink proteins to DNA. Ten million cells were resuspended in 500 µL of SDS lysis buffer (2 mL for CTCF) and incubated on ice for 15 minutes. After sonication to shear DNA, lysates from $2x10^6$ cells (PAX5) or $4x10^6$ cells (CTCF) were diluted in 450 µL of the ChIP dilution buffer (8 mL for CTCF) for further immunoprecipitation or stored at 4°C to be directly uncrosslinked and purified (DNA input fraction). Magnetic beads (80 µL, Dynabeads Protein G, Invitrogen) were washed twice with 1 mL of blocking solution (1X PBS; 5 mg/mL BSA; 0.008% of sonicated Salmon sperm DNA solution; protease inhibitors). Half of the beads were incubated 1 h at room temperature in 60 µL of the blocking solution with 2 µg of goat polyclonal anti-PAX5 antibody (Santa Cruz, Santa Cruz, CA), 10 µg of mouse polyclonal anti-CTCF antibody (Rockville, MD), or without antibody. The beads were then washed twice with 1 mL of the blocking solution, added to the diluted chromatin solution and incubated overnight at 4°C. They were then washed twice with 500 µL of each of the following washing solutions: low salt solution, high salt solution, LiCl solution, and finally TE. The eluate was then resuspended in 200 µL of 5% Chelex solution and incubated 10 min at 100°C to reverse protein-DNA crosslinks. The immunoprecipitated DNA was purified by proteinase K treatment, RNAse A digestion, followed by phenol/chloroform extraction, ethanol precipitation, and resuspension in 50 µL water.

For PAX5 ChIP assays, purified DNA was analyzed by PCR with specific primers for the first exon of the hTERT gene to generate a 178 bp fragment (hTERT exon1: forward: 5'-

CAGCCCCTCCCCTTCCTTTC-3'; reverse: 5'- GCAGCACCTCGCGGTAGTG-3'), the second exon of the hTERT gene as a 143 bp fragment (hTERT exon2: forward: 5'-CCAGCGTGCGCAGCTACCT -3'; reverse: 5'- GGGAGCCACCAGCACAAAGA-3'), the CD19 gene as a 135 bp positive control (CD19: forward: 5'-ACCACCGCCTTCCTCTCTG-3'; reverse: 5'-TGGCATGGTGGTCAGACTCT-3'), and finally the KRAS gene as a 162 bp negative control (KRAS: forward : 5'-GCCTGCTGAAAATGACTG-3'; reverse: 5'-GGTCCTGCACCAGTAATATG-3'). Quantitative PCR was performed using SybrGreen-ER kit (Invitrogen) with the following PCR conditions: 95°C for 5 min for initial denaturation, then 45 cycles of 94°C for 30 s, 54°C (CD19 and KRAS) or 62°C (hTERT) for 45 s, and 72°C for 60 s.

For CTCF ChIP assays, purified DNA was analyzed by PCR with specific primers for the amplification of the first exon of the hTERT gene (hTERT exon1). The human CTCF-binding site N, a MYC insulator site (MYC-N) was used as positive control and a CTCF non-binding site G of MYC (MYC-G) was used as a negative control (38, 39).

Immunohistochemistry (IHC)

Four µm paraffin sections were cut and mounted on coated slides. Slides were dewaxed and rehydrated in a xylene-ethanol series. hTERT IHC was performed using a rabbit polyclonal antibody (EST21-A; Alpha Diagnostic International, San Antonio, Texas). Antigen retrieval was performed using a pressure cooker for 2 min in 10 mM sodium citrate buffer pH 6. The slides were washed with PBS, incubated for 2 h at room temperature, and then overnight at 4°C with the anti-TERT antibody, diluted 1:50 in Dako Diluent Antibody (Dako, Glostrup, Denmark) containing 0.5 M NaCl. After washing, the EnVision+ System-HRP (AEC) (Dako) was used according to the manufacturer's instructions. Slides were then counterstained with hematoxylin.

Immunohistochemical studies of PAX5 and CD3 were performed using a PAX5 mouse monoclonal antibody (BD Biosciences Pharmingen, San Jose, CA) and a CD3 mouse monoclonal antibody (Novocastra, Newcastle, UK). PAX5 antibody was diluted 1:50 in Dako Antibody Diluent (Dako) containing 0.5 M NaCl, and CD3 antibody was used undiluted. After washing, the EnVision+ Peroxidase rabbit (Dako) was used according to the manufacturer's instructions, and the results were visualized with DAB+ substrate-chromagen solution (Dako).

Transient transfection

Normal fibroblast BJ cells were seeded at a concentration of 10^5 cells/well in a 12-well plate 24 h before transfection. Jet PEI transfection reagent (2 µl) (Polyplus-transfection, Illkirch, France) was used to transfect 2 µg of a PAX5 expression plasmid (phPAX5, a kind gift from Pr. M. Busslinger, Research Institute of Molecular Pathology, Vienna, Austria) (30). Cells treated the same way but without plasmid were used as a transfection control. Extraction of total RNA and DNase treatment were performed 48 h after transfection.

Transfection of siRNA

A double-stranded annealed Stealth RNAi oligonucleotide targeting PAX5 was designed by Invitrogen software (sense, 5'-GAGGAUAGUGGAACUUGCUCAUCAA-3'). A non-specific fluorescent siRNA (Invitrogen) was used as a control. Transfection of siRNA oligonucleotides in Raji cells was performed with Amaxa Nucleofector (Amaxa Biosystems, Cologne, Germany) according to the manufacturer's protocol. To transfect $4x10^6$ cells, 130 pmol of siRNA was used. To determine the effect of PAX5 silencing on hTERT expression, cells were harvested at 24 and 48 hours after transfection. The efficiency of RNA silencing was checked by quantitative RT-PCR of PAX5 mRNA and by immunofluorescence staining with PAX5 antibody (BD Biosciences, Erembodegem Belgium).

Quantitative RT-PCR

Total RNA was extracted from cells before and after siRNA treatment using Trizol-LS (Invitrogen) according to the manufacturer's protocol. Quantitative RT-PCRs were performed on a Rotorgene 6000 cycler (Corbett Research, Sydney, Australia). hTERT and PAX5 mRNAs were amplified using the Quantitative RT-PCR Thermoscript One-Step System (Invitrogen). Each reaction included 50 ng of template RNA, 250 nM of FAM-labeled probe, and 500 nM of each primer. The primers and probes are described in supplemental Table S2. Cycling parameters were 60°C for 30 min followed by 95°C for 5 min, and then 45 cycles of 95°C for 15 s and 60°C for 60 s. The positive control mRNAs, CD19 and β-actin, were amplified by the same enzymes, but with 1.25 µM SYTO 9 green fluorescent nucleic acid stain (Invitrogen) instead of the labeled probes. After amplification, a melting curve was acquired by heating from 70°C to 95°C.

The reaction efficiency was determined with a cDNA dilution series. The relative level of each mRNA was calculated on the basis of the two standard curve relative quantification method. Gene expressions were normalized to β-actin and to the cells transfected with the

non-coding siRNA. At least two independent determinations of fold differences were used to calculate the average fold difference values and associated standard deviation.

Table S2 - Primer sequences for quantitative RT-PCR

Gene		Primer sequences (sense and antisense)	PCR product
hTERT		5'-TGACACCTCACCTCACCCAC-3' 5'-CACTGTCTTCCGCAAGTTCAC –3	95 pb
	probe	5’-FAM-ACCCTGGTCCGAGGTGTCCCTGAG-EDQ-3’	
PAX5		5’-TACTCCATCAGCGGCATCCT-3’ 5’-CTCCTGAATACCTTCGTCTCTCTTG-3’	81 bp
	probe	5’-FAM-CCAGCGCCGACACCAACAAGC-BHQ1-3’	
β-actin		5'-AGGCCAACCGCGAGAAGATGAC-3' 5'-GGGATAGCACAGCCTGGATAGCA -3'	87 pb
CD19		5’-GTGGCAACCTGACCATGTCATT -3’ 5’-GACCAGGGCTCTTTGAAGATGA -3’	167 bp

RESULTS

In lymphoid cells, a hypomethylated hTERT promoter allows hTERT expression

To define the methylation status of the *hTERT* promoter in lymphoid tissues, we analyzed 6 primary B-cell lymphomas, 6 primary T-cell lymphomas and 4 non-malignant lymphoid tissues. Four human lymphoid tumor cell lines were also investigated. RT-PCR analysis confirmed that *hTERT* transcripts were present in all the lymphoma tissues and cell lines as well as in the non-neoplastic lymphoid tissues (Fig. 1A). The *β-actin* gene was simultaneously amplified as a control.

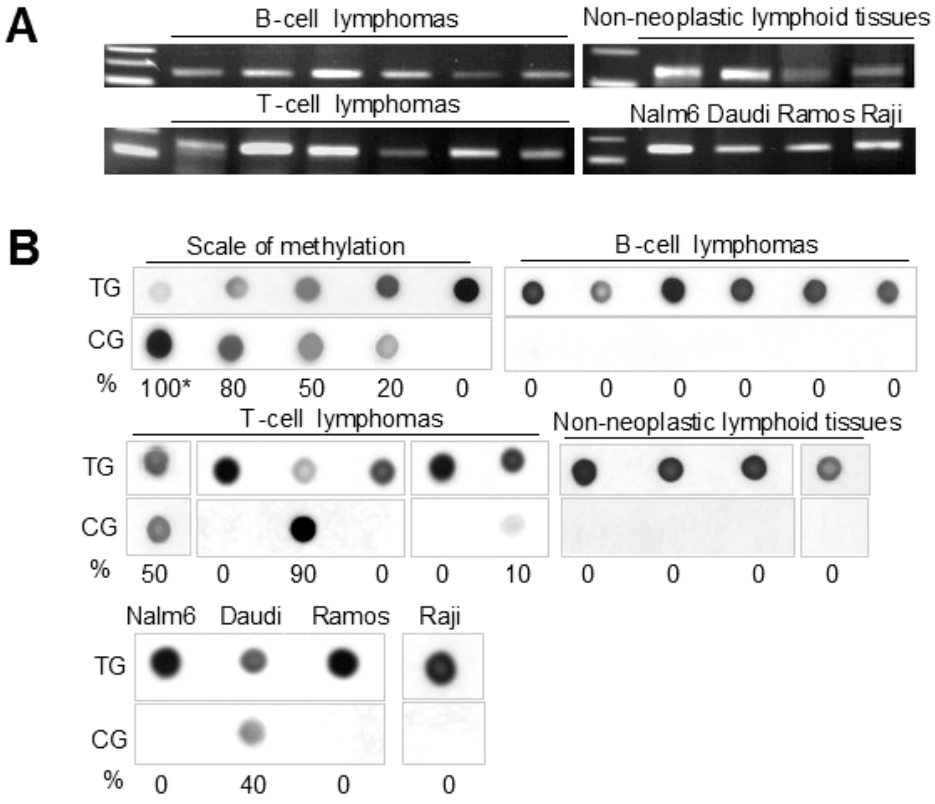


Figure 1. *hTERT* mRNA expression and *hTERT* methylation in lymphoid tissues and cell lines. (*A*) Detection of hTERT expression by RT-PCR in 6 B-cell lymphomas, 6 T-cell lymphomas, 4 non-neoplastic lymphoid tissues, and 4 lymphoid cell lines. (B) Methylation analysis of the hTERT promoter by methyl-sensitive dot blot assay (MS-DBA). Hybridization with a « TG » probes revealed the non-methyl part of the PCR and hybridization with a « CG » probes detected the methyl part of the PCR. A scale of methylation (100%, 80%, 50%, 20%, and 0%) was used to quantify the percentage of methylation found in the different samples. 100* means that the 100% of methylation possesses a residual background with the non-methyl probe. The same samples used for RT-PCR were tested for methylation.

Using MS-DBA, we next explored the methylation status of the *hTERT* promoter. In B-cell lymphomas and non-neoplastic lymphoid tissues, the *hTERT* promoter was unmethylated, while it was hypermethylated in half of the T-cell lymphomas (Fig. 1B). The *hTERT* promoter was methylated in Daudi cells but unmethylated in the other three cell lines (Raji, Ramos, and Nalm6). Direct sequencing and MS-SSCA confirmed the results obtained by MS-DBA (data not shown). To summarize, in some T-cell lymphomas *hTERT* expression goes along with *hTERT* promoter methylation, as is the case for most solid tumors. In transformed B cells and non-neoplastic lymphocytes, however, *hTERT* is expressed in the presence of a hypomethylation promoter. In this situation hTERT expression must be regulated by a methylation-independent mechanism.

Putative PAX5 binding sites are present in the hTERT gene

To determine if transcription factors specific to lymphoid cells might be involved in hTERT regulation, we searched the gene sequence for new transcription factor binding sites. MatInspector revealed two potential binding sites for PAX5, also known as B cell-Specific Activator Protein (BSAP), a transcription factor involved in B-cell differentiation and function (23,24), from +110 to +137 bp and +489 to +516 bp downstream of the ATG translational start site (Fig. 2). This suggested that PAX5 might be involved in the regulation of hTERT transcription in lymphoid cells.

Figure 2. Localization of putative transcription factor binding sites on hTERT sequences from -401 bp to +600 bp flanking the ATG (+1). The main transcriptional start sites are indicated by arrows. The ATG translational start site is highlighted in grey. The exonic regions are underlined. Binding sites for known activators and repressors of hTERT are shown in boxes. Known binding sites of SP1, c-MYC, E2F and CTCF are shown in small boxes. Potential binding sites of PAX5 predicted by the MatInspector program are shown in large grey boxes.

PAX5 binds the hTERT CpG island in vitro and in vivo

To determine if the predicted PAX5 binding sites in the hTERT exon were authentic, we first performed EMSAs using extracts from Raji cells. Two DIG-labeled oligonucleotide probes were used, one representing the PAX5 binding site present on the first hTERT exon. The second, a site on the CD79A promoter, served as a positive control (30,40). A specific band for PAX5 was obtained with the CD79A oligonucleotide (Fig. 3A, lane 1). A similar band was obtained with the hTERT probe (lane 2). A negative control was performed to visualize the background of the reaction (lane 3). To check the specificity of the band, cold competitor oligonucleotides were added to the labeled hTERT probe. A 100-fold molar excess of CD79A and hTERT competitors resulted in almost complete inhibition of PAX5 binding (lanes 4 and 5). The same results were obtained with Nalm6 extracts (Fig. 3A). A 5- to 150-fold increase in the amount of cold CD79A probe also resulted in a progressive inhibition of binding (Fig. 3B). Preincubation of Raji cell extracts with a PAX5-specific antibody resulted in a supershifted band, confirming that PAX5 bound to the site identified in the first exon of hTERT (Fig. 3C). These results demonstrated that PAX5 does bind to the predicted target sequence in the first exon of hTERT.

To determine if PAX5 bound to the hTERT gene under physiological conditions, chromatin immunoprecipitation (ChIP) experiments were performed using Nalm6 and Raji cells. DNA samples isolated from the input, the anti-PAX5-bound, and the no-antibody fractions were analyzed by quantitative PCR. CD19 is a well-known target of PAX5 (29) and was used as positive control. The results indicated an approximately 10-fold enrichment of hTERT exon 1 when normalized with the negative control KRAS gene and around 8-fold enrichment of hTERT exon 2 in both Nalm6 and Raji cells (Fig. 3D). Enrichment of the CD19 gene was about 3 to 4 times greater than that for hTERT, which was not surprising as the binding of PAX5 to the CD19 target sequences is very strong. In the no-antibody fraction, no enrichment was detected with either hTERT or CD19 (data not shown). Thus, PAX5 is bound in vivo to the first and the second exons of hTERT, providing strong evidence that PAX5 could be involved in the transcriptional regulation of the gene in B cells.

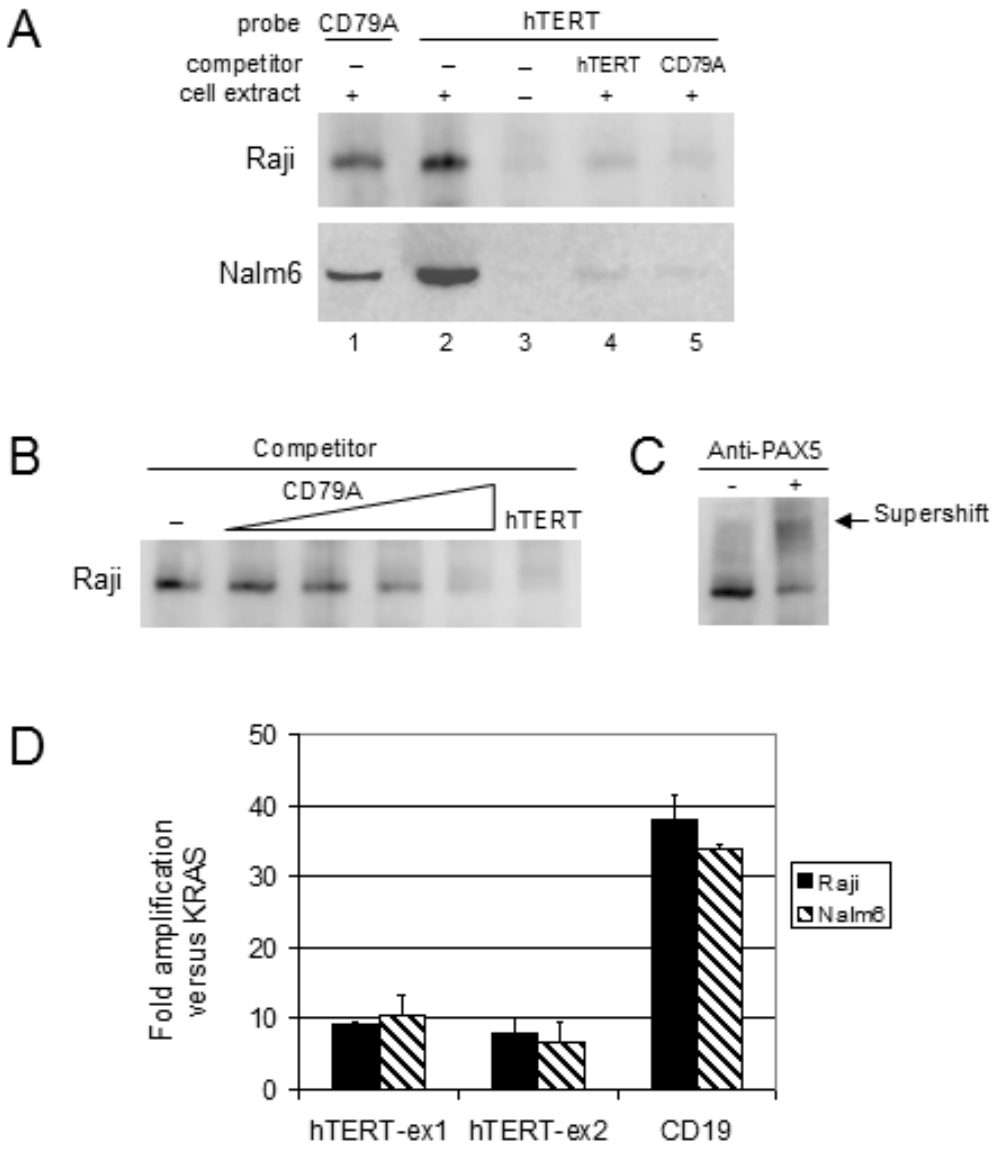


Figure 3. In vitro and in vivo binding of PAX5 in Nalm6 and Raji cells. (A) EMSAs were performed with DIG-labeled oligonucleotides representing PAX5 binding sites on CD79A as a positive control gene (lane 1) and on the hTERT gene (lanes 2-5). A negative control without extract was performed (lane 3). An excess (100x) of unlabeled oligonucleotide (hTERT; CD79A) was used in a competition assay for PAX5 binding to the labeled probes (lanes 4 and 5, respectively). (B) A graduated competition with unlabeled oligonucleotide (CD79A) was performed on Raji cellular extracts, with an excess of 5x, 15x, 50x, and 150x of unlabeled CD79A oligonucleotide and with an excess (150x) of unlabeled hTERT oligonucleotide. (C) A supershift is performed with a PAX5 antibody on Raji cellular extracts. (D) Chromatin immunoprecipitation (ChIP) of PAX5 in Nalm6 and Raji cells. Fold amplification of hTERT and CD19 was calculated versus the KRAS negative control. The bound fraction was compared to the input fraction fixed to 1.

PAX5 does not inhibit binding of CTCF to hTERT in vivo

A possible explanation for the effect of PAX5 on hTERT expression could be that it interferes with CTCF-binding to the hTERT promoter. The CTCF transcription factor was found to be essential for repression of hTERT transcription in a variety of normal somatic cells (6). As PAX5 binding sites lie downstream CTCF target sequences (Fig.2), we therefore performed ChIP analysis to analyze CTCF binding. In Raji cells, hTERT exon 1 was enriched approximately four-fold compared to the negative control, which is in the same range as in the two positive controls, MYCN and H19 (Fig. 4). After transfection with a PAX5 siRNA, ChIP of CTCF was performed but no change in CTCF binding was noticed (Fig. 4). Thus, PAX5 binding to the hTERT exonic region does not block CTCF binding.

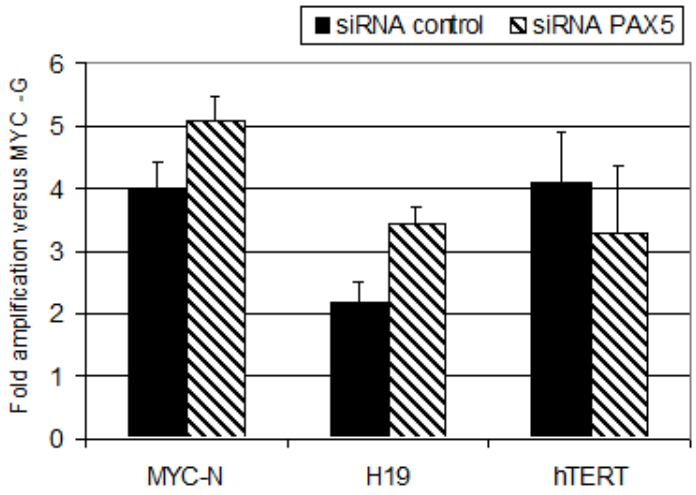


Figure 4. ChIP of CTCF in Raji cells 48 h after transfection with either a control siRNA or a siRNA against PAX5. Fold amplification of hTERT, MYC-N and H19 was calculated versus the MYC-G negative control. The bound fraction was compared to the input fraction fixed to 1.

hTERT and PAX5 have similar patterns of expression in B-cell lymphomas and the B-cell areas of non-neoplastic lymphoid tissues

PAX5 is a specific marker for all stages of B-cell differentiation except for plasma cells (41). In our series, PAX5 mRNA was detected by RT-PCR in all B- and T-cell NHL, as well as in non-neoplastic lymphoid tissues and cell lines.

By IHC of consecutive sections, hTERT and PAX5 were both detected in the same regions of the B-cell lymphomas suggesting they were present in the same tumor cells (Fig.

5A-B). The T cells were identified by CD3 expression. In B-cell lymphomas, the normal T cells did not appear to express either PAX5 or hTERT (Fig. 5A, 5C). In non-neoplastic lymphoid tissues, both PAX5 and hTERT were highly expressed in germinal center B cells and B cells of the mantle zone (Fig. 5G, 5H), whereas CD3+ T cells were PAX5- and hTERT-negative (Fig. 5G-I). In T-cell lymphomas, hTERT was expressed in the CD3+ neoplastic T cells (Fig. 5D, 5F) while PAX5 was not (Fig. 5E-F). The positivity of PAX5 by RT-PCR in the T-cell lymphomas was thus apparently due to the presence of normal B cells in the tumor tissue. In the T-cell lymphomas studied, PAX5 had no role in activating hTERT expression. In summary, PAX5 and hTERT co-localize in normal and malignant B cells, supporting the suggestion that PAX5 might be involved in hTERT activation in these cells.

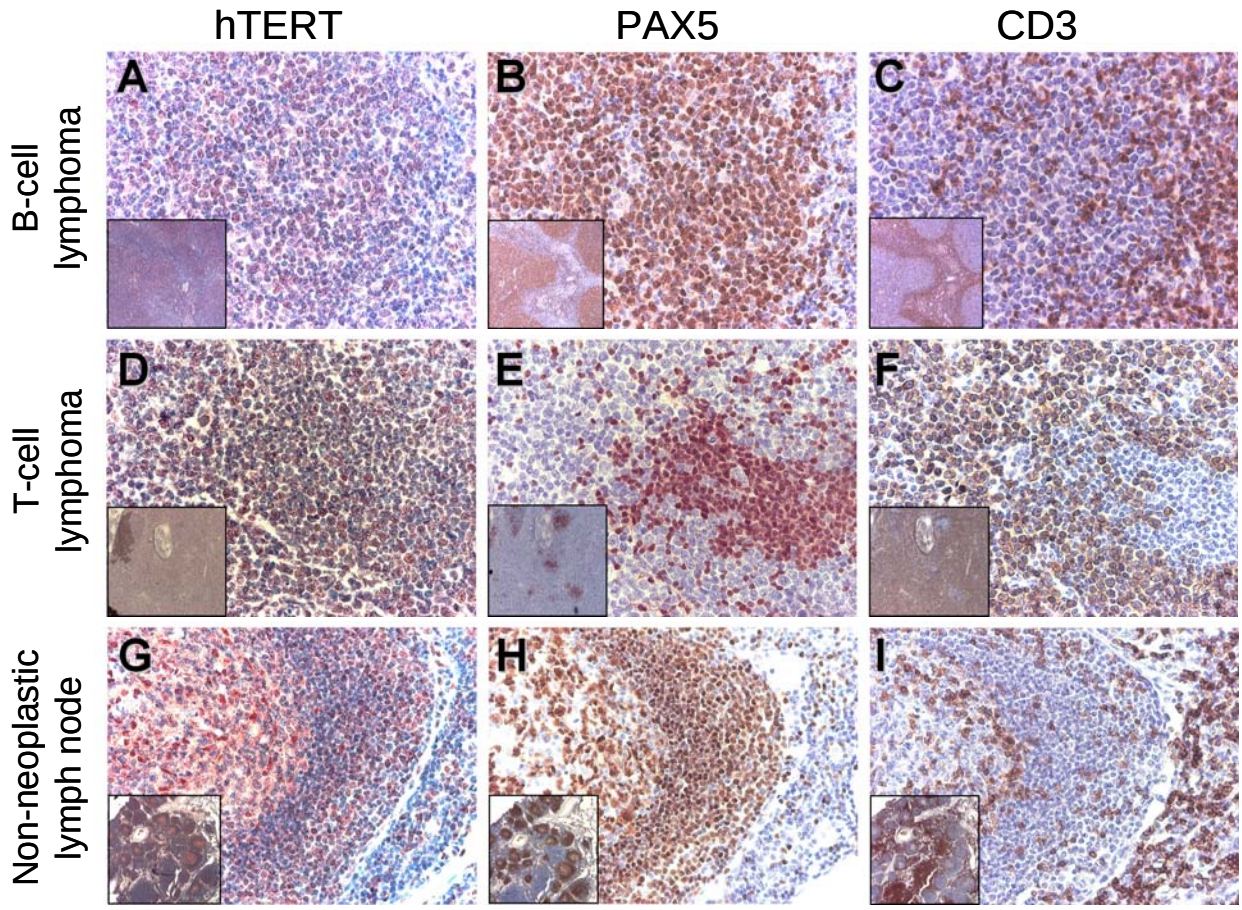


Figure 5. IHC of hTERT, PAX5 and CD3. Representative pictures are shown at high magnification (x 40) and at low magnification (x 2) in the insets. *A*, *B*, *C* were from a B-cell lymphoma; *D*, *E*, *F* were from a T cell lymphoma; *G*, *H*, *I* were from a normal lymph node.

Suppression of PAX5 by siRNA represses hTERT transcription in telomerase-positive cells

To determine if a reduction in PAX5 expression would be associated with a change in hTERT expression, we transfected Raji cells with a PAX5 siRNA. After transfection, transcript levels of PAX5, the down-regulation of PAX5 protein was confirmed by PAX5 immunofluorescence staining, while the level of PAX5, hTERT and CD19 transcripts were monitored by quantitative RT-PCR. Cells transfected with a scrambled siRNA were used as a control. Twenty-four hours after transfection, PAX5 transcript levels were reduced ~50% in association with significant reductions in the levels of CD19 and hTERT expression (~30%) (Fig. 6A). After 48 h, PAX5 transcripts were reduced by 77% in association with reductions of 57% and 64% in transcripts for CD19 and hTERT, respectively. These studies showed that inhibition of PAX5 leads to a strong downregulation of hTERT expression, indicating that PAX5 is essential for hTERT expression in B cells.

PAX5 activates hTERT transcription in normal telomerase-negative cells

To determine if ectopic expression of PAX5 could activate *hTERT* expression in normal telomerase-negative cells, we transfected normal BJ fibroblasts with a *PAX5* expression plasmid. RT-PCR analyses of *PAX5* transcripts 40 h post-transfection confirmed the efficacy of transfection (Fig. 6B). RT-PCR analyses of transcripts for *CD19*, an established target of PAX5, showed substantial expression in the transfected cells. In addition, the transfected cells expressed *hTERT* transcripts at high levels. Non-transfected and mock-transfected BJ cells did not express transcripts of *PAX5*, *CD19*, or *hTERT*. This experiment showed that ectopic expression of PAX5 was sufficient to activate *hTERT* transcription in normal somatic cells.

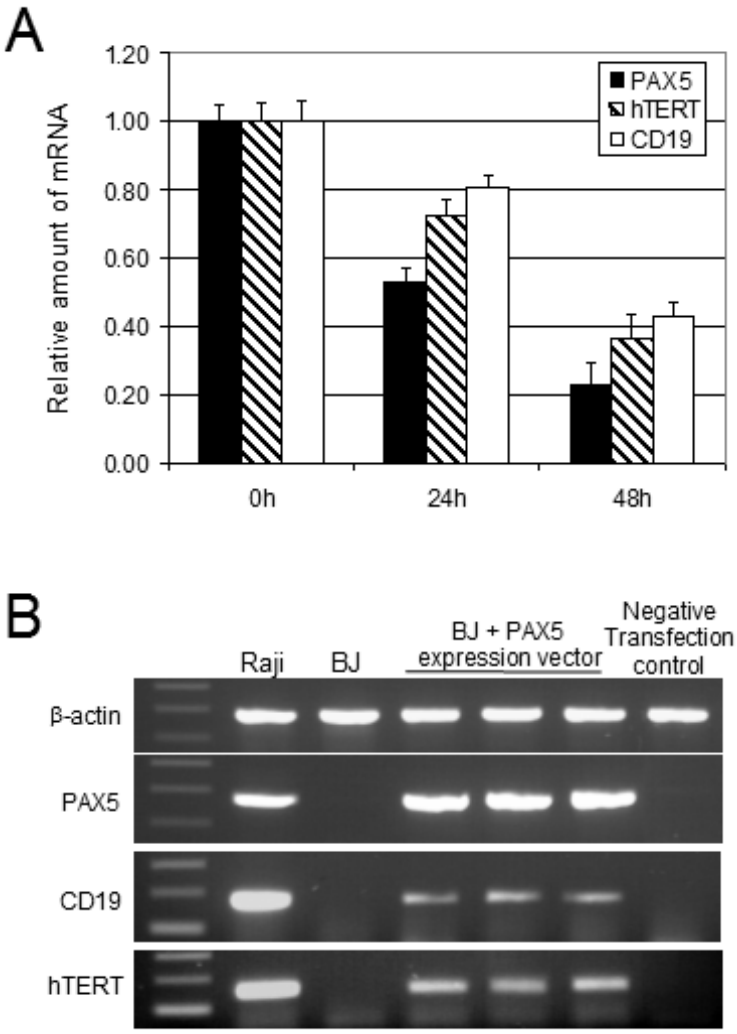


Figure 6. The effect of activation or inactivation of PAX5. (*A*) Quantitative RT-PCR of *PAX5, hTERT*, and *CD19* after transfection of a *PAX5* siRNA into Raji cells. Quantitations were performed 24 and 48 h after transfection. The relative amounts of each mRNA were normalized to the amounts of mRNA after transfection of a scrambled siRNA compared with *β-actin*. (*B*) Detection of *PAX5, CD19*, and *hTERT* expression by RT-PCR before and after transfection of a *PAX5* expression plasmid into telomerase- and PAX5-negative BJ cells and in Raji positive control cells. *β-actin* was used as a control for the RT-PCR. Triplicates of the transfection experiments are shown.

DISCUSSION

In the present study, we showed that hTERT is transcribed in association with the unmethylated 5' region in B cells, B-cell lymphomas and B-cell lymphoma cell lines, defining a novel methylation-independent mode of hTERT regulation. EMSA and ChIP assays identified two binding sites in hTERT for the B cell-specific transcription factor PAX5. These sites lie downstream of the ATG translational start site and are located in the first exon and at the beginning of the second exon of hTERT. Moreover, in B cells, decreasing PAX5 expression resulted in a significant reduction in hTERT expression. Importantly, we showed that ectopic expression of PAX5 in telomerase-negative normal cell lines is sufficient to activate hTERT expression. Taken together, these data strongly implicate PAX5 as a key factor in the transcriptional regulation of hTERT expression in B cells.

hTERT is a new PAX5 target, which has no direct link to B-cell differentiation, in contrast to the well-known PAX5 target genes. These other target genes were identified through a study in which a large panel of genes, known to be important for B lymphopoiesis, was chosen for comparative expression analysis in wild-type and PAX5-deficient pre-B cells (30). Among the principal targets of PAX5, three genes - CD79A, CD19 and PDCD1 (PD-1) - code for cell surface molecules involved in signal transduction, while the products of two other target genes, MYCN (N-Myc) and LEF1, are nuclear transcription factors (29,30).

PAX8, which belongs to the same subgroup of PAX proteins as PAX5, has been implicated in the activation of hTERT in glioma (22). PAX8 failed to activate the hTERT promoter in telomerase-negative primary cell lines and other factors seem to be necessary for the expression of hTERT. On the contrary, activation of PAX5 was sufficient to initiate the transcription of hTERT in telomerase-negative primary cell lines. Apparently, the action of PAX5 on hTERT is very different from that of PAX8. PAX8 mainly seems to act on the formation of the transcription complex, whereas the major role of PAX5 in transcriptional activation does not seem to recruit basal transcription machinery, but is likely to modulate the structure of local chromatin, allowing other sequence-specific factors to activate transcription. Indeed, PAX5 can activate transcription through association with chromatin effector enzymes such as DAXX, CREB binding protein (CBP), and GCN5, which possess histone acetyltransferase (HAT) activity. PAX5 can also interact with BRG1, a catalytic component of the Swi/Snf chromatin remodeling complexes (42). On the other hand, CTCF directly binds

to SIN3A, which condenses chromatin and prevents transcription by recruitment of histone deacetylase (HDAC) activity (43). Therefore, the simultaneous binding of CTCF and PAX5 on hTERT exons might produce opposing effects on chromatin: the recruitment of histone modification and nucleosome remodeling activities by PAX5 might antagonize chromatin-mediated transcriptional repression by CTCF. Additional studies need to be performed to more accurately understand how CTCF and PAX5 interact in regulating hTERT expression.

In summary, we describe a methylation-independent mechanism of hTERT regulation that occurs in telomerase-positive B cells. In these cells, hTERT is a novel target of PAX5, which is essential for B-cell development and function. According to our data, in B cells, PAX5 also participates in cellular mechanisms underlying cell immortality by upregulating hTERT gene expression.

ACKNOWLEDGEMENTS

This work was supported by grants from the Swiss National Science Foundation (Grants 3100A0-101732 and 3100A0-113505) and in part by the Intramural Research Program of the NIH, National Institute of Allergy and Infectious Diseases.

REFERENCES

1. Meeker, A. K. and Coffey, D. S. (1997) *Biochemistry. (Mosc)* **62,** 1323-1331
2. Meyerson, M., Counter, C. M., Eaton, E. N., Ellisen, L. W., Steiner, P., Caddle, S. D., Ziaugra, L., Beijersbergen, R. L., Davidoff, M. J., Liu, Q., Bacchetti, S., Haber, D. A., and Weinberg, R. A. (1997) *Cell* **90,** 785-795
3. Nakamura, T. M., Morin, G. B., Chapman, K. B., Weinrich, S. L., Andrews, W. H., Lingner, J., Harley, C. B., and Cech, T. R. (1997) *Science* **277,** 955-959
4. Poole, J. C., Andrews, L. G., and Tollefsbol, T. O. (2001) *Gene* **269,** 1-12
5. Goueli, B. S. and Janknecht, R. (2003) *Oncogene* **22,** 8042-8047
6. Renaud, S., Loukinov, D., Bosman, F. T., Lobanenkov, V., and Benhattar, J. (2005) *Nucleic. Acids. Res.* **33,** 6850-6860
7. Dessain, S. K., Yu, H. Y., Reddel, R. R., Beijersbergen, R. L., and Weinberg, R. A. (2000) *Cancer. Res.* **60,** 537-541
8. Devereux, T. R., Horikawa, I., Anna, C. H., Annab, L. A., Afshari, C. A., and Barrett, J. C. (1999) *Cancer. Res.* **59,** 6087-6090
9. Guilleret, I., Yan, P., Grange, F., Braunschweig, R., Bosman, F. T., and Benhattar, J. (2002) *Int. J. Cancer* **101,** 335-341
10. Nomoto, K., Maekawa, M., Sugano, K., Ushiama, M., Fukayama, N., Fujita, S., and Kakizoe, T. (2002) *Jpn. J. Clin. Oncol.* **32,** 3-8
11. Renaud, S., Loukinov, D., Abdullaev, Z., Guilleret, I., Bosman, F. T., Lobanenkov, V., and Benhattar, J. (2007) *Nucleic. Acids. Res.* **35,** 1245-1256
12. Bechter, O. E., Eisterer, W., Dlaska, M., Kuhr, T., and Thaler, J. (2002) *Exp. Hematol.* **30,** 26-33
13. Widschwendter, A., Muller, H. M., Hubalek, M. M., Wiedemair, A., Fiegl, H., Goebel, G., Mueller-Holzner, E., Marth, C., and Widschwendter, M. (2004) *Gynecol. Oncol.* **93,** 407-416
14. Zinn, R. L., Pruitt, K., Eguchi, S., Baylin, S. B., and Herman, J. G. (2007) *Cancer Res.* **67,** 194-201
15. Weng, N. P. (2002) *Springer. Semin. Immunopathol.* **24,** 23-33
16. Underhill, D. A. (2000) *Biochem. Cell Biol.* **78,** 629-638
17. Busslinger, M., Klix, N., Pfeffer, P., Graninger, P. G., and Kozmik, Z. (1996) *Proc. Natl. Acad. Sci. U. S. A* **93,** 6129-6134
18. Dorfler, P. and Busslinger, M. (1996) *EMBO J.* **15,** 1971-1982
19. Nutt, S. L., Morrison, A. M., Dorfler, P., Rolink, A., and Busslinger, M. (1998) *EMBO. J.* **17,** 2319-2333
20. Muratovska, A., Zhou, C., He, S., Goodyer, P., and Eccles, M. R. (2003) *Oncogene* **22,** 7989-7997

21. Robson, E. J., He, S. J., and Eccles, M. R. (2006) *Nat. Rev. Cancer* **6,** 52-62
22. Chen, Y. J., Campbell, H. G., Wiles, A. K., Eccles, M. R., Reddel, R. R., Braithwaite, A. W., and Royds, J. A. (2008) *Cancer Res.* **68,** 5724-5732
23. Barberis, A., Widenhorn, K., Vitelli, L., and Busslinger, M. (1990) *Genes Dev.* **4,** 849-859
24. Nutt, S. L., Urbanek, P., Rolink, A., and Busslinger, M. (1997) *Genes Dev.* **11,** 476-491
25. Zhang, W., Piatyszek, M. A., Kobayashi, T., Estey, E., Andreeff, M., Deisseroth, A. B., Wright, W. E., and Shay, J. W. (1996) *Clin. Cancer Res.* **2,** 799-803
26. Torlakovic, E., Torlakovic, G., Nguyen, P. L., Brunning, R. D., and Delabie, J. (2002) *Am. J. Surg. Pathol.* **26,** 1343-1350
27. Mhawech-Fauceglia, P., Saxena, R., Zhang, S., Terracciano, L., Sauter, G., Chadhuri, A., Herrmann, F. R., and Penetrante, R. (2007) *J. Clin. Pathol.* **60,** 709-714
28. Fitzsimmons, D., Hodsdon, W., Wheat, W., Maira, S. M., Wasylyk, B., and Hagman, J. (1996) *Genes Dev.* **10,** 2198-2211
29. Kozmik, Z., Wang, S., Dorfler, P., Adams, B., and Busslinger, M. (1992) *Mol. Cell. Biol.* **12,** 2662-2672
30. Nutt, S. L., Morrison, A. M., Dorfler, P., Rolink, A., and Busslinger, M. (1998) *Embo Journal* **17,** 2319-2333
31. Schebesta, M., Pfeffer, P. L., and Busslinger, M. (2002) *Immunity.* **17,** 473-485
32. Fuxa, M., Skok, J., Souabni, A., Salvagiotto, G., Roldan, E., and Busslinger, M. (2004) *Genes Dev.* **18,** 411-422
33. Roessler, S., Gyory, I., Imhof, S., Spivakov, M., Williams, R. R., Busslinger, M., Fisher, A. G., and Grosschedl, R. (2007) *Mol. Cell Biol.* **27,** 579-594
34. Guillou, L., Coindre, J. M., Gallagher, G., Terrier, P., Gebhard, S., Somerhausen, N. D., Michels, J. J., Jundt, G., Vince, D. R., Collin, F., Trassard, M., Le Doussal, V., and Benhattar, J. (2001) *Hum. Pathol.* **32,** 105-112
35. Clement, G. and Benhattar, J. (2005) *J. Clin. Pathol.* **58,** 155-158
36. Bianco, T., Hussey, D., and Dobrovic, A. (1999) *Hum. Mutat.* **14,** 289-293
37. Bian, Y. S., Yan, P., Osterheld, M. C., Fontolliet, C., and Benhattar, J. (2001) *Biotechniques* **30,** 66-72
38. Pugacheva, E. M., Tiwari, V. K., Abdullaev, Z., Vostrov, A. A., Flanagan, P. T., Quitschke, W. W., Loukinov, D. I., Ohlsson, R., and Lobanenkov, V. V. (2005) *Hum. Mol. Genet.* **14,** 953-965
39. Renaud, S., Pugacheva, E. M., Delgado, M. D., Braunschweig, R., Abdullaev, Z., Loukinov, D., Benhattar, J., and Lobanenkov, V. (2007) *Nucleic. Acids. Res.* **35,** 7372-7388
40. Wheat, W., Fitzsimmons, D., Lennox, H., Krautkramer, S. R., Gentile, L. N., McIntosh, L. P., and Hagman, J. (1999) *Mol. Cell. Biol.* **19,** 2231-2241
41. Jensen, K. C., Higgins, J. P. T., Montgomery, K., Kaygusuz, G., van de Rijn, M., and Natkunam, Y. (2007) *Mod. Pathol.* **20,** 871-877

42. Gregory, P. D., Wagner, K., and Horz, W. (2001) *Exp. Cell. Res.* **265,** 195-202
43. Lutz, M., Burke, L. J., Barreto, G., Goeman, F., Greb, H., Arnold, R., Schultheiss, H., Brehm, A., Kouzarides, T., Lobanenkov, V., and Renkawitz, R. (2000) *Nucleic. Acids. Res.* **28,** 1707-1713

CHAPTER 4

Detection of hTERT promoter methylation by real-time methylation-sensitive high resolution melting: a potential cancer biomarker in biological fluids

Stéphanie Bougel, Gabrielle Gallagher, Jeanne-Chantal de Flaugergues, Robert Janzer and Jean Benhattar

Institute of Pathology, Centre Hospitalier Universitaire Vaudois and University of Lausanne, CH-1011 Lausanne, Switzerland

Manuscript

ABSTRACT

Background: Sensitive and specific cancer biomarkers are needed for screening, primary diagnosis, and follow-up. hTERT expression might be such a marker but its use is hampered by proliferating normal lymphocyte cells. hTERT methylation, characterizing most cancer cells, might be an alternative as proliferating lymphocytes do not have hTERT methylation (or have unmethylated hTERT promoter). The aim of this study was to develop a specific, sensitive, quantitative, and fast method for detection of hTERT methylation and to explore its use as a cancer biomarker in a biological fluid, using cerebrospinal fluid (CSF) as a model.

Materials and methods: hTERT methylation levels were assayed by several quantitative techniques including methylation-sensitive dot blot assay (MS-DBA), methylation-sensitive high resolution melting (MS-HRM), and a newly developed real-time MS-HRM assay. We used MS-HRM assays for the analysis of 50 CSF specimens from 45 patients including 22 CSFs from 18 patients with a known malignancy suspected for leptomeningeal metastasis. Cytological analysis served as a gold standard.

Results: MS-HRM assays both allowed hTERT methylation quantification in CSF samples. The MS-HRM detected samples containing more than 10% of hTERT methylated alleles whereas real-time MS-HRM detected methylation below 10%. PCR products were obtained from 46 CSF samples (92%). hTERT methylation was only detected in the CSF from patients with a known malignancy.

Conclusion: The real-time MS-HRM analysis is a fast, sensitive, and specific technique for methylation assessment in many diagnostic and research applications. We have tested this approach on CSF and proof of concept has been obtained.

Key words: hTERT, metastasis, DNA methylation, cerebrospinal fluids, HRM, biomarker

INTRODUCTION

The inheritance of information on the basis of gene expression levels is known as epigenetics, as opposed to genetics, which refers to information inherited on the basis of gene sequence. The most commonly occurring epigenetic event in mammalian genome is DNA methylation of the CpG dinucleotides. In contrast to the relative paucity of CpGs in the genome as a whole, these dinucleotides can be clustered in small stretches of DNA termed "CpG islands" and are often associated with the promoter regions. Abnormal patterns of DNA methylation have been recognized in cancer cells. They exhibit both loss of methylation in the CpG-depleted regions, where most CpG dinucleotides should be methylated, and gains in methylation of CpG islands in gene promoter regions (Bird and Wolffe, 1999; Herman and Baylin, 2003). Methylation of CpG islands in gene promoter regions is associated with aberrant silencing of transcription and is a mechanism for inactivation of tumor-suppressor genes, in addition to mutation (Jones and Laird, 1999; Jones, 1999). In carcinogenesis, methylation of promoter CpG islands tends to be an early event, and therefore the detection of methylation holds promise as a tool for early cancer detection (Laird, 2003; Shi *et al.*, 2007). Methylation of genes has already been used to detect tumor cells, such as methylation of APC and hTERT in esophageal cancers (Clement *et al.*, 2006), MGMT in glioblastoma (Hegi *et al.*, 2005) or RASSF1A in breast cancers (Shinozaki *et al.*, 2005). However, in body fluids such as blood, urine, or cerebrospinal fluid (CSF) sputum, tumor-derived material is hard to detect because of the presence of material from normal cells. Therefore, only highly sensitive methods of cancer detection are suitable for these materials.

Telomerase activity is detected in about 90% of human cancers, but not in most normal cells. The expression of its catalytic subunit, hTERT (human telomerase reverse transcriptase), has been shown to be a biomarker in hepatocellular, colorectal, and esophageal carcinoma (Gertler *et al.*, 2002; Miura *et al.*, 2007; Gertler *et al.*, 2008). Normal proliferating progenitor cells and activated lymphocytes, often infiltrated tumor tissues and in some normal tissues, also express hTERT and have an active telomerase complex (Weng, 2002). In body fluids, including CSF, proliferating lymphocytes can be the cause of hTERT expression (Kleinschmidt-DeMasters *et al.*, 1998; Braunschweig *et al.*, 2001; Lee, 2005), which limits its use as a diagnostic tool in cancer. In contrast, hTERT promoter methylation is correlated with hTERT expression in most telomerase-positive tumors (Devereux *et al.*, 1999; Dessain *et al.*, 2000; Guilleret *et al.*, 2002; Nomoto *et al.*, 2002), while the promoter is unmethylated in telomerase positive normal cells, including activated lymphocytes (Bougel, submitted).

Therefore, the use of hTERT methylation as a biomarker might circumvent the confounding effect of activated lymphocytes.

The finding of malignant cells in the cerebrospinal fluid is essential for the diagnosis of leptomeningeal metastasis (LM). Because early treatment of LM may prevent neurological deterioration, it is important to establish the diagnosis as early as possible. CSF cytology is the only examination that directly verifies the presence of malignancy in CSF (Aboulafia *et al.*, 1996). Nevertheless, cytological diagnosis is often difficult to establish by morphology alone, especially in cases with low numbers of cells (Schinstine *et al.*, 2006). PCR techniques are more sensitive than cytology if there is a specific genetic marker, such as immunoglobulin gene rearrangements for lymphoma (Gleissner *et al.*, 2002) or KRAS mutation in lung or colorectal cancers (Swinkels *et al.*, 2000). However, most of these markers characterize a limited set of cancers and so, as yet, no single tumor marker allows detection of LM in CSF.

Most methods used for DNA methylation analysis discriminate methylated and unmethylated sequences after bisulfite modification of the target DNA and followed by PCR amplification. Real-time PCR methods allowed the development of new sensitive and quantitative techniques derived from Methylation-specific PCR (MSP), such as MethyLight, quantitative analysis of methylated alleles (QAMA) or Sensitive Melting Analysis after Real Time (SMART-MSP) (Kristensen *et al.*, 2008). In the SMART-MSP methodology, sensitive melting analysis using high resolution melting (HRM) is performed immediately after the real-time PCR in a closed-tube system, and allow to avoid false-positive results referred for MSP analysis (Rand *et al.*, 2002; Brandes *et al.*, 2007). Methylation sensitive HRM (MS-HRM) analysis has been advocated as the method of choice for methylation assessment (Wojdacz and Dobrovic, 2007).

The aim of this study was to develop a specific, sensitive, quantitative and fast method for detection of low level of hTERT methylation and to explore its potential use as a cancer biomarker in the diagnosis of metastasis in CSF. As the number of tumor cells can vary, a method is needed with a detection level of as low as 1% of methylated DNA in a non-methylated background. In this study, we developed real-time methyl-sensitive high resolution melting in combination with TaqMan analysis (real-time MS-HRM) in order to attain a very low level of detection of methylation, which could be applicable to clinical samples.

MATERIALS AND METHODS

Control samples

Hela cells were used as 100% hTERT methylated standard and placental tissue as a 0% hTERT methylated standard. The range of methylated and unmethylated allele dilutions was created by mixing genomic DNAs before bisulfite modification to obtain methylated/unmethylated hTERT template ratios of 0, 1, 10, 20, 50, 80, and 100%. Each of our experimental runs included a range of methylated/unmethylated standards. Human tumor cell lines (SW480, OE33, CO115 cells) and human lymphoid tissues were tested.

Patients

All available 50 CSF specimens were collected from 45 patients seen at the Centre Hospitalier Universitaire Vaudois (CHUV) of Lausanne and at regional hospitals between 2005 and 2007. All CSF samples were immediately processed for cytological analysis (J. C. de Flaugergues and R. Janzer). Residual CSF samples were 1-2 ml in volume and were stored at 4°C for 1-4 days before processing for hTERT methylation analysis. No patient had CSF specially and solely for this study.

Eighteen patients with a known malignancy were suspected for LM. A total of 22 CSFs were investigated. For all these patients the primary tumor was also investigated for hTERT methylation analysis. The primary tumors included 11 breast cancers, 3 medulloblastomas, 2 lung adenocarcinomas, 1 prostate carcinoma and 1 colon cancer. The tumor tissues came from the files of the Institute of Pathology of Lausanne. The use of human tissues for this study was done according to the guidelines of the local ethical committee.

In addition, 28 CSFs from 27 patients with non-neoplastic diseases (including inflammatory diseases and viral syndromes) or with a B-cell lymphoma were used as negative controls.

DNA extraction and bisulfite modification

DNAs were extracted by using the DNeasy Tissue Extraction Kit (Qiagen, Hilden, Germany) with some modifications. 360 µL of ATL buffer and 40 µL (600 ng) of proteinase K were added to 1-2 ml of CSF. After evaporation in a Speedvac to a residual volume of 400 µL, DNA extraction was performed as recommended by the manufacturer. Bisulfite modification of the genomic DNA was performed with the EpiTect Bisulfite Kit (Qiagen). The starting

amount of DNA was 1 µg for the controls, but variable amounts of CSF DNA (10 to 500 ng) were used depending on the number of cells contained in each sample.

hTERT methylation analyses

Three methods were performed to analyze hTERT promoter methylation. All the analyses were performed twice. Methylation-sensitive dot blot assay (MS-DBA) was performed as previously described (Clement and Benhattar, 2005).

Methylation-sensitive high resolution melting (MS-HRM) was carried out sequentially on a Rotor-Gene 6000 (Corbett Life Science, Sydney, Australia). PCR amplification was performed in a 20 µL total volume containing 2.5 mM $MgCl_2$, 250 µM of dNTP, 200 nM of each primer, 5 µM SYTO 9 dye (Invitrogen, Carlsbad, CA), 5% DMSO, 1 U Platinium Taq polymerase (Invitrogen), and 2 µL of bisulfite-modified DNA template. DNA was amplified using the following primers: forward 5'- CGCCCTAAAAACAACCCTAAATCTC -3'; reverse 5'- AGGGAGGGGTTATGATGTGGAG -3'. The amplification consisted of 5min at 95°C, followed by 7 cycles of 10s at 95°C, 15s at 55°C and 15s at 72°C, and finally 33 cycles of 10s at 95°C and 30s at 60°C. High resolution melting (HRM) analysis was performed at the temperature ramping and florescence acquisition setting recommended by the manufacturer (temperature ramping from 70–95°C, rising by 0.1°C/2s). The 97-bp amplified fragment contained 5 CpG sites.

Real-time methylation-sensitive high resolution melting (real-time MS-HRM), comprising both HRM and real-time TaqMan PCR analysis, was performed. The TaqMan MGB probe was designed to contain four CpG sites that were always found methylated in cancers (Guilleret *et al.*, 2002; Guilleret and Benhattar, 2004). This probe was labeled with TET and can only detect the methylated allele. PCR amplification and high resolution melting analysis were carried out sequentially on a Rotor-Gene 6000 with the same mix as for MS-HRM analysis, but with the addition of 200 nM of labeled probe. The program of amplification consisted of 5min at 95°C, followed by 40 cycles of 10s at 95°C and 30s at 60°C. A 107-bp hTERT-bisulfite modified fragment was amplified using the following primers: forward 5'- G***CG***TC***CG***AACCTAAAAACAACCCTA -3', reverse 5'- TT***CG***AGGGAGGGGTTATGATGTG -3'; and the internal labeled probe: 5'- TET-***CG***ACCAAAAAAT***CG***C***CG***CA***CG***CA-BHQ1 -3'. The length of PCR product was minimized for equivalent amplification of methylated and unmethylated alleles. High resolution melting (HRM) analysis was performed as described for MS-HRM analysis. The reaction efficiency was determined with a DNA dilution series. The evaluation of the

methylation was calculated as the ratio between methylation part (M) monitored by the Taqman MGB probe and the totality of the PCR amplification (M+U) measured by the SYTO9. The ratio was calculated on the basis of the two standard curve relative quantification method. The melting curves of the HRM analysis were normalized by the software which allows a direct comparison of the samples even with different starting fluorescence levels.

RESULTS AND DISCUSSION

1- Sensitivity of the MS-DBA and MS-HRM assays

A methyl-sensitive Dot blot assay (MS-DBA) was first performed on a mixture of genomic DNAs with different levels of hTERT methylation (Figure 1). The results show that this technique is sensitive and quantitative, but insufficiently specific as a residual background signal that does not reflect methylation can be detected in unmethylated samples (Figure 1, lane 2). MS-DBA is an efficient technique for screening large numbers of samples, but it not specific enough to distinguish 1-2% of methylation from unmethylated samples.

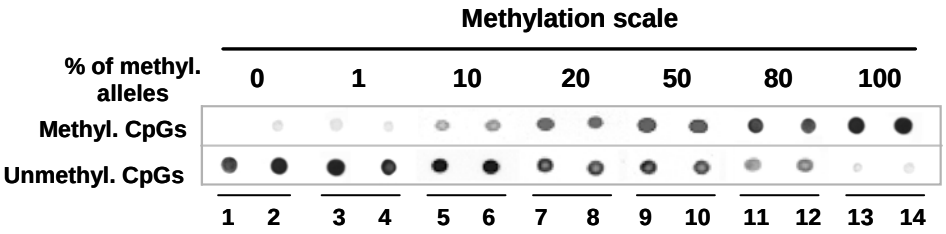


Figure 1. hTERT methylation by methyl-sensitive dot blot assay. Bisulfite DNA containing a known level of hTERT methylation were amplified by PCR and dot blot analysis was performed. Dots hybridized with an oligoprobe specific for either the methylated DNA (Methyl. CpGs) or the unmethylated DNA (Unmethyl. CpGs). The experiments were performed twice for each level of hTERT methylation.

MS-HRM was previously described as a sensitive and specific method for the detection of MGMT methylation (Wojdacz and Dobrovic, 2007). MS-HRM assay also gives a quality control to avoid false-positive results caused by incomplete conversion or false priming due to less stringent PCR conditions (Wojdacz and Dobrovic, 2007). We therefore applied this method to the detection of hTERT methylation. As the hTERT promoter is included in a CpG-rich region, we added a three base tail at one primer to obtain CpG-free primers with a sufficiently high Tm. HRM relies upon the precise monitoring of the change of fluorescence intensity as a DNA duplex melts. Standard DNA mixtures containing 0, 1, 10, 20, 50, 80 and 100 % of hTERT methylation were tested. The melting curves obtained by HRM analysis were unique and characteristic of each standard sample. An evaluation of the extent of methylation can be achieved by a comparison of the shapes of normalized melting profiles between standards and samples (Figure 2A). The HRM analysis was accurate for a

range of 10% to 100% methylation and gave reproducible and specific results. MS-HRM analysis confirmed the hTERT methylation status obtained by MS-DBA of different cell lines (SW480, OE33, CO115 cells) and tissues (3 human lymphoid tissues), which were respectively 100% and 0% of methylation (Figure 2B). The simplicity and the rapidity of HRM procedure in the closed-tube system could be helpful for diagnosis and is a significant advantage compared to MS-DBA assay.

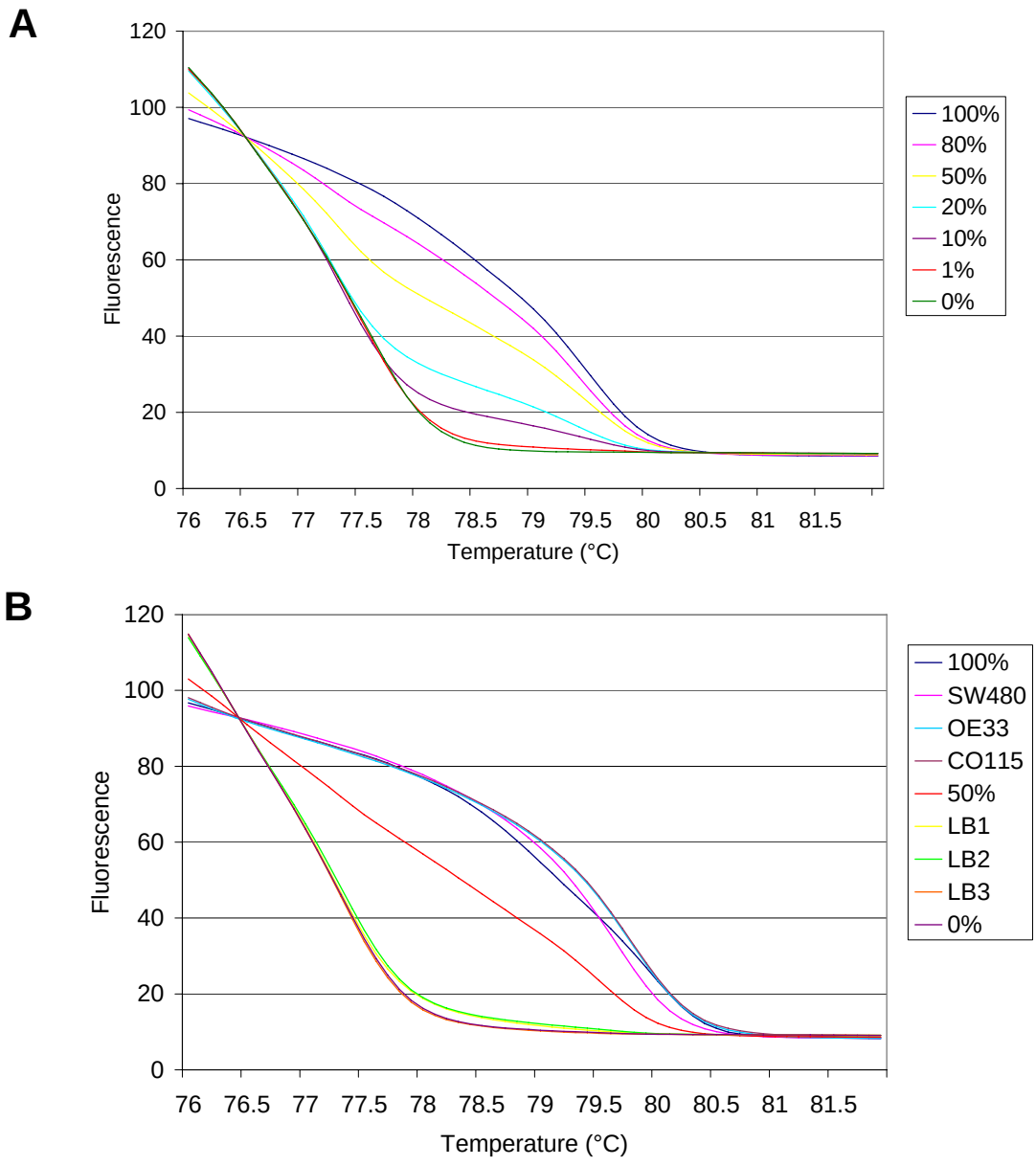


Figure 2. MS-HRM analysis of hTERT methylation. (A) MS-HRM profiles obtained with mixtures of bisulfite DNA containing a known level of hTERT methylation (0, 1, 10, 20, 50, 80, and 100%). Each HRM curve is specific for a standard sample. (B) MS-HRM profiles obtained with bisulfite-DNA from standards (100, 50, 0% of hTERT methylation), from cell lines (SW480, OE33, CO115 cells), and from 3 human lymphoid tissues (LB1, LB2, LB3). Before the HRM step, all the PCR reactions were performed with an annealing temperature of 60°C.

In order to increase the sensitivity of MS-HRM, PCR primers with CpGs were selected to induce a PCR bias towards the methylated templates. This is acceptable if a limited number of CpGs is included in the primers, particularly if they are placed away from the 3′ end (Dobrovic *et al.*, 2002). Moreover, in order to reduce potential false-positive or false-negative signals, hTERT methylation was simultaneously investigated by HRM and TaqMan MGB probe analysis in a same closed-tube. We coined the term real-time MS-HRM for this approach. In this method, TaqMan assay covers methylation of CpG sites by the fluorescent probe, and HRM scans all of the CpGs flanked by the primers-binding to the target sequence, regardless of the methylation status of CpGs in the primer-binding side. Standard curves for the assay are shown in Figure 3.

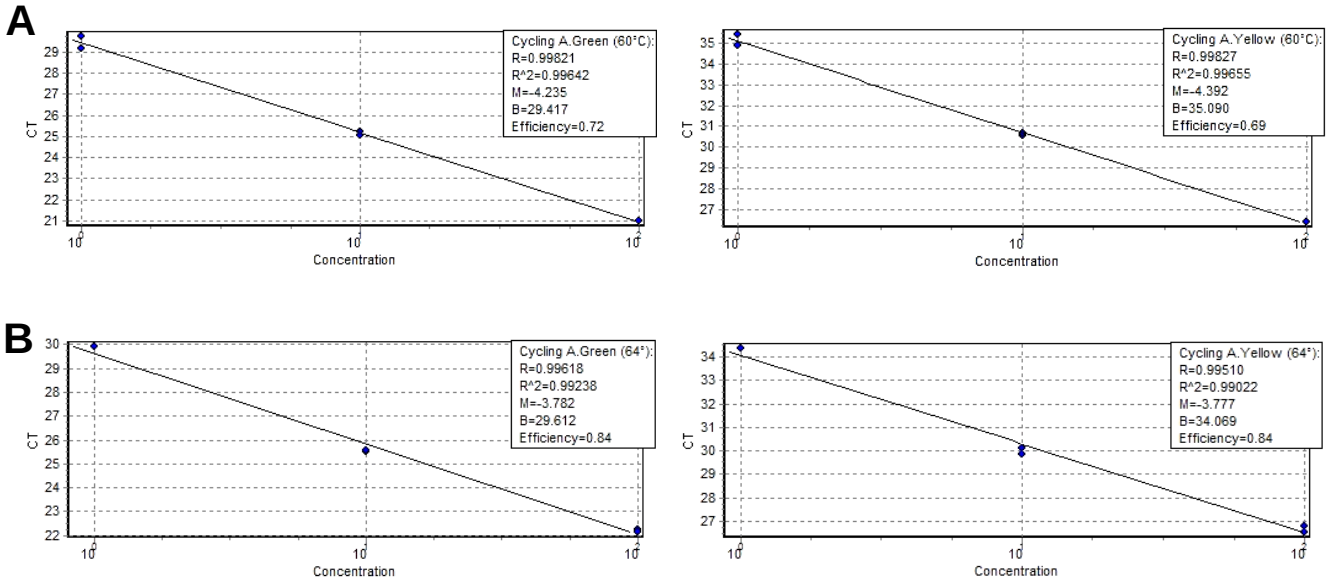


Figure 3. Parameters of the standard curves (coefficient of correlation (R^2) and efficiency) of real-time MS-HRM assays. Assays were performed with dilutions of fully methylated control DNA of Hela cells. The green channel detects the amplification of both methylated and unmethylated strands by SYTO9 dye. The yellow channel detects the amplification of methylated strand by the TET-labeled probe. (A) Annealing temperature of PCR reaction = 60°C. (B) Annealing temperature of PCR reaction = 64°C.

The TaqMan MGB probe had a high specificity and the background signal on unmethylated targets was negligible. Moreover, it was possible to detect methylation of DNA mixtures containing 10% or 20% of methylation with high resolution (Figure 4A). Increasing of the annealing temperature of PCR amplification allowed higher sensitivity. At 64°C, samples containing 1% methylated template could be easily detected by HRM analysis as well as TaqMan analysis (Figure 4B and 4C respectively). In contrast, samples with more than 20% methylation could not be differentiated from a fully methylated sample. The real-time MS-HRM performed at 64°C with primer containing CpG, was more sensitive than both MS-HRM and MS-DBA assays.

Thus, MS-HRM and real-time MS-HRM are both suitable to quantitatively analyze hTERT methylation when variable amounts of methylated-tumor DNA are present in the analyzed samples. The MS-HRM will allow detection of samples containing more than 10% of hTERT methylated alleles and the real-time MS-HRM of samples containing less than 10% hTERT methylated alleles.

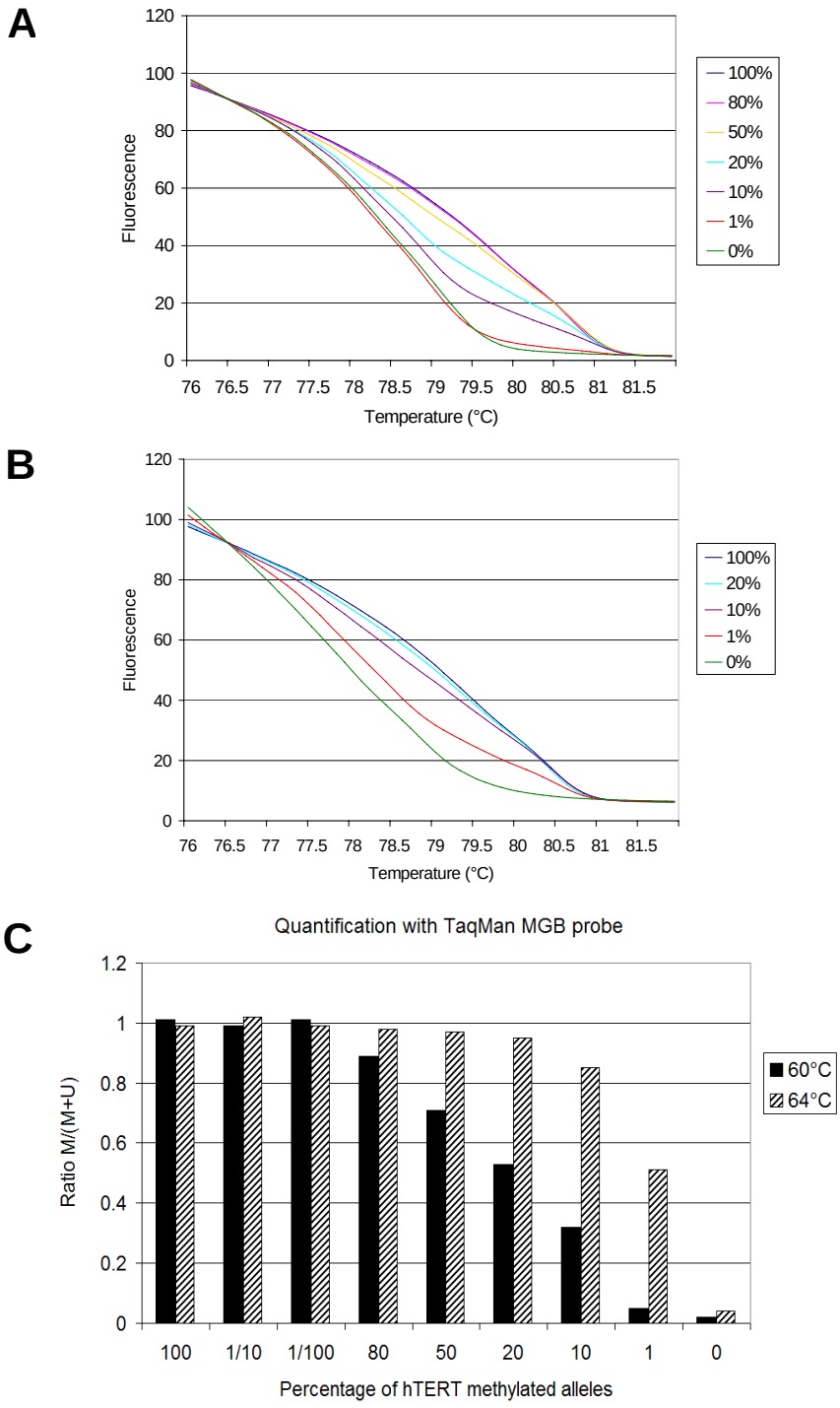


Figure 4. Real-time MS-HRM analysis of hTERT methylation from DNA mixtures containing a known level of hTERT methylation. (A) MS-HRM profiles obtained at the annealing temperature of 60°C for the PCR reaction. (B) MS-HRM profiles obtained at the annealing temperature of 64°C for the PCR reaction. (C) Comparison of hTERT methylation quantification by TaqMan probe when using an annealing temperature of 60°C or 64°C. M: methylated sequences. U: unmethylated sequences. The evaluation of the methylation was calculated as the ratio between methylation part (M) monitored by the Taqman MGB probe and the totality of the PCR amplification (M+U) measured by the SYTO9. The ratio was calculated on the basis of the two standard curve relative quantification method. The 1/10 and 1/100 samples represent the dilution ratio of fully methylated DNA.

2- Application of the hTERT MS-HRM assays to clinical specimens

The applicability of the hTERT MS-HRM and real-time MS-HRM assays was tested on a panel of 50 CSF samples from a total of 45 patients. Among those, 22 CSFs were suspected for leptomeningeal metastasis and 28 CSFs were non-neoplastic samples. CSF samples were first processed for cytological analysis. For patients with an identified primary tumor, CSF cytological diagnosis was positive for malignancy when atypical large cells with prominent nucleoli were present in significant number. CSF was negative for malignancy when only inflammatory cells were found. Primary tumors were tested for the occurrence of hTERT promoter methylation for reasons of comparison.

Of the 50 investigated CSFs, the DNA extracted and bisulfite-modified from 46 (92%) samples was amplifiable by PCR. Four non-neoplastic CSFs were not amplified probably because of the limited number of collected cells.

The 28 CSFs from 27 patients with non-neoplastic diseases, including inflammatory diseases and viral syndromes, or with B-cell lymphomas were used as negative controls. In cytologically non-malignant CSFs, no hTERT methylation was observed (Table 1).

Table 1. Cytology and hTERT methylation for the detection of leptomeningial metastasis in CSF

	Cytological diagnosis		**Total**
	Positive	**Negative**	
hTERT Methylated	12	0	12
hTERT Unmethylated	3	28*	31
Total	15	28	43

*4 tumors and 24 negative control samples.

Of 18 patients suspected for leptomeningeal metastasis, 3 patients (16%) had an unmethylated primary tumor. The corresponding CSFs from these 3 patients were also unmethylated, even if tumor cells were identified by cytology. Of the 19 CSF samples from the other 15 patients with a methylated primary tumor, 15 CSFs were positive for malignancy by cytological analysis, and 4 negative. None of the 4 negative samples were hTERT methylated by MS-HRM analysis (Table 1). MS-HRM assays detected hTERT methylation in

12 of the 15 cytologically positive samples (Table 1). Interestingly, the level of hTERT methylation correlated with the percentage of tumor cells estimated by cytology. Methylation of 3 samples was only detectable by the most sensitive approach (real-time MS-HRM), which indicates that the number of tumor cells is relatively low in these samples. All the results were reproducible between replicates.

hTERT methylation and cytological analyses were concordant in 84% (16/19) of the CSF samples from patients with an hTERT methylated primary tumor. In the 3 negative samples, the cytological analysis identified between 5 to 10% atypical cells, which should have been easily detectable by real-time MS-HRM. Sample variation could explain the discrepancy between the cytology and the methylation analysis, like aggregate formation, but also hTERT unmethylated metastasizing cells from an hTERT methylated primary tumor. The possibility of cytological overdiagnosis should also be considered.

In summary, the real-time MS-HRM analysis is a fast, sensitive and specific technique for hTERT methylation detection. The combination of TaqMan technology with HRM provides a double in-tube method meaning that the analysis takes place without the PCR product leaving the tube that it was amplified in, which could be suitable for diagnostic applications. In our series, bisulfite-modified DNA was amplified successfully from 92% (46/50) of the CSF specimens, which confirms the overall relatively good preservation of DNA in CSF (Pine *et al.*, 2005). According our data (Table 1), the sensibility was 80%, and the specificity was 100%. The positive prospective values and the negative prospective values were 100% and 90%, respectively. Therefore, hTERT methylation through MS-HRM analysis could provide a useful contribution to the diagnosis of leptomeningeal metastasis. A larger study is needed to confirm our findings and to determine whether hTERT methylation could become a powerful cancer biomarker for the detection of leptomeningeal metastasis in CSF.

ACKNOWLEDGEMENTS

This work was supported by the Swiss National Science Foundation (Grant 3100A0-113505).

REFERENCES

Aboulafia DM, Taylor LP, Crane RD, Yon JL and Rudolph RH. (1996). Carcinomatous meningitis complicating cervical cancer: a clinicopathologic study and literature review. *Gynecol Oncol,* **60,** 313-318.

Bird AP and Wolffe AP. (1999). Methylation-induced repression--belts, braces, and chromatin. *Cell,* **99,** 451-454.

Brandes JC, Carraway H and Herman JG. (2007). Optimal primer design using the novel primer design program: MSPprimer provides accurate methylation analysis of the ATM promoter. *Oncogene,* **26,** 6229-6237.

Braunschweig R, Guilleret I, Delacretaz F, Bosman FT, Mihaescu A and Benhattar J. (2001). Pitfalls in TRAP assay in routine detection of malignancy in effusions. *Diagn Cytopathol,* **25,** 225-230.

Clement G and Benhattar J. (2005). A methylation sensitive dot blot assay (MS-DBA) for the quantitative analysis of DNA methylation in clinical samples. *Journal of Clinical Pathology,* **58,** 155-158.

Clement G, Braunschweig R, Pasquier N, Bosman FT and Benhattar J. (2006). Alterations of the Wnt signaling pathway during the neoplastic progression of Barrett's esophagus. *Oncogene,* **25,** 3084-3092.

Dessain SK, Yu H, Reddel RR, Beijersbergen RL and Weinberg RA. (2000). Methylation of the human telomerase gene CpG island. *Cancer Res,* **60,** 537-541.

Devereux TR, Horikawa I, Anna CH, Annab LA, Afshari CA and Barrett JC. (1999). DNA methylation analysis of the promoter region of the human telomerase reverse transcriptase (hTERT) gene. *Cancer Res,* **59,** 6087-6090.

Dobrovic A, Bianco T, Tan LW, Sanders T and Hussey D. (2002). Screening for and analysis of methylation differences using methylation-sensitive single-strand conformation analysis. *Methods,* **27,** 134-138.

Gertler R, Doll D, Maak M, Feith M and Rosenberg R. (2008). Telomere length and telomerase subunits as diagnostic and prognostic biomarkers in Barrett carcinoma. *Cancer,* **112,** 2173-2180.

Gertler R, Rosenberg R, Stricker D, Werner M, Lassmann S, Ulm K, *et al*. (2002). Prognostic potential of the telomerase subunit human telomerase reverse transcriptase in tumor tissue and nontumorous mucosa from patients with colorectal carcinoma. *Cancer,* **95,** 2103-2111.

Gleissner B, Siehl J, Korfel A, Reinhardt R and Thiel E. (2002). CSF evaluation in primary CNS lymphoma patients by PCR of the CDR III IgH genes. *Neurology,* **58,** 390-396.

Guilleret I and Benhattar J. (2004). Unusual distribution of DNA methylation within the hTERT CpG island in tissues and cell lines. *Biochem Biophys Res Commun,* **325,** 1037-1043.

Guilleret I, Yan P, Grange F, Braunschweig R, Bosman FT and Benhattar J. (2002). Hypermethylation of the human telomerase catalytic subunit (hTERT) gene correlates with telomerase activity. *Int J Cancer,* **101,** 335-341.

Hegi ME, Diserens AC, Gorlia T, Hamou MF, de TN, Weller M, *et al*. (2005). MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med,* **352,** 997-1003.

Herman JG and Baylin SB. (2003). Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med,* **349,** 2042-2054.

Jones PA. (1999). The DNA methylation paradox. *Trends Genet,* **15,** 34-37.

Jones PA and Laird PW. (1999). Cancer epigenetics comes of age. *Nat Genet,* **21,** 163-167.

Kleinschmidt-DeMasters BK, Hashizumi TL, Sze CI, Lillehei KO, Shroyer AL and Shroyer KR. (1998). Telomerase expression shows differences across multiple regions of oligodendroglioma versus high grade astrocytomas but shows correlation with Mib-1 labelling. *J Clin Pathol,* **51,** 284-293.

Kristensen LS, Mikeska T, Krypuy M and Dobrovic A. (2008). Sensitive Melting Analysis after Real Time-Methylation Specific PCR (SMART-MSP): high-throughput and probe-free quantitative DNA methylation detection. *Nucleic Acids Research,* **36**.

Laird PW. (2003). The power and the promise of DNA methylation markers. *Nat Rev Cancer,* **3,** 253-266.

Lee WY. (2005). Limitations of detection of malignancy in pleural effusions using ELISA-based TRAP assay: comparison with cytological examination. *Cytopathology,* **16,** 227-232.

Miura N, Maruyama S, Oyama K, Horie Y, Kohno M, Noma E, *et al*. (2007). Development of a novel assay to quantify serum human telomerase reverse transcriptase messenger RNA and its significance as a tumor marker for hepatocellular carcinoma. *Oncology,* **72 Suppl 1,** 45-51.

Nomoto K, Maekawa M, Sugano K, Ushiama M, Fukayama N, Fujita S, *et al*. (2002). Methylation status and expression of human telomerase reverse transcriptase mRNA in relation to hypermethylation of the p16 gene in colorectal cancers as analyzed by bisulfite PCR-SSCP. *Jpn J Clin Oncol,* **32,** 3-8.

Pine SR, Yin C, Matloub YH, Sabaawy HE, Sandoval C, Levendoglu-Tugal O, *et al*. (2005). Detection of central nervous system leukemia in children with acute lymphoblastic leukemia by real-time polymerase chain reaction. *J Mol Diagn,* **7,** 127-132.

Rand K, Qu W, Ho T, Clark SJ and Molloy P. (2002). Conversion-specific detection of DNA methylation using real-time polymerase chain reaction (ConLight-MSP) to avoid false positives. *Methods,* **27,** 114-120.

Schinstine M, Filie AC, Wilson W, Stetler-Stevenson M and Abati A. (2006). Detection of malignant hematopoietic cells in cerebral spinal fluid previously diagnosed as atypical or suspicious. *Cancer,* **108,** 157-162.

Shi H, Wang MX and Caldwell CW. (2007). CpG islands: their potential as biomarkers for cancer. *Expert Rev Mol Diagn,* **7,** 519-531.

Shinozaki M, Hoon DS, Giuliano AE, Hansen NM, Wang HJ, Turner R, *et al*. (2005). Distinct hypermethylation profile of primary breast cancer is associated with sentinel lymph node metastasis. *Clin Cancer Res,* **11,** 2156-2162.

Swinkels DW, de Kok JB, Hanselaar A, Lamers K and Boerman RH. (2000). Early detection of leptomeningeal metastasis by PCR examination of tumor-derived K-ras DNA in cerebrospinal fluid. *Clin Chem,* **46,** 132-133.

Weng NP. (2002). Regulation of telomerase expression in human lymphocytes. *Springer Seminars in Immunopathology,* **24,** 23-33.

Wojdacz TK and Dobrovic A. (2007). Methylation-sensitive high resolution melting (MS-HRM): a new approach for sensitive and high-throughput assessment of methylation. *Nucleic Acids Research,* **35**.

CHAPTER 5

Conclusion and Perspectives

Telomerase adds telomeric repeat sequences to the end of chromosomes, and prevents the loss of telomere and cellular senescence. The catalytic subunit of telomerase, hTERT, is the limiting factor for its activation (Bodnar *et al.*, 1998; Counter *et al.*, 1998). An abundance of regulatory models involved in the transcriptional control of hTERT has been suggested after identification of numerous activators and repressors that bind to the hTERT 5' regulatory region, and implication of CpG methylation and histone acetylation (Devereux *et al.*, 1999; Cong and Bacchetti, 2000; Dessain *et al.*, 2000; Poole *et al.*, 2001; Takakura *et al.*, 2001; Ducrest *et al.*, 2002; Guilleret *et al.*, 2002; Goueli and Janknecht, 2003; Lv *et al.*, 2003; Pardal *et al.*, 2003; Goueli and Janknecht, 2004; Nishi *et al.*, 2004; Yatabe *et al.*, 2004; Pendino *et al.*, 2006; Renaud *et al.*, 2007; Cairney and Keith, 2008). Numerous investigations have attempted to elucidate the regulation of hTERT transcription, its transport to the nucleus, the assembly of the telomerase complex, its recruitment to the telomere, and the role of post-translational modifications of hTERT protein. No single mechanism can explain silencing of telomerase in most somatic cells and its reactivation in tumor cells. This thesis illustrates the complexity of the hTERT transcriptional regulation in normal and tumor cells.

In telomerase-negative somatic cells, telomeres shorten with each cell division, resulting in replicative senescence and cell death. In general, stem cells and other actively replicating cells are telomerase-positive and are an exception to this rule (Figure 1).

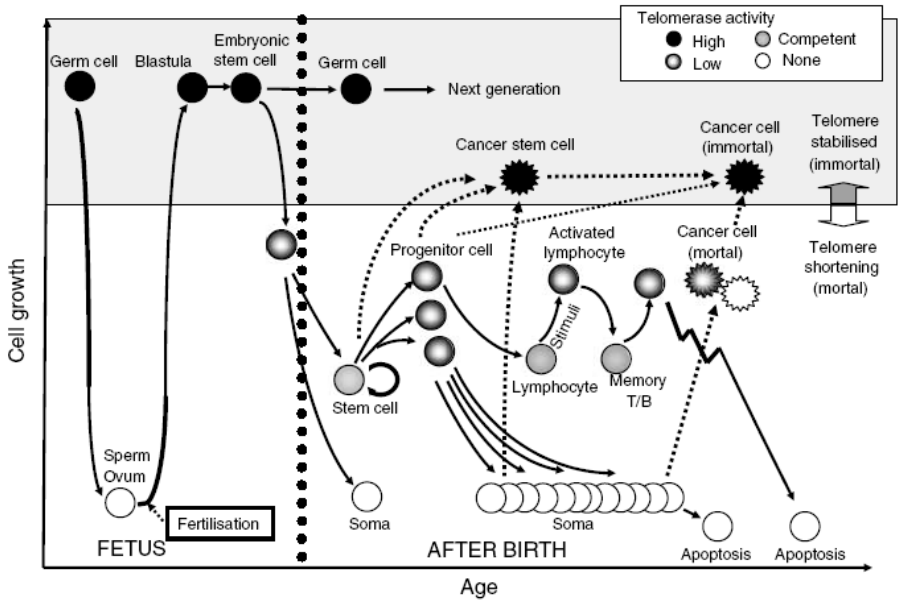


Figure 1. Telomere and telomerase dynamics in human cells (cited from Hiyama and Hiyama, 2007).

In germ cells, telomerase is expressed in order to maintain telomere length from generation to generation (Blasco *et al.*, 1997). Stem cells demonstrate proliferative capacity and therefore have to preserve telomere length through numerous cell divisions. In fact, human adult stem cells including skin, intestinal crypt, mammary epithelial, pancreas, neuronal, adrenal cortex, hematopoietic, kidney, and mesenchymal stem cells exhibit low levels of telomerase activity (Hiyama and Hiyama, 2007). Upon activation lymphocytes also develop telomerase activity, whereas naïve or memory cells exhibit very low telomerase activity.

The discovery of the implication of CTCF in the transcriptional regulation of hTERT in part explained the mechanism of silencing of telomerase in most somatic cells and its reactivation in neoplastic cells (Renaud *et al.*, 2005). In telomerase-negative cells, binding of CTCF to the hTERT 5' regulatory region inhibits hTERT transcription and prevents telomerase activation. In telomerase-positive cells, CTCF cannot exert its inhibitory activity because of methylation-dependent and -independent mechanisms, and as a result hTERT transcription occurs. In this study, we identified new factors implicated in both methylation-dependent and -independent mechanisms of the regulation of hTERT expression.

First, we aimed to identify factors involved in the regulation of hTERT expression when methylation is present in the 5' region of the gene, which is the case in most telomerase-positive cancer cells. A particular pattern of methylation was shown to be crucial for establishing hTERT expression at a basal level, through prevention of CTCF inhibitor binding and hypomethylation of a short region of the CpG islands surrounded by highly methylated border regions (Renaud *et al.*, 2007). Usually, CpG islands are unmethylated in normal cells (Weber *et al.*, 2007), while hypermethylation of these sequences silences gene expression, for example of tumor suppressor genes as is found in cancer cells (Ehrlich, 2002). DNA methylation patterns are "interpreted" by the methyl-CpG binding domain family of proteins. The potential involvement of MBD proteins in the unconventional regulation of hTERT expression by methylation was therefore investigated. ChIP experiments and ChIP on chip analysis indicate that MBD2 specifically binds to the methylated CpG island of hTERT. MBD2 depletion by RNAi did not induce MeCP2 or MBD1 binding to this methylated region in HeLa cells, but increased hTERT expression. The specific transcriptional repression of hTERT through MBD2 was confirmed in breast, liver, and neuroblastoma cancer cell lines, and thus represents an additional element in the control of hTERT expression. We showed that repression of hTERT transcription by DNA methylation in most carcinoma cells is

mediated by one MBD protein, as for other genes like BRCA1, although the MBD2 affect is not sufficient to completely arrest hTERT expression.

We decided to focus on methylation-independent mechanisms of telomerase activation through hTERT expression (Bechter *et al.*, 2002; Widschwendter *et al.*, 2004; Zinn *et al.*, 2007). We first characterized the methylation status of the hTERT gene in normal lymphocytes and lymphomas. The hTERT gene was found to be completely demethylated in telomerase-positive B cells. We also found that the B-cell specific transcription factor PAX5 is necessary for activation of hTERT expression. PAX5 binding sites were identified by EMSA and ChIP experiments, from +110 to +137 bp and +489 to +516 bp downstream of the ATG translational start site, in close proximity to the CTCF binding sites. PAX5 and CTCF were shown to simultaneously bind to the hTERT gene. We determined that ectopic expression of PAX5 in telomerase-negative cells is sufficient to activate hTERT transcription whereas depletion of PAX5 by RNAi leads to repression of hTERT transcription in B cells. In summary, we demonstrated that hTERT transcription, in normal and neoplastic telomerase-positive B cells, does not require DNA methylation, but is dependent on PAX5.

Understanding how hTERT is activated in lymphoid cells is of interest because solid tumors are often invaded by inflammatory cells (Ruegg, 2006). Among them, B lymphocytes exhibit variable levels of telomerase activity depending on their developmental stage (Norrback *et al.*, 1996; Hu *et al.*, 1997; Igarashi and Sakaguchi, 1997; Weng *et al.*, 1997). Their presence can lead to telomerase activity or hTERT expression in non tumor cell containing samples, which hampers their uses in cancer diagnosis (Cunningham *et al.*, 1998; Yang *et al.*, 1998; Dikmen *et al.*, 2003). On the contrary, hTERT methylation is highly correlated with the presence of cancer cells and is not detected in telomerase-positive normal B cells. Therefore, we studied hTERT methylation as a potential cancer biomarker. The developed method for hTERT methylation detection relies on MS-HRM technology, which was improved by the addition of a TaqMan probe in order to attain a very low level of detection of methylation. Our results suggest that, hTERT methylation holds promise as a sensitive and highly specific biomarker for cancer cells, for example in the detection of leptomeningial metastasis in cerebrospinal fluids (CSF).

In summary, we have investigated the implication of MBD2 and PAX5 in the regulation of hTERT transcription in telomerase-positive cells, and we revealed an interesting diagnostic approach that uses hTERT methylation as a cancer biomarker. These results

complete an earlier model elaborated in our laboratory and allow the development a general understanding of how hTERT transcription is regulated by CTCF.

Our results suggest that different telomerase-positive cell types use different mechanisms to inhibit CTCF effects. In addition, normal and cancerous telomerase-positive cells from a same tissue origin apparently use the same mechanism to eliminate the inhibitory effect of CTCF. In germ cell tumors, BORIS has been directly involved in the activation of hTERT transcription through competition with CTCF binding, which alleviates the CTCF block (Renaud S., manuscript in preparation). BORIS is also expressed in normal germ cells and probably allows these cells to keep a very high level of telomerase, which is required to preserve the correct telomere length for the daughter cells. Likewise, preliminary results have shown that hTERT methylation also occurs in the stem cell niches located at the base of the colon crypt (unpublished data). Thus, it seems that epithelial stem cells and carcinoma cells both use DNA methylation to block the inhibitory effects of CTCF and allow hTERT transcription. We will explore this possibility in further studies. Furthermore, we still do not know if the cancer cells develop directly from stem or progenitor cells without loss of hTERT methylation or if a step of hTERT remethylation occurs in preneoplastic somatic cells. Finally, we identified PAX5 as a factor involved in the activation of hTERT transcription, in both normal and neoplastic telomerase-positive B cells.

All these results are summarized in a multifaceted model of hTERT regulation by CTCF described in Figure 2.

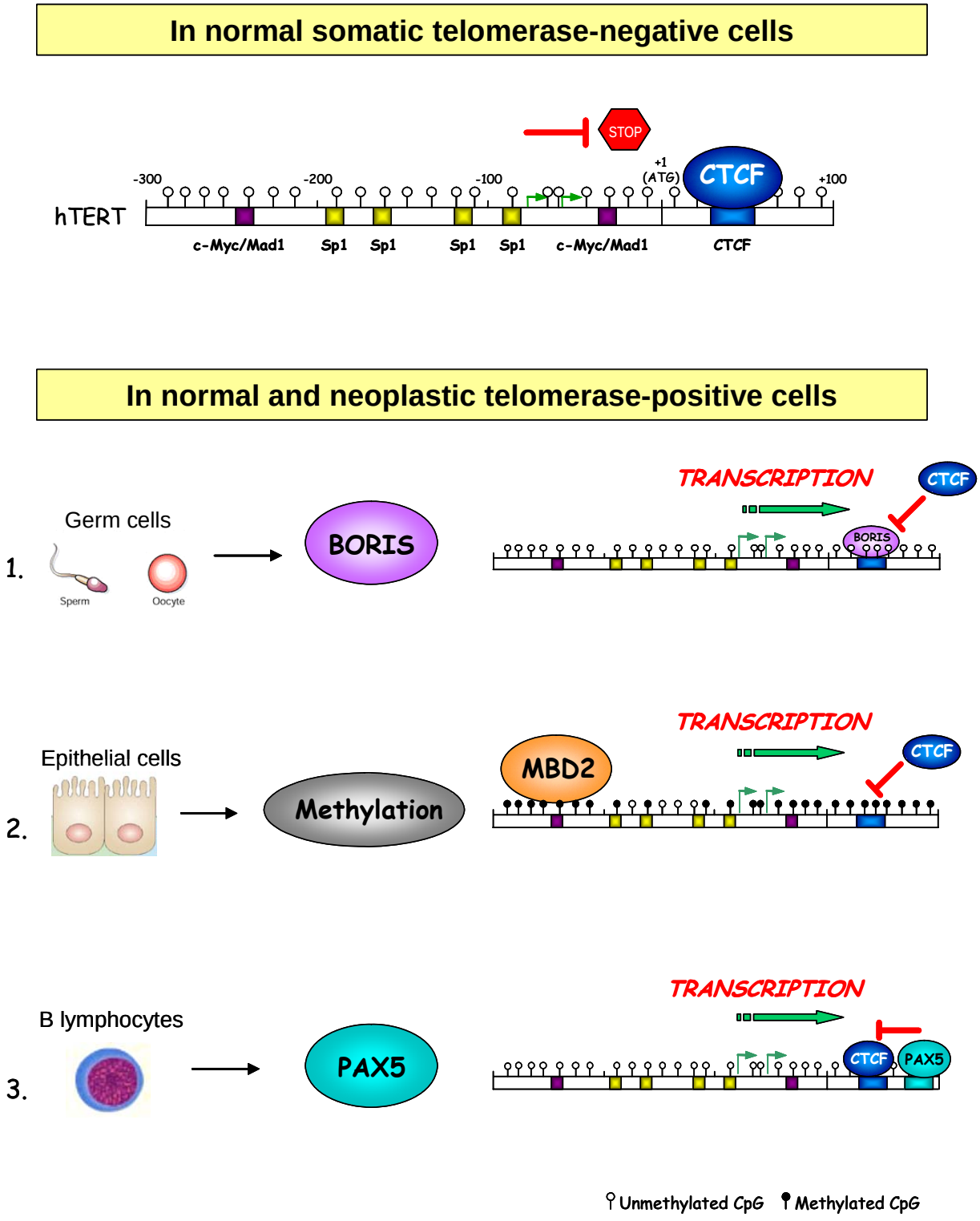


Figure 2. Hypothetic model of hTERT transcriptional regulation. In normal cells, the hTERT gene is not methylated, thus CTCF can inhibit the transcription. In telomerase-positive normal or cancerous cells, CTCF inhibition is counteracted, thus hTERT expression is allowed. In germ cells, BORIS factor prevents the binding of CTCF, allowing transcription of hTERT. In epithelial cells, hTERT gene are hypermethylated, except in a region upstream of the transcription start site. The methylation releases CTCF and some transcription of hTERT occurs. In B cells, PAX5 factor probably inhibits the repressor effect of CTCF by an unknown mechanism. Green arrows represent the transcriptional start sites; empty circles represent unmethylated CpG sites and solid circles the methylated ones.

It is important to note that CTCF is not the only factor implicated in the complex regulation of hTERT: other factors are necessary to activate hTERT transcription. For instance, we have already observed that some ALT-telomerase-negative osteosarcoma cells do not express hTERT in spite of a profile of hTERT methylation that is permissive for hTERT transcription in other cell lines. hTERT CpG island methylation appears to be an important factor for hTERT activation, but not sufficient by itself. The hTERT promoter activation by the transcription factors present in these cells could be insufficient to counteract the inhibition imposed by binding of MBD2 to the methylated promoter (unpublished data). Another study suggests that cells activate ALT pathway because they lack some factors that enable the activation of hTERT transcription (Stewart, 2005). Likewise, around 50% of the immortalized cell lines used hTERT methylation to avoid CTCF binding, but other immortalized cells are unmethylated and do not express the BORIS factor. It would be interesting to investigate PAX5 expression or find other specific factors allowing hTERT expression in immortalized cells.

To better explain the mechanism of our proposed model (Figure 2), further investigations are needed to understand how the binding of CTCF to the proximal hTERT exonic region can inhibit hTERT transcription. The association of CTCF with histone deacetylase suggests that the transcriptional repression might occur through chromatin condensation (Lutz *et al.*, 2000). In the regulation of the H19/Igf2 imprinted region, CTCF emerges as a mediator of long-range interactions that form a special conformation to sequester a gene into a loop of silent chromatin. The Chromosome Conformation Capture (3C) assay has highlighted that on the unmethylated maternal allele of Igf2, CTCF associates to create a boundary that blocks the access of the enhancers to the promoters (Murrell *et al.*, 2004; Kurukuti *et al.*, 2006). Thus, CTCF might interact with the transcriptional machinery or with histone deacetylases to allow the inhibition of the hTERT promoter, and a mechanism by chromatin looping should be studied in order to detect if CTCF creates an inactive loop to block hTERT transcription.

Several points remain to be clarified, such as how the pattern of methylation of hTERT CpG island is established during tumorigenesis, or how hTERT expression is regulated in other normal cells such as embryonic stem cells or in neuronal precursor cells which also express telomerase (Haik *et al.*, 2000).

To further explore the regulation of hTERT in lymphoid cells, it would be necessary to study the relationship between CTCF and PAX5 and determine how PAX5 manages to block CTCF inhibition on the hTERT gene. As the function of PAX5 factor depends on its interactions with different other proteins, it would be of interest to identify partners in the complex associated with PAX5.

We have demonstrated that in some T cell lymphomas regulation of hTERT is methylation-independent, without expression of PAX5 or BORIS factors (unpublished data). It might erase other tissue-specific factors that could prevent the effect of the CTCF repressor.

The role of other PAX proteins in the regulation of hTERT expression has to be investigated more closely. PAX proteins are essential in early development for tissue specificity and their deregulated expression is correlated with different types of cancer (Muratovska *et al.*, 2003; Robson *et al.*, 2006). Pax gene expression is involved in enhancing survival and proliferation of cancer cells, as tumor decrease is induced after deletion of Pax gene. As PAX8 factor, which has been involved in the activation of hTERT and hTR promoters in glioma (Chen *et al.*, 2008), other members of PAX5 family could influence hTERT transcription in a tissue-specific manner. For instance, PAX2, which is normally expressed during kidney development, is a sensitive and highly specific marker for renal cell carcinoma (Gokden *et al.*, 2008). Implication of PAX2 in the hTERT regulation and its effect on CTCF has to be explored. Moreover, PAX5 was revealed to maintain the proliferative and tumorigenic phenotype of neuroblastoma (Baumann Kubetzko *et al.*, 2004). Therefore, it would be interesting to determine the involvement of PAX5 in the regulation of hTERT in neuroblastoma, and determine the methylation profile of the 5'region of hTERT in this cancer. Recently, anti-PAX5 immune responses were revealed of interest in the targeting of many malignancies (Yan *et al.*, 2008). Therefore, PAX5 is a new promising target for cancer immunotherapy. This supports the hypothesis that PAX transcription factors could play a key role in the regulation of telomerase activity and could be the center of new investigations in cancer therapy (Muratovska *et al.*, 2003).

A larger study has to be performed to confirm the use of hTERT methylation as a specific cancer biomarker for leptomeningeal metastasis detection and to determine the sensibility and the specificity of this new marker.

We studied hTERT methylation in cancer diagnosis in CSF, but the method could also be explored in other samples, including blood. It has been demonstrated that cell-free DNA circulating in blood (cirDNA) exhibits the same tumor specific alterations as the DNA from tumor tissues, such as mutation and promoter hypermethylation (Anker *et al.*, 2003; Swaminathan and Butt, 2006; Fleischhacker and Schmidt, 2007). Alterations in DNA methylation patterns are the manifestation of an early and common dysregulation in tumorigenesis (Miyamoto and Ushijima, 2005). The applicability of methylation markers to cancer diagnosis and evaluation of treatment efficacy have already been evaluated in cirDNA for breast cancer (Laktionov *et al.*, 2004; Martinez-Galan *et al.*, 2008; Rykova *et al.*, 2008). Methylation analysis of cirDNA samples from gastric cancer patients were performed on MGMT, p15, and hMLH1 genes, and allowed the detection of cancer with a sensitivity of 75% and a specificity of 54% (Kolesnikova *et al.*, 2008). The analysis of hTERT methylation in blood from cancer patients could help diagnose different kinds of cancer, and potentially could have a significant impact on screening, early diagnosis, and monitoring therapy of cancer.

The hTERT regulatory model proposed here is the only one that takes into account the epigenetic status of the gene and some involved transcriptional factors. Transcriptional gene regulation study requires not only the one-by-one analysis of transcriptional factors, but also a general overview of epigenetic events. Our studies underline the fact that genetic and epigenetic regulations can never be dissociated. Our results allow a better understanding of the hTERT regulation in normal somatic cells and tumor cells, and can open the way to the elaboration of new diagnostic approaches and new anti-telomerase strategies in cancer treatment.

REFERENCES

Anker P, Mulcahy H and Stroun M. (2003). Circulating nucleic acids in plasma and serum as a noninvasive investigation for cancer: time for large-scale clinical studies? *Int J Cancer,* **103,** 149-152.

Baumann Kubetzko FB, Di PC, Maag C, Meier R, Schafer BW, Betts DR, *et al*. (2004). The PAX5 oncogene is expressed in N-type neuroblastoma cells and increases tumorigenicity of a S-type cell line. *Carcinogenesis,* **25,** 1839-1846.

Bechter OE, Eisterer W, Dlaska M, Kuhr T and Thaler J. (2002). CpG island methylation of the hTERT promoter is associated with lower telomerase activity in B-cell lymphocytic leukemia. *Experimental Hematology,* **30,** 26-33.

Blasco MA, Lee HW, Hande MP, Samper E, Lansdorp PM, DePinho RA, *et al*. (1997). Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. *Cell,* **91,** 25-34.

Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, *et al*. (1998). Extension of life-span by introduction of telomerase into normal human cells. *Science,* **279,** 349-352.

Cairney CJ and Keith WN. (2008). Telomerase redefined: integrated regulation of hTR and hTERT for telomere maintenance and telomerase activity. *Biochimie,* **90,** 13-23.

Chen YJ, Campbell HG, Wiles AK, Eccles MR, Reddel RR, Braithwaite AW, *et al*. (2008). PAX8 regulates telomerase reverse transcriptase and telomerase RNA component in glioma. *Cancer Res,* **68,** 5724-5732.

Cong YS and Bacchetti S. (2000). Histone deacetylation is involved in the transcriptional repression of hTERT in normal human cells. *J Biol Chem,* **275,** 35665-35668.

Counter CM, Meyerson M, Eaton EN, Ellisen LW, Caddle SD, Haber DA, *et al*. (1998). Telomerase activity is restored in human cells by ectopic expression of hTERT (hEST2), the catalytic subunit of telomerase. *Oncogene,* **16,** 1217-1222.

Cunningham VJ, Markham N, Shroyer AL and Shroyer KR. (1998). Detection of telomerase expression in fine-needle aspirations and fluids. *Diagn Cytopathol,* **18,** 431-436.

Dessain SK, Yu H, Reddel RR, Beijersbergen RL and Weinberg RA. (2000). Methylation of the human telomerase gene CpG island. *Cancer Res,* **60,** 537-541.

Devereux TR, Horikawa I, Anna CH, Annab LA, Afshari CA and Barrett JC. (1999). DNA methylation analysis of the promoter region of the human telomerase reverse transcriptase (hTERT) gene. *Cancer Res,* **59,** 6087-6090.

Dikmen E, Kara M, Dikmen G, Cakmak H and Dogan P. (2003). Detection of telomerase activity in bronchial lavage as an adjunct to cytological diagnosis in lung cancer. *Eur J Cardiothorac Surg,* **23,** 194-199.

Ducrest AL, Szutorisz H, Lingner J and Nabholz M. (2002). Regulation of the human telomerase reverse transcriptase gene. *Oncogene,* **21,** 541-552.

Ehrlich M. (2002). DNA methylation in cancer: too much, but also too little. *Oncogene,* **21,** 5400-5413.

Fleischhacker M and Schmidt B. (2007). Circulating nucleic acids (CNAs) and cancer--a survey. *Biochim Biophys Acta,* **1775,** 181-232.

Gokden N, Kemp SA and Gokden M. (2008). The utility of Pax-2 as an immunohistochemical marker for renal cell carcinoma in cytopathology. *Diagn Cytopathol,* **36,** 473-477.

Goueli BS and Janknecht R. (2003). Regulation of telomerase reverse transcriptase gene activity by upstream stimulatory factor. *Oncogene,* **22,** 8042-8047.

Goueli BS and Janknecht R. (2004). Upregulation of the Catalytic Telomerase Subunit by the Transcription Factor ER81 and Oncogenic HER2/Neu, Ras, or Raf. *Mol Cell Biol,* **24,** 25-35.

Guilleret I, Yan P, Grange F, Braunschweig R, Bosman FT and Benhattar J. (2002). Hypermethylation of the human telomerase catalytic subunit (hTERT) gene correlates with telomerase activity. *Int J Cancer,* **101,** 335-341.

Haik S, Gauthier LR, Granotier C, Peyrin JM, Lages CS, Dormont D, *et al*. (2000). Fibroblast growth factor 2 up regulates telomerase activity in neural precursor cells. *Oncogene,* **19,** 2957-2966.

Hiyama E and Hiyama K. (2007). Telomere and telomerase in stem cells. *Br J Cancer,* **96,** 1020-1024.

Hu BT, Lee SC, Marin E, Ryan DH and Insel RA. (1997). Telomerase is up-regulated in human germinal center B cells in vivo and can be re-expressed in memory B cells activated in vitro. *J Immunol,* **159,** 1068-1071.

Igarashi H and Sakaguchi N. (1997). Telomerase activity is induced in human peripheral B lymphocytes by the stimulation to antigen receptor. *Blood,* **89,** 1299-1307.

Kolesnikova EV, Tamkovich SN, Bryzgunova OE, Shelestyuk PI, Permyakova VI, Vlassov VV, *et al*. (2008). Circulating DNA in the blood of gastric cancer patients. *Ann N Y Acad Sci,* **1137,** 226-231.

Kurukuti S, Tiwari VK, Tavoosidana G, Pugacheva E, Murrell A, Zhao ZH, *et al*. (2006). CTCF binding at the H19 imprinting control region mediates maternally inherited higher-order chromatin conformation to restrict enhancer access to Igf2. *Proceedings of the National Academy of Sciences of the United States of America,* **103,** 10684-10689.

Laktionov PP, Tamkovich SN, Rykova EY, Bryzgunova OE, Starikov AV, Kuznetsova NP, *et al*. (2004). Cell-surface-bound nucleic acids: Free and cell-surface-bound nucleic acids in blood of healthy donors and breast cancer patients. *Ann N Y Acad Sci,* **1022,** 221-227.

Lutz M, Burke LJ, Barreto G, Goeman F, Greb H, Arnold R, *et al*. (2000). Transcriptional repression by the insulator protein CTCF involves histone deacetylases. *Nucleic Acids Res,* **28,** 1707-1713.

Lv J, Liu H, Wang Q, Tang Z, Hou L and Zhang B. (2003). Molecular cloning of a novel human gene encoding histone acetyltransferase-like protein involved in transcriptional activation of hTERT. *Biochem Biophys Res Commun,* **311,** 506-513.

Martinez-Galan J, Torres B, Del MR, Munoz-Gamez JA, Martin-Oliva D, Villalobos M, *et al*. (2008). Quantitative detection of methylated ESR1 and 14-3-3-sigma gene promoters in serum as candidate biomarkers for diagnosis of breast cancer and evaluation of treatment efficacy. *Cancer Biol Ther,* **7,** 958-965.

Miyamoto K and Ushijima T. (2005). Diagnostic and therapeutic applications of epigenetics. *Jpn J Clin Oncol,* **35,** 293-301.

Muratovska A, Zhou C, He S, Goodyer P and Eccles MR. (2003). Paired-Box genes are frequently expressed in cancer and often required for cancer cell survival. *Oncogene,* **22,** 7989-7997.

Murrell A, Heeson S and Reik W. (2004). Interaction between differentially methylated regions partitions the imprinted genes Igf2 and H19 into parent-specific chromatin loops. *Nature Genetics,* **36,** 889-893.

Nishi H, Nakada T, Kyo S, Inoue M, Shay JW and Isaka K. (2004). Hypoxia-inducible factor 1 mediates upregulation of telomerase (hTERT). *Mol Cell Biol,* **24,** 6076-6083.

Norrback KF, Dahlenborg K, Carlsson R and Roos G. (1996). Telomerase activation in normal B lymphocytes and non-Hodgkin's lymphomas. *Blood,* **88,** 222-229.

Pardal R, Clarke MF and Morrison SJ. (2003). Applying the principles of stem-cell biology to cancer. *Nat Rev Cancer,* **3,** 895-902.

Pendino F, Tarkanyi I, Dudognon C, Hillion J, Lanotte M, Aradi J, *et al*. (2006). Telomeres and telomerase: Pharmacological targets for new anticancer strategies? *Curr Cancer Drug Targets,* **6,** 147-180.

Poole JC, Andrews LG and Tollefsbol TO. (2001). Activity, function, and gene regulation of the catalytic subunit of telomerase (hTERT). *Gene,* **269,** 1-12.

Renaud S, Loukinov D, Abdullaev Z, Guilleret I, Bosman FT, Lobanenkov V, *et al*. (2007). Dual role of DNA methylation inside and outside of CTCF-binding regions in the transcriptional regulation of the telomerase hTERT gene. *Nucleic Acids Res,* **35,** 1245-1256.

Renaud S, Loukinov D, Bosman FT, Lobanenkov V and Benhattar J. (2005). CTCF binds the proximal exonic region of hTERT and inhibits its transcription. *Nucleic Acids Res,* **33,** 6850-6860.

Robson EJ, He SJ and Eccles MR. (2006). A PANorama of PAX genes in cancer and development. *Nat Rev Cancer,* **6,** 52-62.

Ruegg C. (2006). Leukocytes, inflammation, and angiogenesis in cancer: fatal attractions. *J Leukoc Biol,* **80,** 682-684.

Rykova EY, Tsvetovskaya GA, Sergeeva GI, Vlassov VV and Laktionov PP. (2008). Methylation-based analysis of circulating DNA for breast tumor screening. *Ann N Y Acad Sci,* **1137,** 232-235.

Stewart SA. (2005). Telomere maintenance and tumorigenesis: an "ALT"ernative road. *Curr Mol Med,* **5,** 253-257.

Swaminathan R and Butt AN. (2006). Circulating nucleic acids in plasma and serum: recent developments. *Ann N Y Acad Sci,* **1075,** 1-9.

Takakura M, Kyo S, Sowa Y, Wang Z, Yatabe N, Maida Y, *et al*. (2001). Telomerase activation by histone deacetylase inhibitor in normal cells. *Nucleic Acids Res,* **29,** 3006-3011.

Weber M, Hellmann I, Stadler MB, Ramos L, Paabo S, Rebhan M, *et al*. (2007). Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. *Nat Genet,* **39,** 457-466.

Weng NP, Granger L and Hodes RJ. (1997). Telomere lengthening and telomerase activation during human B cell differentiation. *Proc Natl Acad Sci U S A,* **94,** 10827-10832.

Widschwendter A, Muller HM, Hubalek MM, Wiedemair A, Fiegl H, Goebel G, *et al*. (2004). Methylation status and expression of human telomerase reverse transcriptase in ovarian and cervical cancer. *Gynecologic Oncology,* **93,** 407-416.

Yan M, Himoudi N, Pule M, Sebire N, Poon E, Blair A, *et al*. (2008). Development of cellular immune responses against PAX5, a novel target for cancer immunotherapy. *Cancer Res,* **68,** 8058-8065.

Yang CT, Lee MH, Lan RS and Chen JK. (1998). Telomerase activity in pleural effusions: diagnostic significance. *J Clin Oncol,* **16,** 567-573.

Yatabe N, Kyo S, Maida Y, Nishi H, Nakamura M, Kanaya T, *et al*. (2004). HIF-1-mediated activation of telomerase in cervical cancer cells. *Oncogene,* **23,** 3708-3715.

Zinn RL, Pruitt K, Eguchi S, Baylin SB and Herman JG. (2007). hTERT is expressed in cancer cell lines despite promoter DNA methylation by preservation of unmethylated DNA and active chromatin around the transcription start site. *Cancer Research,* **67,** 194-201.