Biotechnology and Molecular Biology Review Vol. 6(6), pp. 118-125, July 2011
Available online at http://www.academicjournals.org/BMBR
ISSN 1538-2273 ©2011 Academic Journals

Review

Telomerase: Roles in aging, cancer and hereditary disease

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Accepted 12 November, 2010

Telomerase is an enzyme that adds DNA sequence repeats ("TTAGGG" in all vertebrates) to the 3' end of DNA strands in the telomere regions, which are found at the ends of eukaryotic chromosomes. This region of repeated nucleotide repeats called telomeres contain condensed DNA material and prevents constant loss of important DNA from chromosome ends. As a result, every time the chromosome is copied only a couple of telomeres are lost, which causes no damage to the organism. Telomerase is a reverse transcriptase that carries its own RNA molecule, which is used as a template when it elongates telomeres, which are shortened after each replication cycle. The existence of a compensatory shortening of telomere (telomerase) mechanism was first predicted by Soviet biologist Alexey Olovnikov in 1973, who also suggested the telomere hypothesis of aging and the telomere's connections to cancer. Telomerase was discovered by Carol W. Greider and Elizabeth Blackburn in 1984 in the ciliate Tetrahymena. Together with Jack W. Szostak, Greider and Blackburn were awarded the 2009 Nobel Prize in Physiology or Medicine for their discovery. This review focus on role of telomerase in various diseases.

Key words: Telomere, chromatin, aging, cancer, hereditary diseases.

INTRODUCTION

Telomeres, specialized structures at eukaryotic chromosome ends, were originally defined based on the observation that naturally occurring chromosome ends behave differently from induced double stranded DNA breaks (Muller, 1938). The protective function of telomeres is due to the formation of a nucleoprotein complex, which in mammals is comprised of 6 protein subunits (TRF1, TRF2, hRAP1,POT1, TIN2 and TPP1) called shelter in (de Lange, 2005), complex with the telomeric hexa nucleotide TTAGGG repeats. The shelter in complex sequesters, the end of the DNA molecule and prevents it from activating DNA damage pathways (de Lange, 2005). This is achieved by organizing telomeres into lariat structures, called telomere loops or t-loops, formed by invasion of the single-stranded overhang into the duplex telomeric repeats, thereby sequestering the chromosome end and providing a chromosome “cap” (Griffith et al., 1999).

The integrated view of telomere structure that is currently accepted came about piecemeal, as the protein components of the shelter in complex were identified and their interactions and activities were characterized. TRF1 was the first human telomeric binding protein to be biochemically isolated, cloned and characterized (Chong et al., 1995). The related telomeric binding protein TRF2, was identified shortly thereafter by virtue of sequence homology with TRF1 (Broccoli et al., 1997; Bilaud et al., 1997). Both proteins bind to telomeric repeats as homodimers via myb type DNA binding domains located at the car boxy-terminus (Broccoli et al., 1997). TIN2 and hRAP1 were identified in yeast two-hybrid screens asTRF1 (Kim et al., 1999) and TRF2 (Li et al., 2000) interacting proteins, respectively.

Pot1 was identified and cloned based on sequence homology with the end binding proteins of the ciliates *Oxytrichia nova* and *Euplotes crassus* (Baumann and Cech, 2001). POT1 is a single-stranded DNA binding protein that specifically binds to the G-rich strand of telomeric DNA (Baumann and Cech, 2001) through its

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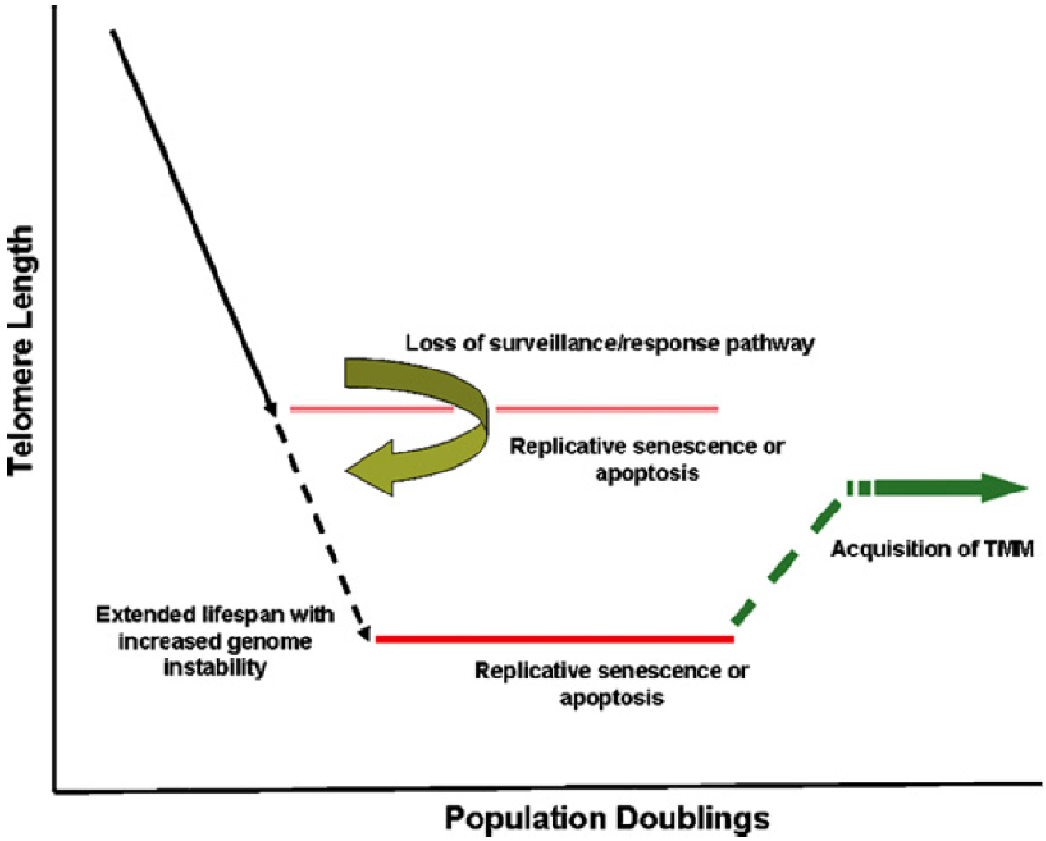

Figure 1. Telomere attrition limits the proliferative potential of cells. Loss of end- capping function leads to cell cycle arrest or apoptosis which can be bypassed by mutation of surveillance and response pathways. Continued cell division with dysfunctional telomeres lead to increased genome instability. Acquisition of a telomere maintenance mechanism stabilizes telomeric DNA arrays and supports unlimited cell division.

oligonucleotide/oligosaccharide binding motif, a motif common to telomeric end binding proteins. TPP1 was identified simultaneously by three laboratories as a TIN2 interacting protein that also interacts with TRF2 and POT1, linking the internal double-stranded telomeric binding proteins to the factor binding at the physical end of the chromosome (Houghtaling et al., 2004; Ye et al., 2004; Liu et al., 2004). Telomere dynamics are central to the processes of human aging and tumor genesis (Figure 1). Due in part to sequence loss as a result of replication, telomeres become shorter with each cell division (Harley et al., 1990). Eventually, in sufficient telomeric DNA remains to recruit enough protein to form a functional telomeric complex. The uncapped chromosome end resembles a double-stranded DNA break (d'Adda di Fagagna et al., 2003; Takai et al., 2003), a highly unstable entity that can give rise to chromosome rearrangements and which activates the cellular DNA damage response, resulting in replicative senescence or apoptosis. Human aging is a complex phenotype arising from a variety of factors. One aspect of aging is the accumulation of tissue damage, due at least in part to an inability to undergo indefinite cellular renewal.

This limited self-renewal capacity is in part a result of the finite division potential of most human cell types. The restricted proliferative capacity of human cells is recapitulated in vitro by primary fibroblasts that irreversibly exit from the cell cycle and undergo replicative senescence after a fixed number of divisions (Hayflick, 1965), driven largely by dysfunctional telomeres. Accumulating evidence supports a model linking telomere attrition with aging phenotypes *in vivo*. For example, premature aging progeroid syndromes such as Werner Syndrome and disorders that involve renewal cell depletion such as Dyskeratosis Congenita have been linked to aberrations in components required for telomere homeostasis (Crabbe et al., 2007; Wong and Collins, 2006; Savage et al., 2006; Savage et al., 2008). Proliferation-associated telomere attrition acts as a tumor suppressor mechanism by limiting the total number of divisions any given cell can undergo.

However, if a mutation in a surveillance or response pathway prevents the cell from responding appropriately to critically shortened telomeres, then continued cellular

proliferation will lead to elevated chromosome instability (Chin et al., 1999). Infact, passage through telomere crisis, a period characterized by a heightened rate of genome rearrangements arising from loss of telomere function, may be a common feature of human cancer cells (DePinho and Polyak, 2004). Ultimately, for a tumor to arise, the initiating cell(s) must circumvent the telomere length-dependent limitation on subsequent cellular divisions. In most carcinomas, this is achieved through the inappropriate activity of a specialized reverse transcriptase called telomerase (Shay and Bacchetti, 1997), which uses an RNA template to copy telomeric sequences *de novo* onto the 3′ ends of existing DNA molecules. A second pathway, called Alternative Lengthening of Telomeres (ALT), relies upon a recombination-based mechanism and is observed in certain classes of malignancies, most notably sarcomas (Costa et al., 2006; Johnson et al., 2005; Ulaner et al., 2003) and glioblastoma multiforme (Hakin-Smith et al., 2003).

In addition to its role in maintaining telomere length, telomerase may also impact on telomere stability by contributing to end-capping. The first hint that telomerase might provide a protective function came from careful observation of telomere length in cells engineered to over-express hTERT (Zhu et al., 1999). As expected, hTERT conferred unlimited replicative potential upon these cells. Surprisingly however, telomere length continued to decrease during the first-40 population doublings of these cells and stabilized at a length shorter than that observed in control cultures without telomerase. Thus, telomerase supported the presence of shorter telomeres by contributing in some way to telomere capping. In agreement, mutant alleles of telomerase that do not affect proliferation-associated sequence loss are capable of significantly extending the lifespan of human primary fibroblasts (Kim et al., 2003) suggesting the presence of attenuated hTERT, which protects shortened telomeres and delays the activation of the cellular senescence response.

Furthermore, in yeast deficient for telomerase, the telomeres are much more likely to fuse to a double strand break regardless of starting telomere length (Chan and Blackburn, 2003). Characterization of the structure of the fusion products indicated that the telomeres involved, had undergone dramatic sequence loss. Recent work suggests that similar short telomeres are present in human cancer cells and may be protected by telomerase (Xu and Blackburn, 2007). Telomerase has also been proposed to have telomere maintenance independent roles, enhancing proliferation (Sarin et al., 2005) and attenuating the DNA damage response (Masutomi et al., 2005). Early studies in the budding yeast *Saccharomyces cerevisiae* demonstrated that telomeres are organized into higher order chromatin structures that repress the expression of genes placed in proximity (Gottschling et al., 1990). Similar studies in mammalian cells initially failed to find convincing evidence of a heterochromatin-like structure at telomeres which affected gene expression (Bayne et al., 1994; Sprung et al., 1996).

However, as molecular markers diagnostic of chromatin states were identified,it became clear that mammalian telomeres also contained epigenetic marks consistent with heterochromatin. Recent studies have demonstrated that telomeric chromatin organization plays a critical role in determining telomere structure which impacts telomere length regulation (Benetti et al., 2007; Garcia-Cao et al., 2004; Gonzalo et al., 2005). These studies provide a molecular link between telomeric chromatin structure, telomere length regulation and processes implicated in human aging and tumorigenesis. Here, we discuss progress in understanding the role of telomeric chromatin in aging and human disease.

TELOMERIC CHROMATIN

Much of our understanding of the organization of telomeric chromatin and its effects on telomere metabolism are derived from studies in model organisms. In particular, studies in *S. cerevisiae* have been very informative and have provided the foundation for many of the subsequent studies in mammalian systems. Any discussion of telomeric chromatin must distinguish between the telomeric DNA sequences, e.g., those sequences that interact with specific proteins to form the chromosome end-capping structure and sub-telomeric sequences which are located internally adjacent to the telomeric repeats. Sub-telomeric regions are often repetitive, and may contain shared sequence elements among more than chromosome end and are highly variable (Riethman, 2008; Louis, 1995).

Accumulating evidence, discussed subsequently, suggest the sub-telomeric regions may regulate telomere length, structure, and affect expression of adjacent genes. A great deal has been learned about how perturbation of telomeric chromatin affects telomere structure, length regulation and function by studying mouse models and has been recently reviewed (Blasco, 2007). Alterations in the level of epigenetic modifications, such as tri-methylation of H3K9 and H4K20 via knockout of the relevant modifying enzymes led to increased telomere length without effect on telomere end-capping function (Garcia-Cao et al., 2004). The major caveat to these experiments is that the effect of reducing chromatin-modifying enzymes on chromatin structure is not limited to the telomere. Thus, while it is straightforward to document alterations in telomeric chromatin and associate this with changes in telomere length, it is much more difficult to assess the indirect contributions to the phenotype as a result of non-telomeric changes in chromatin structure throughout the genome. This is exemplified by the increased genome instability caused by deletion of DNA methyl transferases,

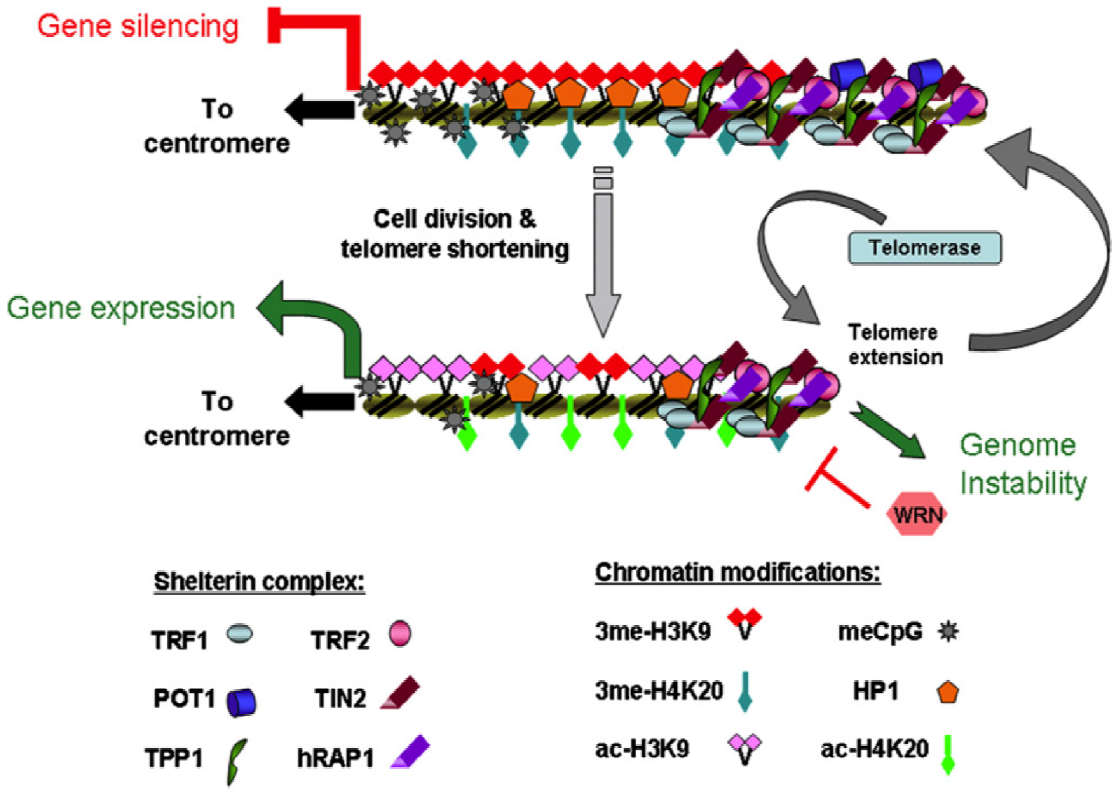

Figure 2. Telomeric DNA is packaged into nucelosomes, depicted as gold bars with black double lines.

which is present at, but not limited to the telomere (Gonzalo et al., 2006).

In an exciting new study, the first report of a histone modification that appears to directly target the telomere was identified. Mammals have seven Sir2 homologs termed SIRT1-7. Mice rendered nullizygous for the SIRT6 gene exhibit premature aging and genomic instability (Mostoslavsky et al., 2006); however, the molecular mechanism for these observed phenotypes was unknown. In this recent report, SIRT6 was found to be a histone H3K9 deacetylase that specifically targets the telomere (Michishita et al., 2008). Furthermore, SIRT6 modification of telomeric chromatin is required for stable association of the WRN helicase, which is mutated in the adult onset progeria Werner Syndrome. WRN has previously been shown to be essential for telomeric replication, preventing loss of telomeric DNA replicated by lagging strand synthesis (Crabbe et al., 2007).

Studies in the telomerase knock-out mouse demonstrated that as telomeres become shorter, the heterochromatic marks are lost and replaced by marks characteristic of open chromatin, such as increased acetylation of histone tails (Benetti et al., 2007), suggesting that a minimum telomere length is necessary to maintain the appropriate chromatin structure at chromosome ends. This change in telomeric chromatin may also alter the expression of adjacent genes. Historically viewed as gene-poor regions of the genome, recent work suggests that in fact, sub-telomeric regions may contain a higher density of genes than was previously appreciated (Linardopoulou et al., 2005; Riethman et al., 2001), raising the possibility that alterations in gene expression as a function of telomere length contribute to aging phenotypes.

ARE CHANGES IN TELOMERIC CHROMATIN STRUCTURE ASSOCIATED WITH HUMAN DISEASE?

As is apparent from the discussion stated earlier, alterations in telomeric chromatin have the potential to affect human health (Figure 2). Changes in chromatin structure at chromosome ends may affect telomere length regulation and end protection, contributing to genome instability and associated pathologies of premature aging and cancer. These perturbations may also affect expression of neighboring genes with concomitant phenotypic consequences. We discuss the evidence that these mechanisms are at work in human aging, cancer and in hereditary disease, as in Figure 2. Telomeric chromatin contains marks characteristic of heterochromatin such as HP1 (orange pentagram) 3me-H3K9 (red diamonds) and 3me-H4K20 (aqua diamonds). Sub-telomeric DNA is also methylated at CpG di nucleotides (grey suns). This repressive chromatin structure leads to silencing of adjacent genes.

Telomeres are assembled into a higher order structure via interaction with the shelter in complex, composed of TRF1 (light blue circle), TRF2 (pink circle), POT1 (dark blue barrel), TIN2 (brown triangle), TPP1 (green arc), hRAP1 (purple triangle). Upon telomere shortening, these marks are lost and replaced by marks consistent with open chromatin such as ac-H3K9 (pink diamonds) and ac-H4K20 (green diamonds). Changes in chromatin structure may lead to activation of gene expression and perturbation of telomere length regulation, either through increased access of telomerase or, in telomerase deficient cells, by inhibition of replication (e.g, increased H3K9 acetylation inhibiting WRN association with the telomere thereby hindering lagging strand replication resulting in increased genome instability).

Aging

The link between telomere length and aging is now well established. Given the observation that heterochromatic marks are lost at the short telomeres of late generation telomerase null mice (Benetti et al., 2007), one might predict a similar scenario in human cells as they approach replicative senescence, although this is yet to be tested. Changes in expression of telomere-proximal genes in relation to individual telomere length have been studied in the context of young versus senescent human fibroblasts (Ning et al., 2003). In this study, there was no significant correlation between telomere length and gene expression of the 34 telomeric genes analyzed. Indeed roughly equal numbers of genes had increased expression in young and senescent fibroblasts (9 and 7 respectively), with the majority of genes (18 of the 34 tested) exhibiting no change in expression level, regardless of final telomere length or amount of sequence lost.

However, alterations in chromatin structure at specific human telomeres as they become shorter with cellular age have not been assessed. While these experiments would be both labor intensive and quite expensive, they are theoretically feasible. For example, the ChIP-Chip approach used to demonstrate an increase in DNA damage factors at individual telomeres in senescent cells (d'Adda di Fagagna et al., 2003) might also detect differences in the level of heterochromatin marks that could then be correlated to levels of telomere-specific gene expression.

In summary, there is currently very little experimental evidence documenting alterations in telomeric chromatin in human cells with age and the biological consequences of these changes. The exception is the study discussed in detail previously, wherein SIRT6, a telomere-specific H3K9 deacetylase, is required for WRN association at telomeres. Mutation of WRN leads to premature aging, in part as a result of defective telomeric replication (Crabbe et al., 2007). Intriguingly, 22 out of 129 patients (17%) with atypical Werner's syndrome surveyed did not contain mutations in the WRN coding region or in LMNA encoding nuclear scaffold proteins lamin A (Chen et al., 2003), which is mutated in the human premature aging syndrome Hutchinson–Gilford Progeria (Eriksson et al., 2003). These data open the door to the possibility that mutations in pathways regulating the assembly and maintenance of telomeric chromatin can be linked with premature aging.

Cancer

Telomeres are tightly linked to tumor genesis. In particular, acquisition of a telomere maintenance mechanism is critical for the unlimited proliferative potential characteristic of most tumors. Studies using knockout mice imply altered telomeric chromatin might impact the efficiency of telomere maintenance (Benetti et al., 2007; Garcia-Cao et al., 2004; Gonzalo et al., 2005; Gonzalo et al., 2006). Two mechanisms for telomere maintenance have been described in human tumors: (1) telomerase activity and (2) ALT. Telomeric chromatin has been implicated in affecting both mechanisms. It has been suggested that the preferential elongation of short telomeres by telomerase may be a consequence of the more open chromatin, that is, loss of heterochromatic marks, at these telomeres (Blasco, 2007). Consistent with this is the observation that over-expression of twoHP1 isoforms, HP1 and HP1, impairs the association of hTERT with telomeres. Similarly, decreased DNA methylation of the sub telomeric region in DNA methyl transferase deficient mice is associated with characteristics of ALT, including long and hetero-geneously sized telomeres, increased telomeric recombination and the presence of ALT-associated PML bodies (APBs) (Gonzalo et al., 2006). APBs, nuclear structures in which the telomere binding proteins, such as TRF1 and TRF2, and telomeric DNA co-localize with the promyelocytic leukemia (PML) nuclear body (Yeager et al., 1999), are tightly correlated with activation of ALT (Yeager et al., 1999). These data suggest decreased methylation of sub-telomeric regions, might accompany ALT activation although this has not yet been directly tested in human tumors. Alterations in telomeric chromatin may also contribute to tumorigenesis by altering the level of cellular DNA damage.

In the study in which HP1 isoforms were over expressed in human cells, perturbed telomere structures lead to an increase in end-to-end fusions, an associated increased sensitivity to ionizing radiation and suppression of tumorigenicity in a xenograft model. In the simplest model, altering HP1 levels at telomeres inhibits hTERT access, thereby leading to telomere attrition and subsequent loss of end capping function. Again, it is important to note that while telomere phenotypes are observed, global levels of HP1 were altered in these

experiments and may be responsible for the increased radio-sensitivity and suppression of tumorigenecity. In contrast to the HP1 study, perturbation of chromatin structure in mouse cells by reduction of chromatin modifying enzymes is not always associated with changes in telomere stability (Garcia-Cao et al., 2004). This could be a result of longer telomeres characteristic of inbred strains of mice or alternatively reflect the continued association of the shelter in complex with telomeres even in the absence of heterochromatic modification of histones.

Hereditary disease

Telomere associated chromosomal rearrangements are responsible for 3 to 5% of unexplained mental retardation syndromes (Ravnan et al., 2006). The chromosomal rearrangements are likely a consequence of the increased recombination that occurs in these regions (Rudd et al., 2007). The plasticity of the subtelomeric region has been implicated in evolution, specifically environmental adaptation, with the undesired corollary being increased mutation rate within this 0.1% of the genome. The relationship between telomeric chromatin structure and telomeric rearrangements in the context of idiopathic mental retardation has yet to be investigated. However, the observation that decreased subtelomeric or H4K20 methylation is associated with increased recombination (Benetti et al., 2007; Gonzalo et al., 2006) in this region implies that the two processes may be mechanistically linked. The role of TPE in human disease has been postulated for many years.

However, to date, this lingering hypothesis has not been supported by the data. The most widely touted disease possibility driven by TPE is the autosomal dominant Facio-Scapulo-HumoralDystrophy (FSHD). FSHD is associated with a decreased number of D4Z4 units, a 3.3 kb tandemly repetitive sequence located in the subtelomeric region of chromosome 4q (Fisher and Upadhyaya, 1997). Given the association between repetitive DNA and variegated gene expression, as well as the telomeric location of the locus, it is not surprising that TPE was invoked as a potential mechanism underlying the disease (Hewitt et al., 1994) in the early days following identification of this phenomenon in yeast and long before evidence supporting TPE in mammalian cells existed. Demonstration that the 4q telomere localizes to the nuclear periphery with constitutive heterochromatin led credence to the TPE hypothesis (Winokur et al., 1994). The double homeo box 4 gene (DUX4) is located within each D4Z4 repeat unit and has been recently demonstrated to be up regulated, along with its cognate target genes, in myoblasts of patients with FSHD (Tam et al., 2004). Additionally, to date there is no evidence supporting altered chromatin structure at the repetitive locus associated with the disease. In contrast, H4 acetylation levels were found to be similar to that observed in euchromatin, rather than in heterochromatin (Dixit et al., 2007). Despite the absence of direct demonstration of TPE contributing to human pathology, the possibility remains that altered gene expression as a result of telomere proximity may be found to play a role in human disease. To date, Dyskeratosis Congenita is the disease best documented to be linked to alteration of telomere maintenance (Jiang et al., 2003). Dyskeratosis Congenita is a progressive bone marrow failure syndrome. Additional features include mucocutaneous abnormalities, chromosome instability and cancer predisposition. The autosomal dominant form of Dyskeratosis Congenita has been demonstrated to be a result of mutations in telomerase, either the RNA (Kirwan and Dokal, 2008) or TERT (Vulliamy et al., 2001) components, or in the shelterin protein TIN2 (Savage et al., 2008).

Furthermore, anticipation, where onset of the disease becomes progressively earlier and symptoms more severe with each generation, has been documented for Dyskeratosis Congenita (Armanios et al., 2005). The anticipation was correlated with a shorter telomere length in each successive generation. These data support the model wherein the clinical pathological features of Dyskeratosis Congenita arise, as a result of impaired telomere maintenance leading to chromosome instability. Less severe presentation of Dyskeratosis Congenita asaplasticanemia has also been documented (Vulliamy et al., 2004).

Again, affected individuals had mutations in the telomerase RNA gene and disease was associated with severely shortened telomeres, suggesting that pathologies arising as a result of impaired telomere maintenance may be more widespread than previously anticipated. Indeed, mutations in telomerase components have also been documented in a percentage of patients with familial idiopathic pulmonary fibrosis (Fogarty et al., 2003). The patients did not exhibit additional clinical features characteristic of Dyskeratosis Congenita, suggesting that the pathology associated with impaired telomere maintenance may be varied. The factors impacting on clinical features associated with impaired telomere maintenance have yet to be elucidated. It is likely that with increased scrutiny, additional pathologies associated with alterations in telomere structure and maintenance will be identified.

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