

Telomeres, Crisis and Cancer

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Abstract: Eukaryotic chromosomes terminate in specialized nucleic acid-protein complexes known as telomeres. Disruption of telomere structure by erosion of telomeric DNA or loss of telomere binding protein function activates a signal transduction program that closely resembles the cellular responses generated upon DNA damage. Telomere dysfunction in turn induces a permanent proliferation arrest known as senescence. Senescence is postulated to perform a tumor suppressor function by limiting cellular proliferative capacity, thus imposing a barrier to cellular immortalization. Genetic or epigenetic silencing of components of the DNA damage pathway, allows cells to proliferate beyond senescence limits. However, these cells eventually reach a stage of extreme telomere dysfunction known as crisis that is characterized by cell death and the concomitant appearance of cytogenetic abnormalities. Telomeric crisis produces significant chromosomal instability, a hallmark of human cancer, and may thus be relevant to carcinogenesis by increasing the occurrence of genetic alterations that would favor neoplastic transformation. The following review examines the relationship of telomere function during crisis in accelerating chromosomal instability and cancer.

GENETIC INSTABILITY AND CANCER

It is well established that numerous genetic and epigenetic alterations to the mammalian genome are required for neoplastic transformation to occur. This concept is underscored by the observation that the majority of inherited human cancer syndromes stem from loss of function mutations in DNA repair genes [1]. Tumor suppressor proteins have been implicated in the control of many divergent DNA repair processes, suggesting cancer cells are able to select for advantageous genetic changes to produce a fully transformed phenotype following diverse types of genetic lesions. Mutation of this class of tumor suppressor genes, known as DNA caretaker genes [2], results in chromosomal instability syndromes, manifesting in the accumulation of characteristic genetic alterations to the genome. For example, inactivating mutations in mismatch repair genes (MMR) result in the accumulation of point mutations throughout the genome and a predisposition to malignancies of the gastrointestinal epithelium. Cancers arising in individuals with MMR mutations display microsatellite repeat instability (MSI) and diploid karyotypes. Conversely, Breast Cancer 1 and 2, BRCA1 and BRCA2, mutations disrupt repair of DNA double strand breaks (DSBs) by homologous recombination [3, 4] and also affect centrosome function and chromosome segregation [5]. Individuals that inherit inactivating mutations in either BRCA1 or BRCA2 are significantly predisposed to epithelial cancers of the breast and ovary [6]. These tumors are often aneuploid and contain multiple gross chromosomal rearrangements.

Loss of genome integrity control appears to have more broad implications than simply cancer predisposition syndromes, as the majority of sporadic epithelial cancers also display chromosomal instability (CIN). This suggests that genomic instability is a selected phenotype for most cancers, sporadic and inherited cancer predisposition syndromes. Nonetheless, defects in maintaining genome stability do not manifest themselves in a uniform set of cancer phenotypes. Tissue specificity is often observed regarding the types of malignancy found in individuals that have inherited mutations in DNA repair genes. This interplay between genome stability and cancer suggests that different cell types *in vivo* are able to utilize distinct DNA repair mechanisms to control genetic stability. Alternatively, cellular responses to different genetic lesions may vary by tissue type, manifesting in tumor formation in the cell types that can best survive certain genetic aberrations.

CELLULAR RESPONSES TO TELOMERE DYSFUNCTION

The telomerase enzyme synthesizes telomere repeat sequences *de novo* and thus can prevent telomere attrition by extending the telomere prior to the completion of DNA replication by conventional DNA polymerases. However, telomerase is not active in most adult somatic cells due to repression of TERT mRNA expression [7, 8]. This lack of sufficient telomerase catalytic activity results in telomere shortening with each cell division, limiting proliferation of primary human cells to a finite number of cell divisions (50-70 divisions for human fibroblasts) as first described by Hayflick [9]. Cessation of cell proliferation at the Hayflick limit has been termed replicative senescence and correlates with telomere shortening [10] (and reviewed in this issue by Hazel *et al.*).

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Recent evidence indicates that at the senescence stage critically short telomeres lose capping function, inducing a DNA damage response (DDR) that is at least partially dependent on the activity of the PI3-kinase-like kinase (PIKK) ATM. The precise mechanisms that cause dysfunctional telomeres to trigger a DDR are unknown, however, telomere erosion that occurs during normal replicative limits induces all of the hallmarks of a DDR including phosphorylation of ATM substrates and activation of the downstream checkpoint kinases CHK1 and CHK2 [11]. Mutation of ATM also attenuates apoptotic responses to telomere dysfunction caused by dominant negative expression of telomere binding protein TRF2 [12]. Thus disruption of native telomere structure triggers an active DDR that is largely indistinguishable from the response to ionizing radiation (IR) induced DSBs [13] and (reviewed in the issue by Hazel *et al.*).

In agreement with the DNA-damage hypothesis of senescence induction, p53 was demonstrated to mediate the adverse effects of telomere dysfunction on organ homeostasis in mTERC^{-/-} mice [14]. Activation of the p53 pathway increases the expression of the CDK inhibitor p21, an important negative regulator of cell proliferation at senescence [15]. Inactivation of p53 in an mTERC^{-/-} background dramatically reduces the level of apoptosis in the testes and enables late generation mTERC^{-/-} males to be fertile.

The postnatal suppression of telomerase in humans and senescence limits to cell proliferation are believed to function as tumor suppressor mechanisms. In agreement with this hypothesis telomere shortening in mTERC^{-/-} mice limits the growth of tumors in different genetic mouse models of lymphoma, sarcoma, hepatocellular carcinoma, and intestinal carcinoma [16-19] as well in carcinoma in response to carcinogen treatment [17, 20]. Similarly, the transformation of primary mouse and human cells with short telomeres requires telomerase reactivation [16, 21]. Multiple studies demonstrate that telomerase inhibition in human cancer cell lines produces a loss of cell viability [22, 23]. Cellular responses to telomere shortening thus appear to protect an organism against carcinogenesis, although this may also have the negative consequence of limiting the regenerative capacity of highly proliferative organ systems (reviewed by Hazel *et al.* in this issue).

ROLES OF TELOMERIC CRISIS IN CHROMOSOMAL INSTABILITY AND CARCINOGENESIS

Viral oncoproteins such as the SV40 large T antigen positively influence cellular immortalization by binding to and inactivating tumor suppressor proteins p53 and pRb. Ectopic expression of such viral oncoproteins permits cells to bypass senescence induced checkpoints, allowing an

additional 20-30 population doublings until a second checkpoint known as crisis occurs [24]. As opposed to senescence, crisis is characterized by cell death rather than arrest. Crisis is also characterized by the appearance of cytogenetic abnormalities such as chromosomal end to end fusions [25]. These chromosomal aberrations are thought to result from rampant telomere dysfunction and to be the initiating stimulus for cell death during crisis [24].

The biphasic response to telomere attrition defined by the seminal work of Shay and Wright created a conceptual framework for understanding telomere dynamics during the multi-step progression model of cancer. While, inactivation of tumor suppressor proteins allows for an extended proliferative capacity, for cellular immortalization to occur, a mechanism of telomere stabilization must eventually be activated. From crisis, rare clones emerge (approximately 1 in 10⁷ cells) that maintain telomere length by reactivating telomerase. In support of this model, forced expression of exogenous hTERT in primary human cells bypassed senescence and crisis limits [26] [25]. Moreover, unambiguous demonstration that telomere maintenance is a requisite pathway in cellular transformation has been revealed in mouse cells with short telomeres [16] and in primary human diploid cells [21] (see review from Opitz in this issue). The important role of telomerase in telomere stabilization was also demonstrated in experiments showing that telomeres can be maintained at lengths shorter than crisis levels if hTERT is expressed [27]. It may be that the presence of even short telomeric sequences can provide both chromosomal protection and avoidance of DDR in the presence of telomerase.

Telomerase reactivation may not be the only mechanism for maintaining telomere length in cancer cells. Immortalization of viral oncoprotein expressing human diploid fibroblasts occurs by hTERT reactivation in 60-90% of cases or *via* an alternative pathway of telomere maintenance known as ALT (Alternative Lengthening of Telomeres) in the other 10-40% [28, and see review by Stewart in this issue]. Similarly, 85-95% of human tumors exhibit telomerase activity, while corresponding normal tissue does not, suggesting the cell culture model for cellular immortalization is a valid representation of *in vivo* tumor biology with respect to telomerase reactivation [29]. As opposed to telomere maintenance by telomerase, ALT is thought to occur by telomere recombination based mechanisms. In yeast, ALT is dependent on Rad52 mediated recombination [30] and nullizygous mutants for both telomerase and Rad52 are not viable beyond crisis.

In mammalian cells, the recombination proteins necessary for ALT are currently undefined. However, recent work suggests that TRF2 may play a role. A mutant allele of TRF2, TRF2^B induced an intra-telomeric, recombination based deletion of

telomeres [31]. These deletions produced extrachromosomal telomere circles equivalent in size to T-loops, and were elevated in ALT cell lines, suggesting an increased level of recombination at telomeres during ALT may be due to TRF2 dysfunction. They also depended on the HR protein XRCC3, a protein that has been implicated in Holliday Junction resolution. These findings have several important implications and point to the long-held observation, that at an individual telomere level, telomere attrition is not necessarily a linear process. Furthermore, senescence in telomerase negative cells may result from an inability to reset telomere length in cells that have undergone catastrophic deletion of one or more telomeres.

ALT has been reported in a small percentage of tumors [32], primarily in soft tissue sarcomas (Henson *et al.* 2002). ALT is also seen in mouse cells that lack telomerase [33, 34]. Passage of myc plus activated ras transformed mTERC^{-/-}; INK4a^{-/-} MEFs results in telomere shortening and a reduced ability to form tumors in SCID mice [16, 34]. The tumors that did arise were unable to form lung metastasis, however transfection of a genomic fragment of mTERC produced a rescue of this phenotype, allowing lung metastasis after tail vein injection. Passage of these cells in culture revealed a stabilization of telomere length by ALT mechanisms [34]. Recently, additional evidence for ALT was demonstrated *in vivo* [35]. Independent mouse models of cancer stemming from viral oncoprotein expression in keratinocytes or in pancreatic b-cells were crossed onto an mTERC^{-/-} background. Despite telomere attrition in the normal tissues that did not express viral oncoproteins, tumors arose at a similar frequency as in control mice that had wildtype telomerase (mTERC^{+/+}). Furthermore these tumors in the mTERC^{-/-} background were able to maintain telomere length, presumably by telomerase independent mechanisms. An interesting area of future study will be to determine if certain viral oncoproteins are able to increase the probability of ALT activation. This may be plausible, as the HPV viral oncoprotein E6 activates telomerase in epithelial cells by increasing hTERT expression [36], indicating that certain viral oncoproteins are capable of influencing telomere maintenance mechanisms. Together, these data indicate that ALT is an alternative mechanism *in vivo* to telomerase re-activation allowing immortal growth of tumor cells.

As mentioned previously ALT is thought to occur by homologous recombination. HR is an extremely complex process that relies on recognition of identical homologous sequences. Disruption in the fidelity of HR results in illegitimate recombination between similar (homeologous) but not identical (homologous) sequences in the genome. The mismatch repair (MMR) proteins are involved in preventing replication errors and the accumulation

of point mutations throughout the genome [37]. Disruption of MMR activity by mutation results in instability of repetitive elements or microsatellite instability (MSI). This leads to a predisposition to gastrointestinal and other malignancies. MMR genes also ensure the fidelity of HR. Cell lines lacking the full complement of MMR activity display an approximately 10 fold increase in recombination between homeologous sequences [37]. This also appears to affect telomere maintenance by ALT (see Stewart in this issue). Yeast deficient in the MMR gene MSH2 were able to proliferate for extended periods in the absence of telomerase [38]. These strains were able to maintain telomere length by recombination of repetitive subtelomeric elements. Some evidence that this is relevant to human cancer has been reported since inhibition of telomerase in MMR deficient colon cancer cells activated ALT mechanisms to maintain telomere length [39]. Examination of MMR pathway status consequently may be necessary prior to patient selection during clinical trials for telomerase inhibition in human cancers.

Though telomere length maintenance is necessary for cellular immortalization, the accrual of chromosomal abnormalities prior to telomerase reactivation may actually be a mechanism of generating genetic alterations required for tumorigenesis [40]. Telomere dysfunction has several consequences related to genome integrity control. Uncapping of telomeres results in chromosomal fusions that are frequently observed in histologic sections of tumors by the presence of anaphase bridges. This occurs because telomere-telomere fusion events set up a bridge-breakage-fusion cycle during mitosis that may result in both aneuploidy and the genesis of non-reciprocal translocations. Attachment of each centromere of a fused chromosome to the mitotic spindle, results in pulling of fused chromosomes to opposite poles of the cell during anaphase. This induces chromosome breakage and the presence of DNA DSBs that can be ligated into non-homologous sequences in the genome *via* non-homologous end joining (NHEJ) mechanisms. The resultant non-reciprocal translocations are commonly seen in epithelial malignancies. The telomere dysfunction based mechanism to generate these cytogenetic abnormalities is illustrated by the fact that they are a prominent feature of epithelial cancers obtained in tumors derived from mTERC^{-/-} mice [41, see also Hazel *et al.* in this issue].

Initial experimental evidence for telomere crisis playing a role in carcinogenesis is derived from studies in aged telomerase deficient mice [42]. Mice carrying a genetic deletion in the mTERC gene displayed accelerated aging and an increased susceptibility to lymphomas and teratocarcinoma. These mice similarly displayed hallmarks of telomere dysfunction and crisis such as chromosomal end-end fusions and anaphase

bridging chromosomes. Similarly, considerable evidence indicates that telomere dysfunction may play a role in the early stages of human malignancy [43]. Further underscoring the relevance of telomere dysfunction to human cancer is the discovery that telomerase activity is impaired in the human syndrome Dyskeratosis Congenita (DKC) (reviewed in this issue by Mason *et al.*). This rare disease, DKC, is characterized by telomere shortening, premature aging, bone marrow failure, liver cirrhosis, skin lesions, increased frequency of malignancies, and reduced survival. A mutation of hTERT was linked to the autosomal dominant form of this disease [44], providing direct evidence for an influence of telomere shortening on human aging and organ homeostasis. Moreover, the DKC phenotypes are strikingly similar to phenotypes observed in aged mTERT^{-/-} mice.

Recent evidence reveals that telomere dysfunction may be a tumor initiating mechanism in sporadic cancers as well. In situ analysis of sporadic breast cancers was performed for analysis of telomere dysfunction [45]. This study revealed telomere shortening and a sharp increase in subtelomeric chromosomal fusions during breast

carcinogenesis at the transition from ductal hyperplasia to ductal carcinoma *in situ*. Similarly, significant telomere shortening and an increase in anaphase bridges – a sign of telomere dysfunction – were observed at the adenoma-carcinoma transition in human colorectal carcinogenesis [18, 46]. In addition, liver nodules in cirrhotic liver – a precancerous disease stage – showed shortened telomeres specifically associated with hepatocellular carcinoma but not present in benign regenerative nodules [47].

These observations in mice and humans provide clear evidence that telomere dysfunction enhances tumor initiation (see also Hazel *et al.* in this issue). It will be interesting to examine the tumors that arise in Dyskeratosis Congenita to determine what mechanisms they utilize to maintain telomeres. In addition, it appears to be of interest to identify genetic lesions that cooperate with telomere shortening to induce chromosomal instability and cancer initiation. A good candidate in this regard is the tumor suppressor p53, which is a major component of the senescence pathway and the most commonly mutated tumor suppressor known in human cancers. The concomitant deletion of the

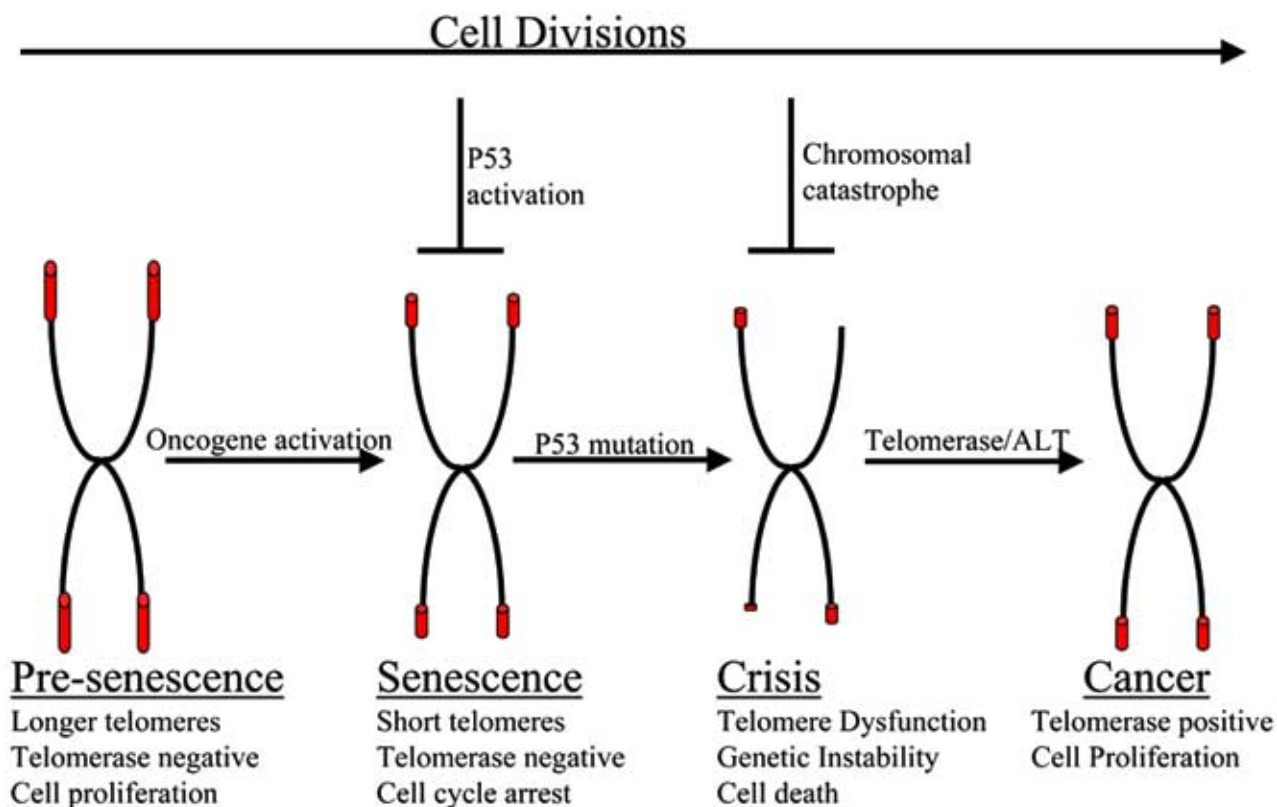


Fig. (1). Telomere length regulation and progression to cancer.

Primary human cells contain telomere lengths that are permissive to proliferation induced by oncogenic stimuli. However, upon critical telomere length shortening a cellular checkpoint known as senescence is activated in a manner dependent upon the tumor suppressor proteins p53 and pRb. Mutation of these tumor suppressor genes enables a precancerous cell to divide approximately 20-30 more times until rampant telomere dysfunction ensues at "crisis." It is thought that stochastic reactivation of telomerase or alternative mechanisms of maintaining telomeres occurs in rare clones at crisis, resulting in cellular immortalization and that this is a requisite event in nearly all cancers.

p53 tumor suppressor in mTERC^{-/-} mice greatly enhanced the cancer-prone phenotype in cell culture based oncogene transformation assays [14]. In mice carrying a heterozygous deletion of one p53 allele (p53^{+/-}) telomere shortening provoked a striking change in the tumor spectrum with a high incidence of epithelial cancers [41]. Notably, epithelial cancers are uncommon in mice with wildtype telomerase suggesting that telomere dysfunction may contribute to the emergence of epithelial cancers [48]. The tumors in mTERC^{-/-}, p53^{+/-} mice showed LOH at the remaining p53 allele [41]. It remains to be tested whether telomere shortening contributed to tumorigenesis in epithelial cell compartments by increasing the rate of LOH of p53 or whether loss of p53 and telomere shortening cooperated to foster chromosomal instability to produce additional genetic lesions necessary for cancer formation. Interestingly, the tumors of p53^{+/-}, mTERC^{-/-} double mutant mice showed high rates of chromosomal instability including non-reciprocal translocations. In addition, the combination of shortened telomeres and loss of p53 function is often observed in human cancer. Studies in conditional p53 deficient mice may further clarify the role of p53 during telomere dysfunction induced carcinogenesis.

An emerging area of future research is to ascertain whether mutation of additional DDR and senescence pathway members cooperate with telomere dysfunction during carcinogenesis. In this respect, it appears that not all components of the DDR that have been implicated in the response to telomere dysfunction behave identically *in vivo*. Lymphoma formation in ATM^{-/-}, mTERC^{-/-} double knockout mice were reduced compared to ATM^{-/-}, mTERC^{+/+} single mutant mice. One possible explanation is that ATM itself has a crucial role for telomere integrity (see above) and that severe telomere dysfunction induced by loss of ATM in combination with telomere shortening impaired tumor formation. A second although not mutually exclusive possibility is that ATM deficiency induces hypersensitivity to ionizing radiation by a failure of ATM mutant cells to repair DSBs, while p53 deficiency results in resistance to apoptosis after DSBs [49]. As telomere dysfunction is recognized in a similar fashion to DSBs, ATM deficiency may sensitize cells containing dysfunctional telomeres to p53-dependent apoptotic responses due to a persistence of uncapped chromosomal termini. In addition, it seems possible that severe telomere dysfunction induces apoptosis by p53-independent signals. It has been suggested from studies in mTERC^{-/-}, p53^{-/-} double knockout mice that p53-independent mechanisms induce defects in organ homeostasis in response to severe telomere dysfunction [14]. In line with this hypothesis severe telomere dysfunction causes cell death in the absence of functional p53 in human cells [23, 24].

In summation telomere dysfunction appears to enhance tumor initiation *via* increasing genetic

instability, however stabilization of telomeres is eventually necessary for tumor progression [50]. The potential chemotherapeutic use of telomerase inhibitors for cancer or conversely activators of telomerase to facilitate regenerative processes will ultimately depend on which of the divergent effects of telomere shortening has a dominant effect on organism survival. The answer to this question will likely be influenced by the disease state in question and the age of the individual being treated.

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ABBREVIATIONS

ALT	=	Alternative Lengthening of Telomeres
MSI	=	Microsatellite repeat instability
BRCA1	=	Breast Cancer 1 gene
BRCA2	=	Breast Cancer 2 gene
CIN	=	Chromosomal instability
DDR	=	DNA damage response
DSBs	=	DNA Double Strand Breaks
DKC	=	Dyskeratosis Congenita
IR	=	Ionizing radiation
MMR	=	Mismatch repair genes
NHEJ	=	Non-homologous end joining
PIKK	=	PI3-kinase-like kinase

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