[Frontiers in Bioscience 10, 1779-1796, May 1, 2005]

EPIGENETIC CONTROL OF TELOMERASE AND MODES OF TELOMERE MAINTENANCE IN AGING AND ABNORMAL SYSTEMS

Serene R. Lai [1], Sharla M. O. Phipps [1], Liang Liu [1], Lucy G. Andrews [1] and Trygve O. Tollefsbol [1, 2, 3]

[1] Department of Biology, University of Alabama at Birmingham, AL 35294, USA, [2] Center of Aging, University of Alabama at Birmingham, AL 35294, USA, [3] Comprehensive Cancer Center, University of Alabama at Birmingham, AL 35294, USA

TABLE OF CONTENTS

1. ABSTRACT

Epigenetic control provides a mechanism for the reversible silencing of telomerase expression that occurs as a natural consequence of differentiation. Significant overlap between indirect telomerase regulation pathways and cell cycle checkpoint pathways exist, suggesting that these discrete genetic elements (namely, p21, p53, and hTERT) synergistically cooperate to inhibit tumorigenesis. Mutations in these pathways have been known to contribute to cancer formation. However, the incorporation of epigenetic regulatory mechanisms provides another line of defense against these negative occurrences. These proteins are also implicated in the process of senescence, caused in eukaryotic cell lines by telomere shortening. Although the debate continues, there is significant evidence to classify the process of cellular senescence as an *in vitro* model for human aging. In addition, the study of stem cells gives information about the down-regulation of hTERT in the aging process. Diseases such as Werner's syndrome, ATM (ataxia telangiectasia mutated kinase), DKC (dyskeratosis congenita), and atherosclerosis have been linked to aberrant telomerase expression and other aging-related tissue malfunctions could be related to the presence of senescent cells changing the cellular microenvironment. Therefore, restoring telomerase activity as a putative therapeutic strategy necessitates further study to elucidate the intricacies linking genetic and epigenetic modulations of hTERT.

2. TELOMERASE AND TELOMERES

Telomerase is a DNA polymerase that uses an RNA template in a reverse transcription reaction to synthesize DNA termini during the replication process. The nascent DNA synthesized by telomerase is referred to as telomeres, and is essential to counteract the "end-replication problem" in linear chromosomes. Telomeres

also serve to preserve the chromosomal integrity by preventing rearrangements, nuclease degradation, and end-to-end chromosomal fusions (1-2). The telomerase holoenzyme is a ribonucleoprotein complex and is composed primarily of two subunits. In humans, the catalytic subunit of telomerase is hTERT (human telomerase reverse transcriptase), which confers its reverse transcription activity; and the RNA component, *hTR*, that is complementary to the 6-nt telomeric repeat sequence, serves as a template for elongation of the telomeres (3-5).

The hTR component of telomerase is ubiquitously expressed in most cell types including telomerase-negative cells such as differentiated somatic cells (6-7). hTERT, on the other hand, is tightly regulated during differentiation and is almost undetectable in most somatic cells (8). A positive correlation has been found between the amount of *hTERT* mRNA and the activity of telomerase, suggesting that telomerase is regulated at the gene transcriptional level of *hTERT* (9-12).

2.1. Telomere attrition rate

Telomerase is expressed at different levels in various cell types, mostly according to the proliferative ability of the cells. It is highly expressed in stem cells, germ cells, other self-renewing tissues such as keratinocytes (basal epidermal cells), and hematopoetic cells (13-14). As discussed above, hTERT expression is positively correlated with telomerase activity, and thus inhibition of hTERT usually results in telomeric attrition. Telomerase activity is usually repressed in somatic cells, but is reactivated in immortalized cell lines and human cancers (15). Due to this repression of telomerase, normal somatic cells experience telomeric attrition at a mean loss of 30-150 bps of telomeric DNA per replication (16), until a critical minimum telomeric length is reached whereby the cells then experience cellular senescence (17). Consistent with the Hayflick Limit theory, which states that cells have a maximum number of population doublings before undergoing senescence, scientists now propose that telomeres serve as a biological clock of the aging process.

In contrast, it is interesting to note that exogenous expression of hTERT can immortalize cells (18) and similarly restore biological function in senescent cell populations (19). About 70% of immortalized human somatic cell lines (20) and 90 – 95% of human cancer cells express high levels of telomerase, comparable to cells normally expressing telomerase such as germline cells. This shows a strong correlation between telomerase expression, telomere length maintenance and tumorigenesis or immortalization (21-22). Thus, in the normal phenotype, it is important for the cell to be able to strike a balance between telomere maintenance and the rate of telomere attrition. This article discusses the epigenetic regulation of telomerase, factors or mechanisms that may modulate its function in the cell, and the effect on telomeres.

2.2. hTERT epigenetic control

2.2.1. DNA methylation of hTERT promoter

Recent evidence suggests that gene expression can be altered markedly via several diverse epigenetic mechanisms that can lead to permanent or reversible changes in cellular behavior. DNA cytosine methylation is the most prevalent eukaryotic DNA modification and constitutes one of the best understood epigenetic phenomena. There is substantial evidence that DNA methylation plays a critical role in gene regulation during development and cellular differentiation. Currently, there are three families of DNA methyltransferases (DNMTs) identified to be involved in the *in vivo* methylation pathways in both mouse and human models. The DNMT1 family is involved in maintenance methylation because they show a preference towards methylating hemimethylated DNA substrates *in vitro* (23). DNMT1 has also been shown to display some *de novo* methylating activity (24), as is the case with DNMT3a and DNMT3b. The DNMT3 family is considered to be largely responsible for establishing new methylation patterns (25).

There has been much debate about the methylation status and resulting transcriptional activity of the hTERT promoter. Gene promoters that are rich in CpG dinucleotides are highly susceptible to DNA methylation at the cytosine residues. In general, CpG island methylation correlates inversely with gene expression, because it may prevent the binding of methylation-sensitive transcription factors. Retinoic acid induced differentiation of human leukemia (HL60) cells and human teratoma cells resulted in a decreased expression of *hTERT*, and a corresponding increase in hTERT promoter methylation. Interestingly, the methylating patterns at the promoter also changed through the differentiation process (153). In contrast, the promoter of hTERT has been found to be hypermethylated in tumor tissue and cell lines that were telomerase positive, as compared to telomerase negative normal tissues where hypomethylation was observed (26). Treatment with the demethylating reagent 5-aza-2'-deoxycytidine caused reactivation of the hTERT promoter in differentiated HT cells (153), while a similar treatment inhibited its expression in human prostate cancer cells (27).

It is evident that *hTERT* expression is multi-fold and methylation studies of the promoter is cell-type specific possibly based on the expression of activators and repressors, and the relevant chromatin structure. It is plausible that epigenetic changes in the chromatin provides access to these transcriptional factors, and a change in the methylation pattern of the promoter suggests a mechanism in which access to methylation-sensitive transcription factors is regulated. It is also probable that it is the differential binding of these factors that eventually results in the heterogenous expression of hTERT as a result of these epigenetic changes.

2.2.2. Histone modifications by acetylation/methylation

Many genes are not modulated solely by transcriptional factors, but also by accessibility of the transcriptional factors to the promoters. Protein access to DNA is modulated by chromatin structure. As the dynamic chromatin switches between euchromatin and heterochromatin, accessibility increases and decreases respectively. Mainly, chromain structure can be changed by histone modifications such as acetylation and methylation

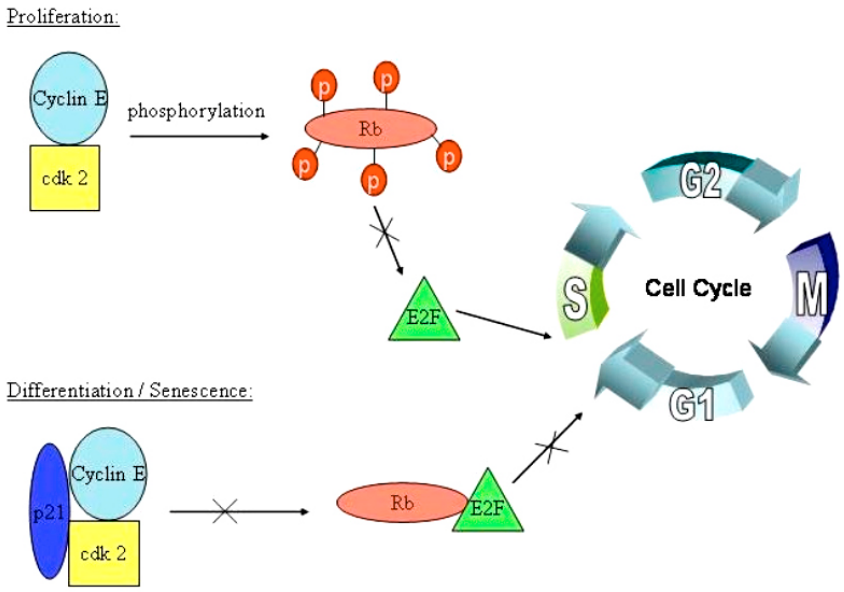

Figure 1. p21, cdk2, cyclin E, pRB, and E2F interaction and the resulting effects on the cell cycle. In proliferating cells, phosphorylation of pRB by cdk2-cyclin E complex prevents binding of pRB to E2F, thus enabling E2F to be involved in the transcription of S-phase related proteins. In differentiated or senescent cells, p21 can act as a CDKI to prevent the downstream phosphorylation of pRB, allowing pRB to sequester E2F, and eventually preventing the entry of the cell into S-phase.

of appropriate amino acid residues. Histone proteins carry basic charges and are wrapped around by negatively charged DNA molecules. The electrostatic attraction helps to sustain a stable association between these two molecules. Acetylation of the histone proteins can neutralize the charge on the histones, and therefore attenuates the attraction force between DNA and histone proteins, leading to an opened chromatin structure. Conversely, deacetylation of histones causes histone tails to tightly associate with DNA, which forms a repressive chromatin structure that is less accessible to transcription factors. Such reversible modifications of the histone proteins can effectively alter the conformation of a given region of chromosomal DNA.

In addition to the acetylation modification, other post-translational modifications of the histone proteins including methylation have also been described to affect transcriptional regulation and gene expression (28). For example, human repetitive elements such as Alu elements contain a high level of H3-Lys9 methylation, which is proposed to play a role in the suppression of recombination among these elements (29). Methylation modification of the histone lysine residues may exist in three different forms: mono-, di- and tri-methylation. G9a histone methyltransferase (HMTase) is indicated to be responsible for all detectable dimethylation and a significant amount of monomethylation within the silent euchromatin, whereas Suv39h1 and Suv39h2 HMTases may direct trimethylation specifically at heterochromatin regions (30). Telomeres are normally enriched in trimethylated H3-Lys9 (31).

Both DNA methylation and histone acetylation have been extensively studied and recognized as global epigenetic mechanisms regulating gene transcription. Observations from the past have revealed a strong link between these two processes. At the *hTERT* promoter, for example, they are found to cooperate with each other at the chromatin level to influence the regulation of *hTERT* expression (32). The different methyl-CpG binding domain proteins such as MeCP2, MBD1, MBD2 and MBD3 may act as mediators between DNA methylation and histone acetylation. They recognize and bind to symmetrically methylated-CpG dinucleotides, recruit histone deacetylase and mediate transcriptional silencing by deacetylating histones in the vicinity of the promoter. The synergistic role between DNA methylation and histone deacetylation events was also documented by studies showing that direct associations between DNMT and HDAC repressor complexes, which are recruited to replication foci through interactions with proliferating cell nuclear antigen (PCNA) (33-36), interact with cell cycle regulator, p21 to regulate *hTERT* expression (see section 2.3).

2.3. Epigenetic modulations of telomerase via indirect pathways

2.3.1. $p21^{WAF1/Cip1/SDI1}$ and p53 cell cycle checkpoint

Since studies have shown that the stable proliferation or senescence of cells is dependent on telomerase activity and telomeric length, it is important to study the relationship of telomerase and cell cycle control mechanisms. The main cell cycle regulatory machinery includes interplay of protein complexes consisting of cyclins, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CDKIs). The phosphorylation of cyclin proteins by their respective CDKs is necessary for the progression of the cell cycle through the G1/S checkpoint into S-phase. $p21^{WAF1/Cip1/SDI1}$ (p21), a CDKI, forms a complex with cyclin E and CDK2 to prevent the downstream phosphorylation of retinoblastoma protein (RB), thereby inhibiting G1/S progression of the cell cycle (Figure 1). p53 is a major player in cell cycle control, and

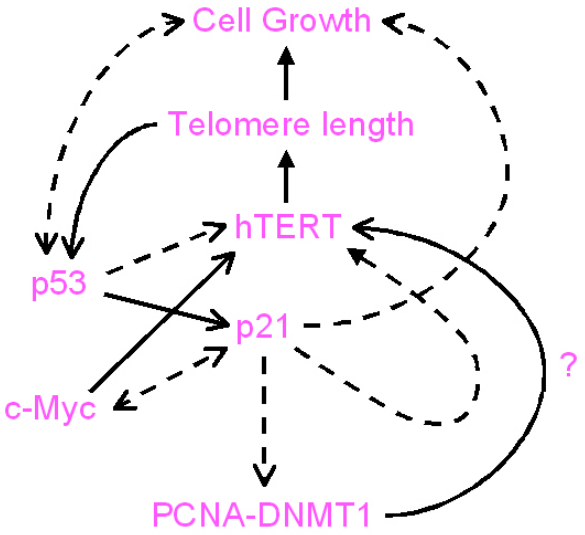

Figure 2. Interlinked pathways involving cell cycle proteins p53 and p21, c-Myc, and potential epigenetic control on hTERT, the telomerase catalytic subunit. Positive regulation is represented by solid arrows, while negative regulation is represented by dashed-arrows.

works in conjunction with p21 to apply the brakes at the G1/S checkpoint.

In 1996, Holt *et al*. demonstrated that telomerase is consistently detectable in immortalized human leukemia (HL60) and cancerous human teratoma (HT1080) cell lines at all stages of the cell cycle (37). This lack of variation in telomerase levels may be explained by the fact that the enzyme has a half-life of about 24 hours (38). In the same study, *all-trans*-retinoic acid differentiated HL60 cells and telomerase-positive contact-inhibited quiescent Swiss fibroblast cell-line NIH3T3 cells displayed minimal telomerase activity, suggesting a mechanism for the down-regulation of the enzyme activity during G_0 phase. Passaging of the contact-inhibited NIH3T3 cells allows the exit of G_0 into G_1 phase, leading to the reactivation of telomerase. This indicates that the inactivation of the enzyme is a reversible process and that telomerase activity does not correlate with DNA synthesis (37). In this section, we will explore the role of epigenetics in the transcriptional regulation of telomerase by cell cycle control proteins.

2.3.2. The p53 / $p21^{WAF1/Cip1/SDI1}$ linked pathways and hTERT

p53 is a tumor suppressor gene that is associated with differentiation, apoptosis, DNA repair and control of cell cycle progression. Mutation of the p53 gene is associated with about 10,000 types of human tumors (39). p53 protein regulates the transcription of many genes, both negatively and positively, thereby influencing the entry processes with which it is associated. The *hTERT* gene has two p53 binding sites at -1877 and -1240 (+1 denotes the start of transcription) upstream of the 5' promoter region. Overexpression of p53 and binding of the protein at these two sites represses the *hTERT* promoter with the assistance of transcription factor Sp1 recruitment (40). CDKI p21 has a p53 responsive element at -2281 / -2262 in the distal region of its promoter. Full transactivation of the p21 promoter is enabled by the binding of p53 protein, Sp1 proteins and related factors at the promoter region (41- 42). The downstream effect of p53 transactivation of the p21 promoter is the negative regulation of the cell cycle, thereby preventing progression past the G1/S checkpoint (43). At the same time, p53 expression leads to the repression of the *hTERT* promoter, thereby effectively shutting down the cell cycle (Figure 2). In mammary epithelial cells, abrogation of p53 function induces cellular immortality, probably through the reactivation of telomerase (40). On the other hand, normal human astrocyte cells (NHAs) senesced upon exogenous p21 expression and could not be induced to reproliferate with hTERT expression, indicating that senescence is directly p53-dependent and that hTERT is a downstream regulating event (44).

2.3.3. Effect of $p21^{WAF1/Cip1/SDI1}$ on hTERT

$p21^{WAF1/Cip1/SDI1}$ (p21) is a CDKI belonging to the CIP/KIP family of negative cell cycle regulators along with $p27^{Kip1}$. The *p21* gene is ubiquitously expressed in mammalian cells and is speculated to be the universal inhibitor of CDKs (45-46). As a major player in cell cycle arrest, p21 is critical for the control of differentiation, senescence, and apoptosis. Overexpression of *p21* induces a correlative repression of *hTERT*, which has been demonstrated in human glioma cells (47) and squamous cell carcinoma cell lines, in which telomerase expression is abnormally elevated (48).The *p21* promoter has been shown to be generally CpG rich and to contain a 78 base pair GC-rich region near the TATA box that is required for induction of the gene (42, 49), indicating possible epigenetic regulation of its expression through changes in DNA methylation. Studies have shown that treatment of human diploid fibroblast HCA2 cells with 5-aza-2-deoxycytidine, a potent DNMT inhibitor, caused a rapid global decrease in methylation followed by an elevation in p21 mRNA and protein levels. The cells were found to be arrested at both G1 and G2 phases of the cell cycle (43). Moreover, transfection of antisense DNMT1 into human breast cancer MDA231 cells revealed a correlation between the decrease in DNMT1 and an increase in p21 protein levels (50). DNMT1 is also known to bind to proliferating cell nuclear antigen (PCNA), an auxillary factor for DNA replication and repair. This complex may be formed as a recruitment mechanism to methylate newly replicated DNA (51). However, upregulation of p21 can cause a competitive inhibitory effect of DNMT1 to the PCNA complex (52), suggesting that p21 may play a role in the epigenetic regulation of DNA methylation (51). The current opinion is that p21 may affect the expression of hTERT, but the exact mechanism by which it acts is not conclusive. Collective data suggests, however, that the regulation of hTERT by p21 is multi-fold and indirect. An example of this interaction would be via epigenetic mechanisms such as through competition for the PCNA complex. Direct regulation of DNA methylation could affect the methylation status of the hTERT promoter and affect its activity. Other examples of hTERT regulation by p21 include G0/G1 cell cycle arrest mechanisms. Specifically, the effect of p21 down-regulation leads to the activation of

Table 1. Summary of telomere protein binding factors

Protein	Binding Properties	Function of Protein at Telomere	Telomerase Activity	References
TRF1	DNA duplex at 5'-TTAGGG) sequence repeat.	Negative feedback loop for telomere length, impede progression at telomere replication fork. Blocks telomerase from binding.	—	64
TRF2	(TTAGGG) sequence repeat; 3' telomere terminal overhang.	Impede progression at telomere replication fork. Blocks telomerase from binding. T-loop formation and stabilization.	—	69-70
Tankyrase	TRF1	ADP-ribosylation of TRF1.	+	75
POT1	TRF1; single-stranded telomere repeat specificity	Negative feedback loop for telomere length. T-loop formation and stabilization.	—	64, 67-68
TIN2	Tankyrase; TRF1; TRF2	Maintain 3' telomere capping. Prevent tankyrase modification of TRF1.	—	77-78, 64
PIP2	TIN2; POT1	Recruitment of POT1 to the TIN2-TRF1 complex.	—	78-79
PINX	hTERT	Sequesters hTERT, preventing telomerase activity.	—	78-79
hEST1A	Single-stranded DNA; hTERT	Recruits telomerase to telomere. Uncaps chromosomal ends.	+	72-73
hRap1	TRF2	Negatively regulate telomere extension.	—	71

This table summarizes the properties of key telomere-associated proteins, such as their binding targets, the downstream functions, and eventual result on telomerase activity

RB and results in the release of transcription factor E2F, which transcribes genes pertinent for S-phase (Figure 1). Evidence has shown this to be sufficient to disrupt p53-dependent repression of hTERT, suggesting that p21 plays an important role in mediating p53-dependent hTERT repression (53). Similarly, the abrogation of E2F1 activity by a dominant negative mutant or inactivation of RB using RNAi and viral oncogenes were also able to abolish p53-dependent hTERT repression (53). This illustrates the importance of cell cycle control on hTERT activity.

2.4. Alternate lengthening of telomeres

Approximately 10% of tumors maintain telomeric lengths via a telomerase-independent pathway referred to as "alternate lengthening of telomeres" (ALT) [54]. More than 80% of ALT cell lines are p53-negative. It is possible that the ALT system utilizes telomeric-DNA recombination. Moreover, p53, which normally suppresses DNA-recombination events, decreases the rate of ALT (56). Among the characteristics of ALT cells that point toward recombination-based models is the fact that these cells lack telomerase activity and yet are able to maintain their telomeres. However, it should be noted that there are occasional tumors that are able to exist devoid of both telomerase and ALT (57). Also, ALT cells possess heterogeneous telomere lengths, suggesting that the telomeres are not undergoing the same rate of loss of telomere lengths per chromosome, and that recombination could be a feasible explanation. Other indications of a recombination-based ALT system include the presence of ALT-associated promyelocytic leukemia bodies (APBs). These APBs consist of a co-localization of promyelocytic leukemia protein (PML) nuclear body, recombination proteins (e.g. RAD proteins), telomere binding proteins (TRF1 and TRF2), and telomeric DNA (58).

Studies have demonstrated that labeling a single telomere in ALT cell lines led to a spread of labeled telomeres, thus supporting the recombination theory, and suggesting that this recombination can occur between telomeres on separate chromosomes (59). In addition, telomere recombination in a mouse model has also been shown between homologous sister chromatids, and subtelomeric ALT of sister chromatid exchange (SCE) occurs at a particularly high frequency (60). Quantification of SCE rate in wild-type mice *in vitro* indicates that regular genomic SCE is superceded by telomeric SCE by 20-fold. This indicates that telomeric DNA is highly susceptible to recombination (54), and that this type of ALT is a normal occurrence in cells, which is probably due to the tandemly repeated TTAGGG sequence homology. An interesting theory that may explain the natural occurrence of ALT could be that this serves to delay clonal senescence. The transfer of a telomere of a chromosome to another telomere could help prolong the onset of senescence due to the fact that the sister chromatid of the donor chromosome still possesses a long telomere, which will enable the recipient chromosome to continue replicating. The result is that the cell is able to delay senescence (54).

Another proposed model of recombination-based ALT is the extension of telomeres via DNA polymerase activity. This type of ALT uses the telomere of a chromosome as a DNA template to extend that of a separate chromosome (56). Since there are genes that have been linked to either increased or decreased rate of ALT such as *p53* and *scid*, it is thus clear that the control of ALT is a multigenic factor (54). It is also plausible to conclude that whichever the method of ALT, cells must maintain telomeric length above a threshold rate of ALT to sustain proliferation. Therefore a combination of genetic mutations could cause ALT to occur above threshold and result in a transformed phenotype with extended telomeres.

2.5. Telomerase recruitment to telomeres – mammalian model

The telomerase recruitment mechanism is an important factor for telomerase mediated telomere extension. Telomere length maintenance is considered homeostatic due to positive and negative feedback mechanisms, as telomere binding proteins serve as positive and negative regulators in this complex network. The complete mechanism of this complex network is still unknown. Proteins involved include: TRF1 (telomere restriction fragment 1), TRF2, hEST1A (human ever shorter telomeres-1A), tankyrases (TRF1 interacting ankyrin-related ADP-ribose polymerase), TIN2 (TRF1 interacting factor 2), PINX1 (Pin2/TRF1 interacting protein), PIP2 (POT1 interacting protein-2), POT1 (protection of telomeres-1) and hRap1 (human repressor activator protein-1) (Table 1, Figure 3). Telomerase recruitment to telomeres by other proteins has been studied in depth in both prokaryotes and eukaryotes (*S. cerevisiae*, *S. pombe*, *D. melanogaster*), and in mammals including

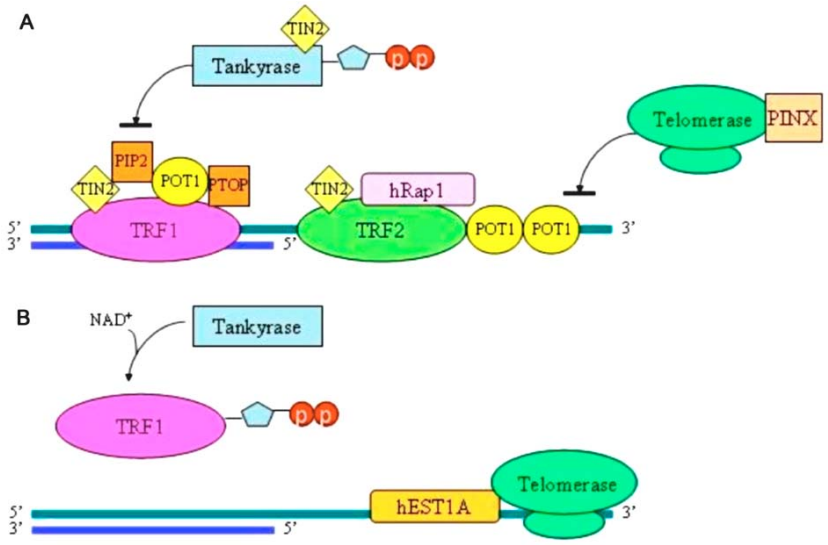

Figure 3. Depiction of telomere binding proteins and proteins affecting telomerase recruitment to telomeres. (A) Negative regulation of telomere extension via feedback loop by TRF1-POT1 complex, with T-loop stabilization by TRF2 and POT1. TRF2-hRap1 complex prevents telomerase access to the terminal 3' end. (B) Positive regulation of telomere extension via tankyrase ADP-ribosylating function to remove TRF1 from the telomeres. hEST1A recruits telomerase holoenzyme by binding to hTERT.

humans. Although there are intrinsic similarities among these models, they are essentially distinct in nature. Therefore in this section, we will only discuss that of the mammalian model.

Both TTAGGG repeat binding factors, TRF1 and TRF2 proteins, bind to duplex telomeric DNA at the TTAGGG repeat array. They are reported to be negative regulators of telomere extension. However, they neither affect the expression of telomerase, nor its enzymatic activity *in vitro* (61). Instead, the binding of TRF1 and TRF2 physically prevents telomerase from adding more telomeric repeats by stalling the addition of telomeric repeats by telomerase at telomere replication forks (64). TRF1 has been demonstrated to be involved in maintaining an equilibrium telomere length in cells, including telomerase-positive human immortal cell lines. Persistent overexpression of TRF1 in human teratoma (HT1080) cells results in a gradual loss of telomere length, while expression of a dominant negative mutant, TRF1, causes the displacement of wildtype TRF1 from telomeres, thus resulting in telomere extension by telomerase (63). It has been hypothesized that the longer the length of the telomere, the more TRF1 proteins are bound, thus serving as a counting mechanism to regulate telomere extension (reviewed in 64). Another protein, POT1, is hypothesized to work in conjunction with TRF1 in its function of telomere length counting. Although POT1 is able to attach to single-stranded telomeric DNA, abrogation of its DNA binding domain does not diminish its recruitment to telomeres (64). However, other novel proteins have been identified such as PTOP (65) and PIP2 (66) which are thought to aid in the process of POT1 recruitment to TRF1. de Lange and colleagues have also shown that TRF1 is important for the recruitment of POT1, since removal of TRF1 from telomeres reduced POT1 binding. Furthermore, abrogation of POT1 single-stranded DNA binding domain results in an enhanced elongation of telomeres by telomerase, indicating that POT1 and TRF1 both cooperate to inhibit telomere extension. This also suggests that POT1 functions downstream of TRF1 in the inhibitory response, possibly by transducing the information of telomere length (67). It is also hypothesized that its inhibitory function could simply be due to physically blocking access of telomerase by binding to the 3'-telomeric overhang (68).

The overexpression of TRF2 also increases the rate of telomere shortening. This protein serves to protect the 3' chromosomal end by remodeling telomeric DNA into a T-loop (69), thus preventing end-fusions (70). Furthermore, it has been postulated that the negative regulation of TRF2 is due in part to hRap1, which is yet another negative regulator of telomere extension. It does not bind directly to telomeric DNA, but is recruited by the negative regulator, TRF2. Mutation of the binding sites of hRap1 to TRF2 induces telomere elongation (71).

hEst1A is a single-stranded DNA binding protein, which is able to bind to the hTERT catalytic subunit to recruit the telomerase holoenzyme. This results in the lengthening of telomeres (72). Overexpression of hEst1A also causes uncapping of chromosomal ends, causing end-to-end fusions (73)

Tankyrases (tankyrase 1 and tankyrase 2) are poly (ADP-ribose) polymerases that can donate or accept ADP-ribose and modify their substrates. Tankyrases 1 and 2 possess 85% homogeneity in their amino acid sequence, and display similar enzymatic properties. They may, however, differ in the downstream effects of their poly ADP-ribosylation (74). It has been demonstrated that these tankyrases can ADP-ribosylate TRF1 to inactivate TRF1

and prevent its attachment to telomeres (75). The ADP-ribosylation activity is found to be dependent on the availability of NAD^+ *in vitro*. Lower levels of NAD^+ were associated with reduced modification of TRF1 even at a higher concentration of tankyrase enzyme. Conversely, tankyrase was able to ADP-ribosylate TRF1 in the presence of higher levels of NAD^+ (75). These data suggest that the inhibitory effect of TRF1 on telomere extension is regulated by ADP-ribosylation. The regulation of TRF1 attachment to telomeres also determines access of telomerase to telomeres for extension.

TIN2 is a protein that can bind to both TRF1 (77) and tankyrase (78), and seems to affect tankryase-TRF1 interaction. In fact, TIN2 prevents tankyrase modification of TRF1, such that TRF1 can exert its inhibitory effect on telomere extension, thus controlling the length of telomeres (64). Further, TIN2 has also been demonstrated to interact with TRF2 to aid in the maintenance of a functionally capped telomere (77).

PINX, another protein that interacts with TRF1, inhibits the elongation of telomeres by directly interacting with telomerase. It has been described as a potent telomerase inhibitor, and a putative tumor suppressor (78). However, the exact mechanism in which it interacts with TRF1 and other telomerase binding proteins is still unknown. *In vitro*, PINX specifically sequesters the hTERT catalytic subunit, inhibiting its function. Overexpression of PINX results in shorter telomeres and decreased telomerase activity. Conversely, deletion of PINX results in longer telomeres and increased telomerase activity *in vitro* (79). Furthermore, a loss of PINX in nude mice causes a higher incidence of tumorigenesis (78), consistent with its role in telomerase inhibition.

3. CELLULAR SENESCENCE AND AGING

3.1. Biological significance

Just as the goal of biomedical research is improving the human condition, the aim of studying human aging focuses on delaying the complex propensities for disease, debilitation, and ultimately death that accompany the aging process. It remains difficult to distinguish the causes from the effects of aging, but studies abound that attempt to classify and explain the molecular and phenotypic changes that constitute aging.

There are many obstacles to researchers engaged in the pursuit of understanding human aging. The duration of such studies, limited longevity of researchers, and decades necessary for the human aging process to develop nearly prohibit these studies from being done *in vivo*. The need for adequate models from which we can extrapolate knowledge of human aging persists, but the choice of such models is far from straightforward.

The short life cycles of animals such as *Drosophila melanogaster* and *Caenorhabditis elegans* or the extensive work done on unicellular *Saccharomyces cerevisiae* make them attractive systems to gerontologists, but it is questionable whether these organisms provide the best possible representations of human aging processes. For example, although many of the genes that modulate aging have been found and studied in *Drosophila* and *C. elegans,* the fact that these animals are comprised of mainly post-mitotic cells and do not get cancer raises questions about how well information garnered from them translates to human physiology. Moreover, the aging phenotypes displayed in yeast and humans are very dissimilar (80).

The study of reptiles and other long-lived animals has been previously overlooked, but now promises to yield interesting findings. 'Anti-aging' pathways apparently unique to reptiles have been identified that could explain increased longevity in these species (82). It is also important to note that some amphibians and fish exhibit "negligible senescence" (83-84) and long-lived birds have high metabolic rates and relatively slow rates of aging (85-86).

Species which are evolutionarily closer to humans arguably should provide data more representative of human aging processes than that from distant relatives. Short-lived mammals such as *Mus musculus* lend fewer clues to human aging because of findings that telomerase-negative mice are normal for multiple generations (87) and overexpression of telomerase in mice does not alter aging as it does in human systems (88). Longer lived mammals that are more closely related to humans such as primates, elephants, and whales can better explain the evolution of longevity and be used to corroborate the data gathered from research on lower life forms.

3.1.1. Senescence

The ideal model system to study human aging is, of course, the humans. The aforementioned limitations of aging studies *in vivo* necessitated the development of *in vitro* aging systems. The study of cell culture to accurately model human biology led to the first formal description of senescence (89). Senescence refers to the limited proliferative capacity of normal cells *in vitro* (reviewed in 90). It is important to make the distinction between terminology associated with senescence and, consequently, relationships to aging. The phrase 'replicative senescence' (RS) generally refers to the state of cells that have been cultured for extended periods of time *in vitro* which renders them incapable of further cell divisions. The causative event of replicative senescence in human cell lines appears to be telomere shortening (91-92). The role of telomere shortening as a putative 'molecular clock' that triggers replicative senescence after a species-dependent number of cell divisions has been extensively studied (93-94), but other data suggest that this particular type of senescence does not represent the most accurate model for human aging *in vivo*. Certain other mammals, such as mice and possibly other rodents, do not exhibit RS due to telomere shortening (96). Moreover, immortal cell lines that are telomerase-negative have been reported (97).

The more general concept of 'cellular senescence' may be more applicable with regards to modeling aging. Cellular senescence encompasses telomeric attrition and any other means by which cells are

forced into the senescent state. Numerous studies have shown that events such as oxidative stress, ionizing radiation, and expression of some signal transduction molecules and cyclin-dependent kinase inhibitors can all lead to cellular senescence (98-100). Busuttil *et al.* (101) propose that the factors leading to genomic instability can also cause senescence and that this link may be associated with human aging. Other factors that can arguably induce senescence include discrete genetic components (102) and the loss of heterochromatin. Studies such as those outlined by Ogryzko *et al*. (103) reinforce the observation that multiple counting mechanisms act to limit the proliferative potential of cells. The addition of sodium butyrate or trichostatin A reversibly inhibit histone deacetylases and activate local transcription, yet cause an irreversible cell cycle exit. The presence of these epigenetic modulators during cell division must, therefore, alter the actions of additional proliferative control mechanism such as DNA methylation. As the loss of CpG methylation is also correlated with a senescent phenotype, it follows that these actions may act in concert in a pathway independent of telomeric length.

The well-characterized senescent phenotype of human diploid fibroblasts generally includes the gradual decrease in growth rate and eventual growth arrest in the G1-S transition (104), larger cell sizes with more diverse morphotypes (105-106) senescence-associated β-galactosidase activity at pH 6 (107), increased percentage of polyploidy and mutations in mtDNA (108-109), decreased ability to express heat shock proteins (110-111), loss of serum-dependent *c-fos* inducibility (112), altered gene expression (such as increased fibronectin, osteonectin, apolipoprotein J, type II (1)-procollagen) (109, 113-115) and increased metalloproteinase activity (116).

3.1.2. Aging

In contrast to senescence, the process of aging is a well-described, strictly *in vivo* phenomenon. Aging was described by Medawar in 1952 as the collection of changes that human beings undergo that progressively renders them more likely to die (117) and encompasses a broad variety of phenotypic changes. These changes can include, but are not limited to, decline in biological functions, decreased ability to maintain homeostasis, decline in cognitive functions (118), reduced hypothalamic antioxidant defenses (119), increased obesity (especially an increase in visceral, relative to subcutaneous, fat deposition [120]), loss of collagen elasticity, accumulation of potentially oxidized biological material in plaques (121), increased insulin resistance, etc. It is often difficult to distinguish these changes from aging-related pathologies such as macular degeneration, atherosclerosis, presbyopia, diabetes, declination in immune function, renal diseases, and cancers.

Aging is known to be associated with universal molecular changes such as telomere shortening (122) and decreased DNA methylation that could potentially be used as counting mechanisms by cells. Slack *et al.* (123) found that SV40-mediated transformation of mouse Balb/c 3T3 cells is dependent on the increased DNA methyltransferase (MeTase) mRNA, protein levels, and MeTase-related global methylation induced by Large T antigen expression. In an examination of differential activity of maintenance and *de novo* DNA methyltransferases during cellular aging, Lopatina *et al.* (124) found a significant decrease in maintenance methylation corresponding to decreased Dnmt1 activity. As Dnmt1 is the major DNA methyltransferase in mammalian cells, these findings help to elucidate the mechanisms responsible for the age-related decline in cytosine methylation as cells age. The fact that manipulations of a relatively small number of genes and pathways can generate an aging phenotype suggests that the widespread effects of aging are controlled by discrete genetic components. Consequently, the genetically-explainable theories of aging center around energy metabolism and caloric restriction (125), free radicals and DNA damage (126-127), and telomerase/senescence (93-94).

3.2. Senescence as an accurate model for aging

Many lines of evidence suggest that the study of telomere shortening and cellular senescence serves as an appropriate model for human aging. First, telomere shortening has been observed *in vivo* (128-129) and the shortening has been correlated to decreased proliferative potential (130-132). Second, senescent cells and senescence-biomarkers can be found *in vivo* and the senescence is associated with pathologies (135). Third, cellular senescence has been described as a mechanism evolved to deter tumorigenesis (136) and there is considerable overlap between cell cycle checkpoint pathways and regulators of telomerase (refer to Section 2.3). Lastly, the study of epigenetic mechanisms that control senescence and the regulation of telomerase have great potential to yield clues in areas such as development, disease, and human aging. Minamino *et al.* (133) observed senescence-associated β-galactosidase staining in atherosclerotic lesions of subjects with ischemic heart disease. Moreover, this group found that inhibiting telomerase in human aortic endothelial cells (HAECs) was enough to induce senescence and that the introduction of telomerase prolonged the lifespan of these cells and inhibited the endothelial dysfunction associated with senescence. Cellular senescence was also found to be associated with the pathogenesis of benign prostatic hyperplasia. The accumulated senescent epithelial cells expressing Il-1α increased the proliferation of nearby non-senescent cells, contributing to this common pathology observed in older men (134). Shortened telomeres in certain mouse systems have even mimicked aging-associated phenomena such as graying hair, reduced stress response, and ulceration (159). Epigenetic manipulations of human cells also alter telomerase expression and shorten their lifespan (refer to Section 2.2).

4. AGING AND TELOMERASE

4.1. Stem cells – a model for aging

Stem cells are generally defined as progenitor cells that are able to differentiate into multiple cell lineages, and are well-known for their self-renewal ability. Moreover, stem cells are able to repopulate the tissue of

origin when transplanted into a damaged recipient (137). The race to study the properties of stem cells and manipulate their development for transplantation therapy has also prompted scientists to turn to stem cells as a model for aging. Since stem cells are less or undifferentiated, they are ideal to study the related genetic response to environmental cues pertinent for cellular commitment into a specific differentiated phenotype, correlating with the onset of aging.

Since *hTERT* expression can be controlled epigenetically and by cell-cycle regulators including the tumor suppressor *p53*, the oncogene c-Myc, the tumor suppressor WT1 (in kidney, gonads, and spleen), and even estrogen (138), this suggests that there is a tight regulation of *hTERT* and its expression is highly responsive to cellular environmental cues. Because of the natural phenomenon of telomeric attrition with each cell division in the absence of telomerase, it is therefore pertinent for highly replicative cells (like stem cells) to maintain the levels of telomerase for chromosomal stability, which immediately implicates *hTERT*. Changes in the lengths of telomeres, secondary to modulations in the levels of telomerase are therefore considered by some scientists as a biological clock for the aging process (139). This section discusses the expression of telomerase in embryonic, adult, and abnormal stem cells, and the use of these cells as tools for aging research.

4.1.1. hTERT and telomerase in embryonic stem cells

Embryonic stem (ES) cells are pluripotent cells that are surgically extracted from an embryo at the blastocyst stage. The cells are derived from the inner cell mass (ICM), the part of the blastocyst that will eventually give rise to all tissue lineages and even some extraembryonic tissues. In association with their ability to remain in the undifferentiated state and their highly proliferative nature (140), human ES (hES) cells express a high level of telomerase activity that is 3.8 to 5.9 times greater than that expressed by MDA, an immortal human breast carcinoma cell line (141).

Embryoid bodies (EB) are formed from ES cells by allowing the aggregation of ES cells cultured in suspension or in methyl cellulose-containing medium. EB formation is characterized by the spontaneous differentiation of the aggregated ES cells, and is a requisite for the development of ES pluripotent cell lineage potential. EB formation therefore provides a powerful tool to study early lineage formation during mammalian development. It has been determined from the expression of developmental markers that the differentiation stages in EB formation are congruent with the stages of the developing embryo such as implantation to early gastrulation, gastrulation, and early organogenesis (142). EB can be further cultured by surgically grafting them into immuno-compromised mice, resulting in the formation of EB-derived teratomas, which could become malignant (143). EB are not transformed cells, and consistent with this fact, they are found to be telomerase-negative, suggesting a reason why embryoid-derived cell cultures are capable of only about 40 population doublings *in vitro* before the onset of senescence (144) due to telomeric attrition. Recent studies have found that a global demethylation occurs as ES cells transition into EB (145), which supports the findings of Shamblott *et al*., 2001 who determined that embryoid-derived cultures were telomerase-negative due to hypomethylation of the *hTERT* promoter which causes reduced hTERT expression and telomerase activity (146). Therefore it appears likely that epigenetic processes such as DNA methylation are involved in the transition of ES cells into EB.

4.1.2. hTERT and telomerase in adult stem cells

Adult stem cells are multipotent, meaning that they are able to differentiate into a restricted number of lineages. There are, however, certain stem cells that give rise to only one type of mature cell type, for example, the corneal stem cell. Adult stem cells can be found in almost every organ in the body except possibly the heart, and are usually in a minority population of 1-2% or less of total cellularity. These adult stem cells exist for the purpose of tissue regeneration or cell renewal, especially during stress or insult (reviewed in 147). The study of adult stem cells may be important to understand how cellular and whole tissue regeneration occurs or fails to do so *in vivo*, as the human body undergoes the aging process.

Although most adult stem cells express low to moderate levels of telomerase, it is surprising that human mesenchymal stem cells (hMSCs) are found to be telomerase-negative. This indicates that they have a replication limit, which was demonstrated when hMSCs were cultured *in vitro* until they reached their lifespan replication limit of about 18.7 population doublings (148). Transducing hMSCs with *hTERT* causes the hMSCs to exhibit characteristics of transformed cells, such as loss of contact inhibition, anchorage independence, and tumor formation in mice (149).

4.1.3. hTERT and telomerase in abnormal stem cells

Germ cell tumors, such as male germ cell tumors (seminomas), consist of abnormal stem cells formed from germ cell progenitors that are able to undergo extensive differentiation to form teratomas (150). Undifferentiated embryonic carcinomas, which are also termed immature teratomas, can give rise to mature teratomas that are either benign or malignant. These abnormal embryonic stem cells, like normal embryonic stem cells, are pluripotent and differentiate into a disorganized array of every type of cell lineages, such that it is not uncommon for teratomas to come complete with hair and teeth. Normal germ cell progenitors and undifferentiated embryonic carcinomas have high levels of telomerase activity. It has been demonstrated that mature teratomas do not express a detectable level of telomerase which could explain why they have limited proliferative capacity (151) even though some mature teratomas possess long telomeres (152). Abnormal stem cells provide a means to study the differentiation state of tumor cells and its relationship to telomerase expression. Also, since there is an overlap of genetic expression between these cells and normal embryonic stem cells, it makes practical sense to study these abnormal stem cells as they are simpler to culture.

Human embryonic teratocarcinoma (HT) cells are transformed stem cells that are totipotent and express high levels of telomerase. The *hTERT* promoter is unmethylated, allowing for telomerase to be highly transcribed in these particular cells (153). When undifferentiated HT cells were treated with retinoic acid to induce differentiation, a progressive methylation of the *hTERT* promoter coupled with a down-regulation of hTERT was observed. Methylation has been shown to be more pronounced in the distal E-box than the proximal E-box of the *hTERT* promoter, suggesting a control mechanism via epigenetic modification during differentiation to prevent c-Myc/Max protein complex from binding at the promoter. Interestingly, silencing of the *hTERT* promoter after initial differentiation due to histone deacetylation was followed by methylation at later stages of differentiation, indicating that methylation change is a mechanism used for stable inactivation of *hTERT* (153).

4.2. Werner syndrome: a premature aging disease associated with telomerase

Werner syndrome (WS) is an autosomal recessive disorder in which patients show symptoms of premature aging such as graying of hair, cataracts, diabetes mellitus, osteoporosis, and atherosclerosis, and they also display a high incidence of malignant tumors, specifically sarcomas. The average lifespan of a patient with WS is 47 years of age (154-155). WS is caused by a mutation in the gene which encodes for a RecQ helicase protein, WRN (156). This helicase also displays a 3' to 5' exonuclease activity, and both functions may work in coordination to remove DNA repair and recombination intermediates. WRN helicase and exonuclease function to resolve the 3' protective intratelomeric D loop structure, releasing the T loop to allow for telomere replication (157). Telomere binding proteins, TRF1 and TRF2 bind to and protect the telomeres from nuclease digestion, thus allowing for regulation of WRN exonuclease activity. Data has shown that WRN colocalizes with ALT-associated PML bodies (APB; see section on alternate lengthening of telomeres) during S phase in ALT cells (157), suggesting that WRN is involved in a mechanism preventing homologous recombination of telomeres in normal cells.

It has been demonstrated that *WRN* null fibroblasts undergo a higher telomere attrition rate, causing premature senescence in the cells. The fact that this accelerated rate of senescence can be rescued by hTERT expression supports the theory that telomere attrition is the key problem in WS (158). Further, knocking out hTR in mice causes an acceleration of the already early onset of premature aging symptoms paralleled in WS (159). Recent evidence has shown that WS cell senescence is p53-dependent and based on telomere-induced lifespan barriers, since p53 and $p21^{WAF1}$ expression is up-regulated in WS fibroblast cells (160). In addition, it is interesting to note that the p53 / $p21^{WAF1}$ pathway also suppresses *hTERT* transcription (see section 2.3), lending to increased telomeric attrition. It could be hypothesized that the lack of telomerase activity in WS cells could promote ALT occurrence, and WRN deficiency cannot resolve homologous recombination in telomeres causing telomeric DNA strand breaks.

The loss of WRN function in WS results in genomic instability due to problems with DNA repair and replication, defects in homologous recombination, chromosomal alterations, problems with telomere maintenance, and a high rate of apoptosis (reviewed in 155).

4.3. Telomerase activity in cancer and other aging-related diseases

As a general rule, mature mammalian systems require significant capacities for tissue repair and, occasionally, regeneration to combat rates of cellular attrition. In light of the extensive tissue proliferation that occurs even under nonpathological conditions, there must be inherent tumor suppressor mechanisms that regulate the growth of potential cancer cells. Indeed, the three principle tumor suppressor pathways, namely proteins encoded by the INK4a/ARF locus, p53, and telomeric attrition all overlap and play important roles in the cellular processes of senescence, tumorigenesis, and aging. Since telomere activity has been shown to correlate with stages of tumorigenesis and metastasis (reviewed in 161), early reactivation of telomerase activity can potentially be used as a prognostic marker for early detection of certain cancers such as primary lung, breast, colorectal, bladder, renal, thyroid, and pancreatic cancers (162-167). The prevalence of telomerase reactivation seen in malignancies points to telomerase inhibition as a therapeutic strategy. Indeed, inhibiting telomerase in cancer cells by such mechanisms as introduction of dominant negative hTERT vectors, antisense *hTERT*, and immune recognition has resulted in marked decreases in cell proliferation and viability (168-170).

The regulation of telomerase by transcriptional and epigenetic mechanisms (reviewed in 171) has important implications in the onset of cancer. The integration of genetic and epigenetic information provides the necessary framework to resolve the links between telomerase, aging, and cancer. It is interesting to note that epigenetic changes are more widely associated with tumorigenesis than specific genetic mutations (172). Moreover, the global hypomethylation that occurs during aging may be the mechanism by which the epigenetic programming is disrupted and risk of cancer is increased. The late onset of common disorders that involve complex genetics can only be examined in light of epigenetic variations, considering the fact that the genome and hence genetic factors are present since birth. Bjornsson *et al.* propose that epigenetic marks add another layer of complexity that could contribute to the variability in disease phenotypes, namely age of onset, response to treatment and environmental stimuli, difference in severity, and rate of progression (173). Thus, it is imperative to study the relationship between the genetic and epigenetic modifications that control telomerase activity. Recent studies even suggest that the down-regulation of *hTERT* by transcriptional repressors precedes epigenetic modifications that stabilize and maintain the silenced state (174-175).

In addition to cancer and senescence-associated tissue malfunctions, telomere dysfunction has also been

linked to a number of aging-related pathologies. The syndrome known as dyskeratosis congenita involves skin and bone marrow failure reminiscent of accelerated aging. Mutations in the protein encoded by DKC1, dyskerin, lead to this disorder characterized by cancer predisposition and more severe manifestations in later generations, suggesting the involvement of telomere shortening. DCK1 is actually a component of the telomerase holoenzyme (reviewed in 176). A second progeroid syndrome, ataxia telangiectasia, is caused by a deficiency of the AT tumor suppressor (ATM, ataxia telangiectasia mutated kinase). ATM is known to be involved in p53 activation induced by DNA damage and the resulting premature telomere shortening produces patients prone to tumor formation, immunodeficiency, neurodegeneration and skin and hair changes (177). Similarly, Fanconi anemia is characterized by bone marrow failure and the propensity to develop acute myeloid leukemias (178). Accelerated telomere attrition is linked to the progression of this disease (179). The severity of atherosclerosis also inversely correlates with telomere length (180). The extensive telomere shortening in the vascular system driven by the need to combat cell loss could actually contribute to atherosclerotic plaque formation (181). Aberrant telomere shortening has also been linked to the development of cirrhosis in the livers of hepatitis patients and even Alzheimer's disease (182-183). Although the restoration of physiological levels of telomerase activity appears an attractive strategy for treating these disorders, care must be taken to prevent oncogenic transformation.

5. CONCLUSION

Epigenetic modulations work in sync with genetic elements to ensure proper cooperation of the tightly-linked telomerase activity and cell proliferation regulatory pathways. Aging studies have concentrated efforts on the activation of telomerase to prevent or delay aging and prolong disease-free survival. Expansion of telomerase-immortalized cells for *in vitro* tissue engineering and reimplantation is a cell-based therapy for aging related diseases that involve the loss of irreplaceable cells. It is unknown whether manipulations of telomere length by processes which activate telomerase would be useful for clinical therapy. Furthermore, the risks of side effects or secondary changes like oncogenic transformation in cells ectopically expressing telomerase must be taken under consideration and studied in appropriate model systems. With increases in knowledge of telomere biology, it might be possible to treat aging-associated ailments before we fully understand the particulars of how telomerase contributes to factors resulting in human aging. Information on the involvement of telomerase in cancer appears to be advancing at a faster rate than knowledge of the association between telomerase and aging. The field of epigenetics is poised to answer many of the long-standing questions regarding hTERT regulation, aging, and cancer. Moreover, epigenetic approaches integrated with information from genetic studies can further explain the effects of common complex diseases and variations of their aging-related phenotypes.

6. ACKNOWLEDGEMENTS

This work was supported in part by the National Science Foundation GK-12 Fellowship (UAB) to Sharla Phipps, the Ovarian SPORE program, and the UAB Postdoctoral Career Development Award to Liang Liu. S.R.L. and S.M.O.P. contributed equally to the manuscript

7. REREFENCES

1. Greider, C. W: Telomeres. *Curr Opin Cell Biol* 3, 3, 444-451 (1991)

2. Greider, C. W. and E. H. Blackburn: Telomeres, telomerase and cancer. *Sci Am* 274, 2, 92-97 (1996)

3. Greider, W., and E. H. Blackburn: Indentification of a specific telomere terminal transferase activity in Tetrahymena extracts. *Cell* 43, 405-413 (1985)

4. Greider, C. W. and E. H. Blackburn: The telomere terminal transferase of tetrahymena is a ribonucleoprotein enzyme with two kinds of primer specificity. *Cell* 51, 887-898 (1987)

5. Greider, C. W. and E. H. Blackburn: A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis. *Nature* 337, 6205, 331-337 (1989)

6. Meyerson, M., C. M. Counter, E. N. Eaton, L. W. Ellisen, P. Steiner, S. D. Caddle, L. Ziaugra, R. L. Baijersbergen, M. J. Davidoff, Q. Liu, S. Bacchetti, D. A. Haber and R. A. Weinberg: hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells during immortalization. *Cell* 90, 785-795 (1997)

7. Nakamura, T., N. G. Morin, K. B. Chapman, R. L. Wienrich, W. H. Andrews, J. Lingner, C. B. Harley and T. R. Cech: Telomerase catalytic subunit homologs from fission yeast and humans. *Science* 277, 955-959 (1997)

8. Matsutomi, K., E. Y. Yu, S. Khurts, I. Ben-Porath, J. L. Currier, G. B. Metz, M. W. Brooks, S. Kaneko, S. Murakami, J. A. DeCaprio, R. A. Weinberg, S. A. Stewart and W. C. Hahn: Telomerase maintains telomere structure in normal human cells. *Cell* 114, 2, 241-253 (2003)

9. Yi,, X. V. M. Tesmer, I. Savre-Train, J. W. Shay and W. E. Wright: Both transcriptional and posttranscriptional mechanisms regulate human telomerase template RNA levels. *Mol Cell Biol* 19, 3989-3997 (1999)

10. Li, M., Y. Wu, Y. R. Liang and X. Y. Wu: Correlation between expression of human telomerase subunits and telomerase activity in esophageal squamous cell carcinoma. *World J Gastroenterol* 9, 11, 2395-9 (2003)

11. Cong, Y-S., J. Wen and S. Bacchetti: Human telomerase and its regulation. *MicroBiol Molec Biol Rev* 66, 407-425 (1999)

12. Ducrest, A. L., H. Szutorisz, J. Lingner and M. Nabholz: Regulation of the human telomerase gene. *Oncogene* 21, 541-552 (2002)

13. Forsyth, N. R., W. E. Wright and J. W. Shay: Telomerase and differentiation in multicelluclar organisms: turn it off, turn it on, and turn it off again. *Differentiation* 69, 188-198 (2002)

14. Mason, P. J: Stem cells, telomerase and dyskeratosis congenital. *Bioessays* 25, 126-133 (2003)

15. Nakayama, J., H. Tahara, E. Tahara, M. Saito, K. Ito, H. Nakamura, T. Nakanishi, T. Ide and F. Ishikawa: Telomerase activation by hTRT in human normal fibroblasts and hepatocellular carcinomas. *Nat Genet* 18, 65 68 (1998)

16. Vaziri H. and S. Benchimol: Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. *Current Biol* 8, 279-282 (1998)

17. Wright, W. E. and J. W. Shay: Two-step mechanism controlling cellular senescence and immortalization. *Exp Geront* 27, 383-389 (1992)

18. Bodnar, A. G., M. Cuellette, M. Frolkis, S. E. Holt, C. P. Chiu, G. B. Morin, C. Harley, J. W. Shay, S. Lichtsteriner and S. E. Wright: Extension of life-span by introduction of telomerase into normal human cells. *Science* 279, 349-352 (1998)

19. Funk, W. D., C. K. Wang, D. N. Shelton, C. B. Harley, G. D. Pagon and W. K. Hoeffler: Telomerase expression restores dermal integrity to *in vitro*-aged fibroblasts in a reconstituted skin model. *Exp Cell Res* 258, 2, 270-278 (2000)

20. Bryan, T. M., A. Englezou and L. Dalla-Pozza: Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. *Nat Med* 3, 1271-1274 (1997)

21. Saldanha, S. N., L. G. Andrews and T. O. Tollefsbol: Assessment of telomere length and factors that contribute to its stability. *Eur J Biochem* 270, 389-403 (2003)

22. Shay, J. W. and A. F. Gazdar: Telomerase in the early detection of cancer. *J Clin Pathol* 50, 106-109 (1997)

23. Bestor, T. H. and V. M. Ingram: Two DNA methyltransferases from murine erythro-leukemia cells: purification, sequence specificity, and mode of interaction with DNA. *Proc Natl Acad Sci USA* 80, 18, 5559-5563 (1983)

24. Gaudet, F., W. M. Rideout III, A. Meissner, J. Dausman, H. Leonhardt and R. Jaenisch: Dnmt1 expression in pre- and postimplantation embryogenesis and the maintenance of IAP silencing. Mol Cell Biol 24, 4, 1640-1648 (2004)

25. Okano, M., D. W. Bell, D. A. Haber and E. Li: DNA methyltransferases Dnmt3a and Dnmt3b. *Mol Cell Biol* 19, 12, 8211-8218 (1999)

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

27. Kitagawa, Y., S. Kyo, M. Takakura, T. Kanaya, K. Koshida, M. Namiki and M. Inoue: Demethylating reagent 5-azacytidine inhibits telomerase activity in human prostate cancer cells through transcriptional repression of hTERT. *Clin Can Res* 6, 7, 2868-2875 (2000)

28. Verreault, A.: *De novo* nucleosome assembly: new pieces in an old puzzle. *Genes Dev* 14, 12, 1430-1438 (2000)

29. Wood, A. and A. Shilatifard: Posttranslational modifications of histones by methylation. *Adv Protein Chem* 67, 201-222 (2004)

30. Kondo, Y. and J. P. Issa: Enrichment for histone H3 lysine 9 methylation at Alu repeats in human cells. *J Biol Chem* 278, 30, 27658-27662 (2003)

31. Rice, J. C., S. D. Briggs, B. Ueberheide, C. M. Barber, J. Shabanowitz, D. F. Hunt, Y. Shinkai and C. D. Allis: Histone methyltransferases direct different degrees of methylation to define distinct chromatin domains. *Mol Cell* 12, 6, 1591-1598 (2003)

32. Garcia-Cao, M., R. O'Sullivan, A. H. Peters, T. Jenuwein and M. A. Blasco: Epigenetic regulation of telomere length in mammalian cells by the Suv39h1 and Suv39h2 histone methyltransferases. *Nat Genet* 36, 1, 94-99 (2004)

33. Liu, L., S. N. Saldanha, M. S. Pate, L. G. Andrews and T. O. Tollefsbol: Epigenetic regulation of human telomerase reverse transcriptase promoter activity during cellular differentiation. *Genes Chromosomes Cancer* 41, 1, 26-37 (2004)

34. Chuang, L. S., H. I. Ian, T. W. Koh, H. H. Ng, G. Xu and B. F. Li: Human DNA-(cytosine-5) methyltransferase-PCNA complex as a target for p21WAF1. *Science* 277, 5334, 1996-2000 (1997)

35. Fuks, F., W. A. Burgers, A. Brehm, L. Hughes-Davies and T. Kouzarides: DNA methyltransferase Dnmt1 associates with histone deacetylase activity. *Nat Genet* 24, 1, 88-91 (2000)

36. Fuks, F., W. A. Burgers, N. Godin, M. Kasai and T. Kouzarides: Dnmt3a binds deacetylases and is recruited by a sequence-specific repressor to silence transcription. *EMBO J* 20, 10, 2536-2544 (2001)

37. Holt, S. E., W. E. Wright and J. W. Shay: Regulation of telomerase activity in immortal cell lines. *Mol Cell Biol* 16,

6, 2932-2939 (1996)

38. Kondo, S., Y. Kondo, G. Li, R. H. Silverman and J. K. Cowell: Targeted therapy of human malignant glioma in a mouse model by 2-5A antisense directed against telomerase RNA. *Oncogene* 16, 3323-3330 (1998)

39. Vogelstein, B., D. Lane and A. J. Levine: The p53 tumour-suppressor gene integrates numerous signals that control life and death. As when a highly connected node in the Internet breaks down, the disruption of p53 has severe consequences. *Nature* 408, 6810, 307-310 (2000)

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

41. Koutsodontis, G., I. Tentes, P. Papakosta, A. Moustakas and D. Kardassis: Sp1 plays a critical role in the transcriptional activation of the human cyclin-dependent kinase inhibitor p21$^{WAF1/Cip1}$ gene by the p53 tumor suppressor protein. *J Biol Chem* 276, 31, 29116-29125 (2001)

42. Prowse, D. M., L. Bolgan, A. Molnár and G. P. Dotto: Involvement of the Sp3 transcription factor in induction of p21$^{Cip1/WAF1}$ in keratinocyte differentiation. *J Biol Chem* 272, 2, 1308-1304 (1997)

43. Young, J. I. and J. R. Smith: DNA Methyltransferase inhibition in normal human fibroblasts induces a p21-dependent cell cycle withdrawal. *J Biol Chem* 276, 22, 19610-19616 (2001)

44. Evans, R. J., F. S. Wyllie, D. Wynford-Thomas and C. J. Jones: A p53-dependent, telomere-independent proliferative life span barrier in human astrocytes consistent with the molecular genetics of glioma development. *Cancer Res* 63, 16, 4854-4861 (2003)

45. Dotto, G. P: More than a break to the cell cycle? *Biochim Biophys Acta* 1471, M43-M56 (2000)

46. Xiong, Y., G. J. Hannon, H. Zhang, D. Casso, R. Kobayashi and D. Beach: p21 is a universal inhibitory of cyclin kinases. *Nature* 366, 6456, 634-5 (1993)

47. Harada, K., K. Kurisu, T. Sadatmoto, H. Tahara, E. Tahara, T. Ide and E. Tahara: Growth inhibition of human glioma cells by transfection-induced p21 and its effects on telomerase activity. *J Neurooncol* 47, 1, 39-46 (2000)

48. Hendersen, Y. C., R. L. Breau, T. J. Liu and G. L. Clayman: Telomerase activity in head and neck tumors after introduction of wild-type p53, p21, p16, E2F-1 genes by means of recombinant adenovirus. *Head Neck* 22, 4, 347-354 (2000)

49. Fang, J-Y. and Y-Y. Lu: Effects of histone acetylation and DNA methylation on p21^{WAF1} regulation. *World J Gasenterol* 8, 3, 400-405 (2002)

50. Nass, S. J., A. T. Ferguson, D. El-Ashry, W. G. Nelson and N. E. Davidson: Expression of DNA methyl-transferase (DMT) and the cell cycle in human breast cancer cells. *Oncogene* 18, 7453-7461 (1999)

51. Chuang, L., I. Hang-In, T-W. Koh, H-H. Ng, G. Xu and B. Li: Human DNA-(cytosine-5) methyltransferase-PCNA complex as a target for p21^{WAF1}. *Science* 277, 1996-2000 (1997)

52. Cayrol, C., M. Knibiehler and B. Ducommun: p21 binding to PCNA causes G1 and G2 cell cycle arrest in p53-deficient cells. *Oncogene* 16, 3, 311-320 (1998)

53. Shats, I., M. Milyavsky, X. Tang, P. Stambolsky, N. Erez, R. Brosh, I. Kogan, I. Braunstein, M. Tzukerman, D. Ginsberg and V. Rotter: p53-dependent downregulation of telomerase is mediated by p21/waf1. *J Biol Chem* [Epub ahead of print] (2004)

54. Bailey, S. M., M. A. Brenneman and E. H. Goodwin: Frequent recombination in telomeric DNA may extend the proliferative life of telomerase-negative cells. *Nucleic Acids Res* 32, 12, 3743-3751 (2004)

55. Henson, J. D., A. A. Neumann, T. R. Yeager and R. R. Reddel: Alternate lengthening of telomeres in mammalian cells. *Oncogene* 21, 598-610 (2002)

56. Zaineb, R., R. Abdul, R. J. Varkonyi, M. Kulp-McEliece, C. Caslini, J. R. Testa, M. E. Murphy and D. Broccoli: p53 differentially inhibits cell growth depending on the mechanism of telomere maintenance. *Mol Cell Biol* 24, 13, 5967-5977 (2004)

57. Bryan, T. M. and R. R. Reddel: Telomere dynamics and telomerase activity in *in vitro* immortalised human cells. *Eur J Cancer* 33, 767-773.

58. Yeager, T., A. Neumann, A. Englezou, L. Huschtscha, J. Noble and R. Reddel: Telomerase-negative immortalized human cells contain a novel type of Promyelocytic leukemia (PML) body. *Cancer Res* 59, 4175-4179 (1999)

59. Durham, M. A., A. A. Neymann, C. L. Fasching and R. R. Reddel: Telomere maintenance by recombination in human cells. *Nat Genet* 26, 447-450 (2000)

60. Cornforth, M. N. and R. L Eberle: Termini of human chromosomes display elevated rates of mitotic recombination. *Mutagenesis* 16, 85-89 (2001)

61. Smogorzewska, A., B. van Steensel, A. Bianchi, S. Oelmann, M. R. Schaefer, G. Schnapp and T. de Lange: Control of human telomere length by TRF1 and TRF2. *Mol and Cell Biol* 20, 5, 1659-1668 (2000)

62. Ohki, R. and F. Ishikawa: Telomere-bound TRF1 and TRF2 stall the replication fork at telomeric repeats. *Nucleic Acids Res* 32, 5, 1627-1637 (2004)

63. van Steensel, B. and T. de Lange: Control of telomere length by the human telomeric protein TRF1. *Nature* 385, 6618, 676-677 (1997)

64. Smogorzewska, A. and T. de Lange: Regulation of telomerase by telomeric proteins. *Annu Rev Biochem* 73, 177-208 (2004)

65. Liu, D., A. Safari, M. S. O'Connor, D. W. Chan, A. Laegeler, J. Qin and Z. Songyang: PTOP interacts with POT1 and regulates its localization to telomeres. *Nat Cell Biol* 6, 7, 673-680 (2004)

66. Ye, J. Z., D. Hockemeyer, A. N. Krutchinsky, D. Loayza, S. M. Hooper, B. T. Chait and T. de Lange: POT1-interacting protein PIP1: a telomere length regulator that recruits POT1 to the TIN2/TRF1 complex. *Genes Dev* 18, 4, 1649-1654 (2004)

67. Loayza, D. and T. de Lange: POT1 as a terminal transducer of TRF1 telomere length control. *Nature* 423, 6943, 926-927 (2003)

68. Loayza, D., H. Parsons, J. Donigian, K. Hoke and T. de Lange: 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, 13, 13241-13248 (2004)

69. Griffith, J. D., L. Comeau, S. Rosenfield, R. M. Stansel, A. Bianchi, H. Moss and T. de Lange: Mammalian telomeres end in a large duplex loop. *Cell* 97, 503-514 (1999)

70. Karlseder, J., A. Smogorzewska, T. de Lange: Senescence induced by altered telomere state, not telomere loss. *Science* 295, 5564, 2446-2449 (2002)

71. Li, B. and T. de Lange: Rap1 affects the length and heterogeneity of human telomeres. *Mol Biol Cell* 14, 12, 5060-5068 (2003)

72. Snow, B. E., N. Erdmann, J. Cruickshank, H. Goldman, R. M. Gill, M. O. Robinson and L. Harrington: Functional conservation of the telomerase protein Est1p in humans. *Curr Biol* 13, 8, 698-704 (2003)

73. Reichenbach, P., M. Hoss, C. M. Azzalin, M. Nabholz, P. Bucher, J. Lingner: A human homolog of yeast Est1 associates with telomerase and uncaps chromosome ends when overexpressed. *Curr Biol* 13, 7, 568-574 (2003)

74. Kaminker, P. G., S-H. Kim, R. D. Taylor, Y. Zebarjadian, W. D. Funk, G. B. Morin, P. Yaswen and J. Campisi: TANK2, a new TRF1-associated poly(ADP-ribose) polymerase, causes rapid induction of cell death upon overexpression. *J Biol Chem* 276, 38, 35891-35899 (2001)

75. Smith, S., I. Giriat, A. Schmitt and T. de Lange: Tankyrase, a poly(ADP-ribose) polymerase at human telomeres. *Science* 282, 5393, 1484-1487 (1998)

76. Kim, S-H., P. Kaminker and J. Campisi: TIN2, a new regulator of telomere length in human cells. *Nat Genet* 23, 4, 405-412 (1999)

77. Kim, S. H., C. Beausejour, A. R. Davalos, P. Kaminker, S. J. Heo and J. Campisi: TIN2 mediates functions of TRF2 at human telomeres. *J Biol Chem* [Epub ahead of print] (2004)

78. Zhou, X. Z. and K. P. Lu: The Pin2/TRF1-interacting protein PinX1 is a potent telomerase inhibitor. *Cell* 107, 3, 347-359 (2001)

79. Lin, J. and E. H. Blackburn: Nucleolar protein PinX1p regulates telomerase by sequestering its protein catalytic subunit in an inactive complex lacking telomerase RNA. *Genes Dev* 18, 4, 387-396 (2004)

80. Gershon, H. and D. Gershon: The budding yeast, saccharomyces cerevisiae, as a model for aging research: a critical review. *Mech Aging Dev* 120, 1-3, 1-22 (2000)

81. Guerin, J. C: Emerging area of aging research: long-lived animals with "negligible senescence." *Ann NY Acad Sci* 1019, 518-520 (2004)

82. Lutz, P. L., H. M. Prentice and S. L. Milton: Is turtle longevity linked to enhanced mechanisms for surviving brain anoxia and reoxygenation? *Exp Gerontol* 38, 7, 797-800 (2003)

83. Finch, C. E: Longevity, Senescence, and the Genome. Chicago University of Chicago Press (1990)

84. Cailliet, G. M., A. H. Andrews, E. J. Burton, D. L. Watters, D. E. Kline and L. A. Ferry-Graham: Age determination and validation studies of marine fishes: do deep-dwellers live longer? *Exp Gerontol* 36, 4-6, 739-764 (2001)

85. Holmes, D. J. and S. N, Austad: Birds as animal models for the comparative biology of aging: a prospectus. *Journals of Gerontol* 50A, B59-B66 (1995)

86. Holmes, D. J., R. Fluckiger and S. N. Austad: Comparative biology of aging in birds: an update. *Exp Gerontol* 36, 4-6, 869-883 (2001)

87. Rudolph, K. L., M. Millard, M. W. Bosenberg and R. A. DePinho: Telomere dysfunction and evolution of intestinal carcinoma in mice and humans. *Nat Genet* 28, 2, 155-159 (2001)

88. Artandi, S. E., S. Alson, M. K. Tietze, N. E. Sharpless, S. Ye, R. A. Greenberg, D. H. Castrillon, J. W. Horner, S. R. Weiler, R. D. Carrasco, R. A. DePinho: Constitutive telomerase expression promotes mammary carcinomas in aging mice. *Proc Natl Acad Sci USA* 99, 12, 8191-8196 (2002)

89. Hayflick, L. and P. S. Moorhead: The serial cultivation of human diploid cell strains. *Exp Cell Res* 25, 585-621

(1961)

90. Hayflick, L: The cell biology of aging. *Clin Geriatr Med* 1, 1, 15-27 (1985)

91. Chiu, D. P. and C. B. Harley: Replicative senescence and cell immortality: the role of telomeres and telomerase. *Proc Soc Exp Biol Med* 214, 99-106 (1997)

92. J. Campisi.Cancer, aging, and cellular senescence. *In Vivo* 14, 183-188 (2000)

93. Bodnar, G., M. Ouellette, M. Frolkis, S. E. Holt, C-P. Chiu, G. B. Morin, C. B. Harley, J. W. Shay, S. Lichtsteiner and W. E. Wright: Extension of life-span by introduction of telomerase into normal human cells. *Science* 279, 349-352 (1998)

94. Harley, C. B: Telomere loss: molecular clock or genetic time bomb? *Mutat Res* 256, 271-282 (1991)

95. Blackburn, E. H: Telomere states and cell fates. *Nature* 408, 6808, 53-56 (2000)

96. Wright, W. E. and J. W. Shay: Telomere dynamics in cancer progression and prevention: fundamental differences in human and mouse telomere biology. *Nat Med* 6, 849-851 (2000)

97. Bryan, T. M. and R. R. Reddel: Telomere dynamics and telomerase activity *in vitro* immortalized human cells. *Eur J Cancer* 33, 767-773 (1997)

98. Serrano, M., A. W. Lin, M. E. Mccurrach, D. Beach and S. W. Lowe: Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4A. *Cell* 88, 593-602 (1997)

99. Robles, S. J. and G. R. Adami: Agents that cause DNA double strand breaks lead to p16(INK4A) enrichment and the premature senescence of normal fibroblasts. *Cell Cycle* 16, 1113-1123 (1998)

100. Zhu, J., D. Woods, M. McMahon and J. M. Bishop: Senescence of human fibroblasts induced by oncongenic raf. *Genes Dev* 12, 2997-3007 (1998)

101. Busuttil, R. A., M. Dolle, J. Campisi and J. Vijg: Genomic instability, aging, and cellular senescence. *Ann NY Acad. Sci* 1019, 245-255 (2004)

102. Berube, N. G., J. R. Smith and O. M. Pereira-Smith: Insights from model systems: the genetics of cellular senescence. *Am J Human Genet* 62, 1015-1019 (1998)

103. Ogryzko, V. V., T. H. Hirai, V. R. Russanova, D. A. Barbie and B. H. Howard: Human fibroblast commitment to a senescence-like state in response to histone deacetylase inhibitors is cell cycle dependent. *Mol Cell Biol* 16, 5210-5218 (1996)

104. Sherwood, S. W., D. Rush, J. L. Ellsworth and R. T. Schimke RT: Defining cellular senescence in IMR-90 cells: a flow cytometric analysis. *Proc Natl Acad Sci USA* 85, 23, 9086-9090 (1998)

105. Bayreuther, K., H. P. Rodemann, P. I. Francz PI and K. Maier: Differentiation of fibroblast stem cells. *J Cell Sci Suppl* 10, 115-130 (1988)

106. Bayreuther, K., H. P. Rodemann, R. Hommel, K. Dittmann, M. Albiez and P. I. Francz: Human skin fibroblasts *in vitro* differentiate along a terminal cell lineage. *Proc Natl Acad Sci USA* 85, 112-5116 (1988)

107. Dimri, G. P., X. Lee, G. Basile, M. Acosta, G. Scott, C. Roskelley, E. E. Medrano, M. Linskens, I. Rubelj O. Pereira-Smith, and J. Campisi: A biomarker that identifies senescent human cells in culture and in aging skin *in vivo. Proc Natl Acad Sci USA* 92, 20, 9363-9367 (1995)

108. T. Matsumura, Z. Zerrudo, L. Hayflick: Senescent human diploid cells in culture: survival, DNA synthesis and morphology. *J Gerontol* 34, 3, 328-334 (1999)

109. P. Dumont, M. Burton, Q. M. Chen, E. S. Gonos, C. Frippiat, J. B. Mazarati, F. Eliders, J. Remacle and O. Toussaint: Induction of replicative senescence biomarkers by sublethal oxidative stresses in normal human fibroblasts. *Free Radical Biol Med* 28, 3, 361-373 (2000)

110. Choi, H. S., Z. Lin, B. S. Li and A. Y. Liu: Age-dependent decrease in the heat-inducible DNA sequence-specific binding activity in human diploid fibroblasts. *J Biol Chem* 265, 29, 18005-18011 (1990)

111. Bonelli, M. A., R. R. Alfieri, P. G. Petronini, M. Bigotti, C. Campanini and A. F. Borghetti: Attenuated expression of 70-kDa heat shock protein in WI-38 human fibroblasts during aging *in vitro*. *Exp Cell Res* 252, 1, 20-32 (1999)

112. Seshadri, T. and J. Campisi: Repression of c-fos transcription and altered genetic program in senescent human fibroblasts. *Science* 247, 4939, 205-209 (1990)

113. Cristofalo, V. J., D. Volker, M. K. Francis and M. Tresini: Age-dependent modifications of gene expression in human fibroblasts. *Crit Rev Eukaryot Gene Expr* 8, 1, 43-80 (1998)

114. Kumazaki, T., R. S. Robetorye, S. C. Robetorye and J. R. Smith: Fibronectin expression increases during *in vitro* cellular senescence: correlation with increased cell area. *Exp Cell Res* 195, 1, 13-19 (1991)

115. Gonos, E. S., A. Derrentzi, M. Kreiborg, G. Agiostratidou, M. Kassem, B. F. Clark, P. S. Jat and S. I. Rattan: Cloning and identification of genes that associate with mammalian replicative senescence. *Exp Cell Res* 240, 1, 66-74 (1998)

116. Campisi, J: In: The molecular basis of cell cycle and growth control. Eds: G. S. Stein and R. Baserga, Wiley-

Liss, NY 348-373 (1999)

117. Medawar, P. B: An Unsolved Problem of Biology. London: HK Lewis (1952)

118. Kinney, B. A., C. J. Miliska, R. W. Steger and A. Bartke: Evidence that Ames dwarf mice age differently from their normal siblings in behavioral and learning memory parameters. *Horm Behav* 39, 277-284 (2001)

119. Hauck, S. J. and A. Bartke: Effects of growth hormone on hypothalamic catalase and Cu/Zn superoxide dismutase. *Free Radic Biol Med* 28, 970-978 (2001)

120. Enzi, G., M. Gasparo, R. R. Biondetti, D. Fiore, M. Semisa and F. Zurlo: Subcutaneous and visceral fat distribution according to sex, age and overweight, evaluated by computed tomography. *Am J Clin Nutr* 44, 739-746 (1986)

121. Merker, K., A. Stolzing and T. Grune: Proteolysis, caloric restriction and aging. *Mech of Ageing and Dev* 122, 595-615 (2001)

122. Harley, B. B., A. B. Futcher and C. W. Greider: Telomeres shorten during ageing of human fibroblasts. *Nature* 345, 458-460 (1990)

123. Slack, A., N. Cervoni, M. Pinard and M. Szyf: DNA methyltransferase is a downstream effector of cellular transformation triggered by simian virus, 40 large T antigen. *J Biol Chem* 274, 10105-10112 (1999)

124. Lopatina, N., J. F. Haskell, L. G. Andrews, J. C. Poole, S. Saldanha and T. Tollefsbol: Differential maintenance and *de novo* methylating activity by three DNA methyltransferases in aging and immortalized fibroblasts. *J Cell Biochem* 84, 324-334 (2002)

125. Weindruch, R. and R. L. Walford: The Retardation of Aging and Disease by Dietary Restriction. IL, Thomas (1988)

126. Beckman, K. B. and B. N. Ames: The free radical theory of aging matures. *Physiolol Rev* 78, 2, 547-581 (1998)

127. Gensler, H. L. and H. Bernstein: DNA damage as the primary cause of aging. *Q Rev Biol* 56, 3, 279-303 (1981)

128. Schulz, V. P., V. A. Zakian, C. E. Ogburn: Accelerated loss of telomeric repeats may not explain accelerated replicative decline of Werner Syndrome cells. *Human Genet* 97, 750-754 (1996)

129. Martin, J. A. and Buckwalter JA: Telomere erosion and senescence in human articular cartilage chondrocytes. *J Gerontol Biol Sci* 56A, B172-B179 (2001)

130. Yang, L., T. Suwa, W. E. Wright, J. W. Shay and P. J. Hornsby: Telomere shortening and decline in replicative potential as a function of donor age in human adenocortical cells. *Mech Ageing Dev* 92, 190-194 (2001)

131. Paradis, V., N. Youssef, D. Dargere, N. Ba, F. Bonvoust, J. Deschatrette and P. Bedossa: Replicative senescence in normal liver, chronic hepatitic C, and hepatocellular carcinomas. *Hum Pathol* 32, 3, 327-332 (2001)

132. Going, J. J., R. C. Stuart, M. Downie, A. J. Fletcher-Monaghan and W. N. Keith: 'Senescence-associated' beta-galactosidase activity in the upper gastrointestinal tract. *J Pathol* 196, 4, 394-400 (2002)

133. Minamino, T., H. Miyauchi, T. Yoshida, Y. Ishida, H. Yoshida and I. Komuro: Endothelial cell senescence in human atherosclerosis: role of telomere in endothelial dysfunction. *Circulation* 105, 13, 1541-1544 (2002)

134. Castro, P., D. Giri, D. Lamb and M. Ittmann: Cellular senescence in the pathogenesis of benign prostatic hyperplasia. *Prostate* 55, 1, 30-38 (2003)

135. Price, J. S., J. G. Waters, C. Darrah, C. Pennington, D. R. Edwards, S. T. Donell and I. M. Clark. The role of chondrocyte senescence in osteoarthritis. *Aging Cell* 1, 1, 57-65 (2002)

136. Campisi, J: Cellular senescence as a tumor-suppressor mechanism. *TRENDS in Cell Biology* 11, 11, S27-S31 (2001)

137. Verfaillie, C. M., M. F. Pera, and P. M. Lansdrop. Stem cells: hype and reality. *Hematology* 369-391 (2002)

138. Poole, J. C., L. G. Andrews and T. O. Tollefsbol: Activity, function, and gene regulation of the catalytic subunit of telomerase (hTERT). *Gene* 269, 1-2, 1-12 (2001)

139. Ahmed, A. and T. Tollefsbol: Telomeres and telomerase: basic science implications for aging. *J Am Geriatr Soc* 49, 8, 1105-1109 (2001)

140. Sperger, J. M, X. Chen, J. S. Draper, J. E. Antosiewicz, C. H. Chon, S. B. Jones, J. D. Brooks, P. W. Andrews, P. O. Brown and J. A. Thomson: Gene expression patterns in human embryonic stem cells and human pluripotent germ cell tumors. *Proc Natl Acad Sci* 100, 23, 13350-13355 (2003)

141. Thomson, J. A., J. Itskovitz-Eldor, S. S. Shapiro, M. A. Waknitz, J. J. Swiergiel, V. S. Marshall and J. M. Jones: Embryonic stem cell lines derived from human blastocysts. *Science* 282, 1145-1147 (1998)

142. Leahy, J-W Xiong, F. Kuhnert and H. Stuhlmann: Use of developmental marker genes to define temporal and spatial patterns of differentiation during embryoid body formation. *J Exp Zool* 287, 67-81 (1999)

143. Yanai, J., T. Doetchman, N. Laufer, J. Maslaton, S. Mor-Yosef, A. Safran, M. Shani and D. Sofer: Embryonic cultures but not embryos transplanted to the mouse's brain

grow rapidly without immunosuppression. *Int J Neurosci* 81, 1-2, 21-26 (1995)

144. Shamblott, M. J., J. Axelman, J. W. Littlefield, P. D. Blumenthal, G. R. H uggins, Y. Cui, L. Cheng and J. D. Gearhart: Human embryonic germ cell derivatives express a broad range of developmentally distinct markers and proliferate extensively *in vitro*. *Proc Natl Acad Sci* 98, 1, 113-118 (2001)

145. Kremenskoy, M., Y. Kremenska, J. Ohgane, N. Hattori, S. Tanaka, K. Hashizume and K. Shiota: Genome-wide analysis of DNA methylation status of CpG islands in embryoid bodies, teratomas, and fetuses. *Biochem. and Biophys. Res Comm* 311, 884-890 (2003)

146. Guilleret, I., and J. Benhattar: Demethylation of the human telomerase catalytic subunit (hTERT) gene promoter reduced hTERT expression and telomerase activity and shortened telomeres. *Exp Cell Res* 289, 2, 326-334 (2003)

147. Preston, S. L., M. R. Alison, S. J. Forbes, N. C. Direkze, R. Poulsom and N. A. Wright: The new stem cell biology: something for everyone. *Mol Pathol* 56, 86-96 (2003)

148. Zimmermann, S., M. Voss, S. Kaiser, U. Kapp, C. F. Waller and U. M. Martens: Lack of telomerase activity in human mesenchymal stem cells. *Leukemia* 17, 6, 1146-1149 (2003)

149. Keith, W. N: From stem cells to cancer: balancing immortality and neoplasia. *Oncogene* 23, 29, 5092-5094 (2004)

150. Hong, W. K., R. E. Wittes, S. T. Hajdu, E. Cvitkovic, W. F. Whitmore and R. B. Golbey: The evolution of mature teratoma from malignant testicular tumors. *Cancer* 40, 2987-2992 (1977)

151. Ahmed, T., G. J. Bosl and S. I. Hajdu: Teratoma with malignant transformation in germ cell tumors in man. *Cancer* 56, 860-863 (1985)

152. Albanell, J., G. J. Bosl, V. E. Reuter, M. Engelhardt, S. Franco, M. A. S. Moore and E. Dmitrovsky: Telomerase activity in germ cell cancers and mature teratomas. *J Nat Can Inst* 91, 15, 1321-1326 (1999)

153. Lopatina, N. G., J. C. Poole, S. N. Saldanha, N. J. Hansen, J. S. Key, M. A. Pita, L. G. Andrews and T. O. Tollefsbol: Control mechanisms in the regulation of telomerase reverse transcriptase expression in differentiating human teratocarcinoma cells. *Biochem and Biophys Res Comm* 306, 650-659 (2003)

154. Yamamoto, K., A. Imakiire, N. Miyagawa and T. Kasahara: A report of two cases of Werner's syndrome and review of the literature. *J Orthopaedic Surgery* 11, 2, 224-233 (2003)

155. Opresko, P. L., W-H. Cheng, C. von Kobbe, J. A. Harrigan and V. A. Bohr: Werner syndrome and the function of the Werner protein; what they can teach us about the molecular aging process. *Carcinogenesis* 24, 5, 791-802 (2003)

156. Yu, C.-E., J. Oshima, Y-H. Fu, E. M. Wijsman, F. Hisama, R. Alisch, S. Matthews, J. Nakura, T. Miki, S. Ouais, G. M. Martin, J. Mulligan and G. D. Schellenberg: Positional cloning of the Werner's syndrome gene. *Science* 272, 5259, 258-262 (1996)

157. Opresko, P. L., M. Otterlei, J. Graakjær, P. Bruheim, L. Dawut, S. Kølvraa, A. May, M. M. Seidman and V. A. Bohr: The Werner syndrome helicase and exonuclease cooperate to resolve telomeric D Loops in a manner regulated by TRF1 and TRF2. *Mol Cell Biol* 14, 763-774 (2004)

158. Wyllie, B. S., C. J. Jones, J. W. Skinner, M. F. Haughton, C. Wallis, D. Wynford-Thomas, R. G. A. Faragher and D. Kipling: Telomerase prevents the accelerated cell ageing of Werner syndrome fibroblasts. *Nat Genet* 24, 1, 16-17 (2000)

159. Chang, S., A. S. Multani, N. G. Cabrera, M. L. Naylor, P. Laud, D. Lombard, S. Pathak, L. Guarente and R. A. DePinho: Essential role of limiting telomeres in the pathogenesis of Werner syndrome. *Nat Genet* 36, 877-882 (2004)

160. Davis, T., S. K. Singhrao, F. S. Wyllie, M. F. Haughton, P. J. Smith, M. Wiltshire, D. Wynford-Thomas, C. J. Jones, R. G. A. Faragher and D. Kipling: Telomere-based proliferative lifespan barriers in Werner-syndrome fibroblasts involve both p53-dependent and p53-independent mechanisms. *J Cell Sci* 116, 1349-1357 (2003)

161. Ahmed, A. and T. Tollefsbol: Telomeres, telomerase, and telomerase inhibition: clinical implications for cancer. *J Am Geriatr Soc* 51, 116-122 (2003)

162. Arai, T., Y. Yasuda, T. Takaya: Application of telomerase activity for screening of primary lung cancer in broncho-alveolar lavage fluid. *Oncol Rep* 5, 405-408 (1998)

163. Bednarek, A. K., A. Sahin, A.J. Brenner: Analysis of telomerase activity levels in breast cancer: positive detections at the *in situ* breast carcinoma stage. *Clin Cancer Res* 3, 11-16 (1997)

164. Ishibashi, K., K. Hirose, H. Kato: Determining the telomerase activity of exfoliated cells in intestinal lavage solution to detect colorectal carcinomas. *Anticancer Res* 19, 2831-2836 (1999)

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

166. Aogi, K., K. Kitahara, I. Buley: Telomerase activity in lesions of the thyroid: applications to diagnosis of clinical

samples including fine-needle aspirates. *Clin Cancer Res* 4, 1965-1970 (1998)

167. Hiyama, B., T. Kodama, K. Shinbara: Telomerase activity is detected in pancreatic cancer but not in benign tumors. *Cancer Res* 57, 326-331 (1997)

168. Misawa, M., T. Tauchi, G. Sashida: Inhibition of human telomerase enhances the effect of chemotherapeutic agents in lung cancer cells. *Int J Oncol* 21, 1087-1092 (2002)

169. Wong, S. C., H. Yu, S.M. Moochhala and J.B. So: Antisense telomerase induced cell growth inhibition, cell cycle arrest and telomerase activity down-regulation in gastric and colon cancer cells. *Anticancer Res* 23, 465-469 (2003)

170. Minev, B., J. Hipp, H. Firat: Cytotoxic T cell immunity against telomerase reverse transcriptase in humans. *Proc Natl Acad Sci USA* 97, 4796-4801 (2000)

171. Liu, L., S. Lai, L.G. Andrews and T.O. Tollefsbol: Genetic and epigenetic modulation of telomerase activity in development and disease. *Gene* 340, 1-10 (2004)

172. Feinberg, A. P. and B. Tycko: The history of cancer epigenetics. *Nat Rev Cancer* 4, 143-153 (2004)

173. Bjornsson, H. T., M.D. Fallin and A.P. Feinberg: An integrated epigenetic and genetic approach to common human disease. *TRENDS in Genetics* 20, 8, 350-358 (2004)

174. Liu, L., S. N. Saldanha, M.S. Pate, L.G. Andrews and T.O. Tollefsbol: Epigenetic regulation of human telomerase reverse transcriptase promoter activity during cellular differentiation. *Genes Chromosomes and Cancer* 41, 26-37 (2004)

175. Mutskov, V. and G. Felsenfeld: Silencing of transgene transcription precedes methylation of promoter DNA and histone H3 lysine 9. *EMBO J* 23, 138-149 (2004)

176. Marrone, A. and P.J. Mason: Dyskeratosis congenita. *Cell Mol Life Sci* 60, 3, 507-517 (2003)

177. Klapper, W., R. Parwaresch and G. Krupp: Telomere biology in human aging and aging syndromes. *Mechanisms of Ageing and Development* 122, 695-712 (2001)

178. Vessey, C. J., C.J Norbury and I. D. Hichson: Genetic disorders associated with cancer predisposition and genomic instability. *Prog Nucl Acids Res Mol Biol* 63, 189-221 (2000)

179. Leteurtre, F., X. Li, P. Guardiola: Accelerated telomere shortening and telomerase activation in Fanconi's anaemia. *Br J Haematol* 105, 883-893 (1999)

180. Okuda, K: Telomere attrition of the human abdominal aorta: relationships with age and atherosclerosis. *Atherosclerosis* 152, 2, 391-398 (2000)

181. Chang, E. and C. B. Harley: Telomere length and replicative agingin in human vascular tissues. *Proc Natl Acad Sci USA* 92, 24, 11190-11194 (1995)

182. Wiemann S. U: Hepatocyte telomere shortening and senescence are general markers of human liver cirrhosis. *FASEB J* 16, 935-942 (2002)

183. Shu, H., W. Fu and M. P. Mattson: The catalytic subunit of telomerase protects neurons against amyloid -peptide-induced apoptosis. *J Neurochem* 75, 117-124 (2000)

Key Words: hTERT, Epigenetics, Alternate lengthening of telomeres, Werner syndrome, Senescence, p21, p53, Telomerase recruitment, Review

Send correspondence to: Dr Trygve O Tollefsbol, Department of Biology, 175 Campbell Hall, 1300 University Boulevard, University of Alabama at Birmingham, Birmingham, AL, 35294-1170, USA, Tel: 205-934-4573, Fax: 205-975-6097, E-mail: Trygve@uab.edu

http://www.bioscience.org/current/vol10.htm