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Dual roles of telomere dysfunction in initiation and suppression of tumorigenesis

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Abstract

Human carcinomas arise through the acquisition of genetic changes that endow precursor cancer cells with a critical threshold of cancer-relevant genetic lesions. This complex genomic alterations confer upon precursor cancer cells the ability to grow indefinitely and to metastasize to distant sites. One important mechanism underlying a cell's tumorigenic potential is the status of its telomere. Telomeres are G-rich simple repeat sequences that serve to prevent chromosomal ends from being recognized as DNA double-strand breaks (DSBs). Dysfunctional telomeres resemble DSBs, leading to the formation of dicentric chromosomes that fuel high degrees of genomic instability. In the setting of an intact p53 pathway, this instability promotes cellular senescence, a potent tumor suppressor mechanism. However, rare cells that stochastically lose p53 function emerge from this sea of genomic instability and progress towards cancer. In this review, we describe the use of mouse models to probe the impact of dysfunctional telomeres on tumor initiation and suppression.

Replicative senescence is due to telomere attrition

More than 40 years ago, Hayflick and Moorhead discovered that normal human diploid fibroblasts (HDFs) cannot grow indefinitely in culture (Hayflick and Moorhead, 1961). Rather, their proliferative capacities are intrinsically limited. After 60-80 population doublings in culture, HDFs stop dividing and adopt a phenotype characterized by large, flat cell size, a vacuolated morphology, inability to synthesize DNA, and the presence of the senescence-associated β -galactosidase (SA- β -gal) marker (Dimri et al., 1995). We now know that the endpoint of this proliferative limit, termed replicative senescence, is due largely to erosion of telomeres, protective structures that cap the end of all eukaryotic chromosomes. Telomeres are composed of TTAGGG repeats that are maintained by telomerase, a specialized ribonucleoprotein complex that includes an RNA template (*Terc*) and a reverse transcriptase catalytic subunit (*Tert*). Telomerase expression is low or absent in most human somatic tissues, while it is robust in germ and stem cell compartments

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(Counter et al., 1995; Wright et al., 1996). In the absence of telomerase, each round of DNA replication is accompanied by telomere shortening due to the failure of DNA polymerase to synthesize fully the extreme terminus of the lagging DNA strand, leading to telomere attrition with each round of replication. A total lifetime loss of ~2-4 kb of telomere length has been observed in human cells (Harley et al., 1990; Allsopp et al., 1992; Harley et al., 1994). Considering that human telomeres are approximately 8-12 kb in length, this loss is significant over the lifespan of humans. Confirmation that telomere dysfunction indeed signals the onset of replicative senescence comes from studies in which telomerase was ectopically expressed in normal human somatic cells (Bodnar et al. 1998; Vaziri and Benchimol 1998). Activation of telomerase results in telomere elongation, bypass of replicative senescence, a normal karyotype and cellular immortalization. These results clearly demonstrated that telomere length determines the proliferative lifespan of HDFs, and that upregulation of telomerase activity (hence telomere length) restores proliferative capacity.

Telomere structure and function

Telomeres consist of tandem arrays of TTAGGG repetitive sequences that terminate in a 3' single-stranded G-rich overhang. The G-rich overhang is generated by postreplicative processing of the C-rich strand, and is the substrate for telomerase-mediated telomere elongation. Analysis of telomeres from a diverse array of organisms revealed that the telomere 3' single-stranded overhang can invade the double-stranded telomeric tracts, displacing the homologous strand of the same telomere. Consequently, a telomere forms a lasso-like structure, termed the 't-loop', with a displacement (D) loop at the invasion site (de Lange, 2004). It has been proposed that the t loop plays a crucial role in sequestering the 3' end of telomeric DNA from being recognized by the DNA damage machinery as DNA double stranded breaks (DSBs) and therefore initiating inappropriate activation of DNA damage checkpoints.

Mammalian telomeres associate with the shelterin complex of six core proteins composed of telomeric-repeat binding factor 1 (TRF1), TRF2, TRF1 interacting protein 2 (TIN2), protection of telomeres 1 (POT1), the POT1 and TIN2 interacting protein TPP1 and the transcriptional repressor/activator protein RAP1 (de Lange, 2005). Proteins that directly bind the double-stranded telomeric repeats include TRF1 and TRF2. TRF1 is a negative regulator of telomere length while TRF2 plays important roles in preventing a DNA damage response (DDR) at telomeres. POT1 belongs to a family of evolutionarily conserved oligosaccharide/ oligonucleotide-binding (OB)-fold containing proteins and specifically recognizes the single-stranded G-overhang (Baumann and Cech, 2002). Shelterin components that do not bind telomeric DNA directly include TIN2, which associates with TRF1 and TRF2 and TPP1, which forms a heterodimer with POT1. RAP1 is recruited to telomeres by TRF2 and negatively regulates telomere length. Telomere end protection is compromised when components of the shelterin complex are perturbed, resulting in inappropriate chromosomal fusions through the non-homologous end joining (NHEJ) or homologous recombination (HR) pathways. For example, depletion of endogenous TRF2 levels either by overexpression of dominant negative TRF2 (TRF2-DN) or through genetic knockout approaches in mouse cells with long telomeres results in massive chromosomal fusions with telomeric sequence at the sites of fusions, suggesting that telomere structure, not length per se, is essential for its proper capping function (van Steensel et al., 1998; Celli and deLange, 2005).

Telomere dysfunction initiates a DNA damage response

Biochemical analyses revealed that senescent HDFs display molecular markers characteristic of cells bearing DNA double-strand breaks (DSBs), suggesting that critically shortened telomeres elicit a potent DDR (reviewed in Jackson, 2003; Verdun and Karlseder, 2007)). These markers of the DDR include phosphorylated γ -H2AX, 53BP1, NBS1, MDC1 and CHK2, among others (d'Adda di Fagagna et al., 2003; Taki et al., 2003). Many of these proteins localize directly to dysfunctional telomeres to form dysfunctional telomere-induced foci (TIFs), and their inactivation in senescent cells restores cell cycle progression into S-phase (d'Adda di Fagagna et al., 2003; Taki et al., 2003). These results suggest that dysfunctional telomeres are recognized by the DNA damage machinery as DSBs that impinges upon p53 and/or pRb tumor suppressor pathways, and expression of their regulators p21 and p16^{Ink4a} to initiate replicative senescence (Wright and Shay 1992; Kamijo et al., 1997; Sage et al., 2003). Dysfunctional telomeres activate upstream checkpoint PI3 kinases, such as ATM (ataxia-telangiectasia mutated) or ATR (ataxia-telangiectasia and Rad3 related) (d'Adda, et al. 2003, 2004; Celli, et al., 2005; Herbig et al., 2004; Denchi, et al. 2007; Churikov and Price, 2007; Guo et al., 2007). Once activated, these kinases phosphorylate downstream factors including CHK1 and CHK2 that in turn phosphorylates p53 (Gire et al., 2004). Phosphorylation of p53 results in the displacement of the murine double minute 2 (MDM2) protein, liberating p53 from degradation and stimulation of the expression of the cyclin-dependent kinase inhibitor p21. p21 inhibits cell cycle progression by inhibiting cyclin-dependent kinases that phosphorylates and inactivates Rb. In addition to promoting cell cycle arrest, dysfunctional telomeres can also activate p53-dependent apoptosis, since mice bearing dysfunctional telomeres show increased apoptosis in proliferative cells (Lee et al., 1999; Rudolph et al., 2000). Therefore, it is not surprising that p53 loss results in a permissive environment that favors proliferation and survival of genomically damaged cells and the eventual progression to cancer.

The role of p16^{Ink4a}-Rb pathway in mediating the telomere DDR is less clear. p16 is another CDKI that is markedly increased in senescent cells and results in pRb hypophosphorylation (Kim and Sharpless, 2006). Expression of hTERT in HDFs prevents p16^{Ink4a} induction, suggesting that hTERT prevents formation of dysfunctional telomeres that would activate p16^{Ink4a} (Bodnar et al., 1998). Support for this hypothesis comes from experiments in which treatment of HDF with TRF2-DN induces p16^{Ink4a} protein levels and entry into senescence, suggesting that in addition to p53, p16^{Ink4a} could be a second effector of the telomere DDR (Jacob and de Lange, 2004). However, compared to the p53-mediated DDR, the kinetics of p16^{Ink4a}-mediated telomere DDR is very slow. Since TIF formation occurs relatively transiently, this slow induction of a p16^{Ink4a}-damage response could potentially explain why TIFs were not observed in normal senescent HDFs with elevated p16^{Ink4a} levels (Herbig et al., 2004). Interestingly, p16 does not appear to be an important effector of the telomere DDR in mouse embryo fibroblasts (MEFs), since MEFs lacking p53 are completely refractory to the effects of TRF2-DN irrespective of p16 levels (Smogorzewska et al., 2002). However, it is not clear whether this is a property of MEFs or a general property of DDR signal transduction wiring difference between mouse and man. It would be important to generate a mouse model to examine how telomeres DDR are perturbed in the setting of p16 deficiency *in vivo*.

Telomere uncapping resulting from dysregulation of components of the shelterin complex also initiates a DDR. For example, inhibition of TRF2 with TRF2-DN elicits a DDR at telomeres and entry into a senescence phenotype indistinguishable from replicative senescence (van Steensel et al. 1998; Karlseder et al., 1999; Smogorzewska et al., 2002). Deletion of POT1 in both human and mouse cells also leads to a DDR at telomeres and rapid entry into a senescent phenotype that is abrogated when p53 is eliminated (Wu et al., 2006).

While the DDR observed in cells possessing either uncapped or shortened telomeres appear to recruit similar DDR factors to chromosomal ends, it is still an open question as to whether the two processes elicit an identical DDR. Interestingly, mammalian telomeres appear to use multiple mechanisms to evade the DDR. While elimination of TRF2 initiates primarily an ATM-dependent DDR, removal of POT1 results primarily in an ATR-mediated DDR at telomeres (Denchi and deLange, 2007; Guo et al., 2007; Churikov and Price, 2007). Since POT1 specifically bind single-stranded telomeric G-overhangs, it is likely that POT1 masks an ATR-dependent DNA damage checkpoint emanating from the single-stranded overhang.

Inactivation of p53 and pRB by antisense oligonucleotides (Hara et al. 1991) or by viral oncoproteins (Shay et al. 1991) can extend replicative potential in HDFs, driving further telomere erosion and culminating in a period of massive cell death and rampant genomic instability termed 'crisis' characterized by multiple chromosomal fusions (Counter et al. 1992). Virally transformed human cells that eliminate *p53* and/or *pRB* function can escape crisis at low frequencies, and invariably adopt telomere-maintenance programs: 80-90% of human tumors possess telomerase activity, while the remainder maintain telomeres via a recombination-mediated process termed ALT (for Alternative Lengthening of Telomeres) (Shay et al., 1993; Kim et al. 1994; Bryan et al. 1997; Shay and Bacchetti 1997). Together, these observations support the view that replicative senescence and crisis provide potent barriers to tumor development and, by extension, that telomere maintenance is an essential aspect of full malignant progression.

Telomere dysfunction results in genomic instability

Cytogenetic analysis by McClintock on breakage and fusion of maize chromosomes provided the first evidence that proper maintenance of telomeres is important for chromosomal integrity and genome stability. Dysfunctional telomeric ends are highly recombinogenic, leading to the improper chromosomal fusions, some of which may end up as dicentric chromosomes. During anaphase, dicentric chromosomes may be pulled to opposite ends of the cell, forming an anaphase bridge if the two kinetochores from the same chromatid are attached to the opposite spindle poles. Subsequent random chromosome breakage will generate broken chromosomes in the daughter cells leading to the breakage-fusion-bridge (BFB) cycle proposed by McClintock. Since the break could occur anywhere along the anaphase bridge, this type of genomic instability may lead to loss of heterozygosity (LOH) in one daughter cell while the other daughter gains extra genetic information: regional chromosome amplification occurs if information from a homologous chromosome is gained or non-reciprocal translocations (NRTs) occur if information is from a different chromosome. Therefore, the occasional formation of dicentric chromosomes fueled by telomere dysfunction may trigger an endless chain of BFB events, constantly generating novel chromosomal variants with each cell division.

The importance of telomere length in regulating genome stability has been extended across many species. Fission yeast engineered without telomerase die after extensive passage, with rare survivors emerging with recombined (circularized) chromosomes to eliminate the need for telomeres entirely (Nakamura et al., 1997) or activate the ALT pathway to regenerate functional telomeres (Lundblad and Blackburn, 1993). Elevated genomic instability characterized by the formation of NRTs via the BFB cycle was observed in telomerase-null *S. cerevisiae* possessing dysfunctional telomeres (Hackett et al., 2001). In late generation mTerc^{-/-} mice, chromosomes isolated from lymphocytes and embryonic fibroblasts exhibited p-arm-to-p-arm chromosomal fusions, a hallmark of telomere dysfunction (Blasco et al., 1997; Lee et al., 1998; Rudolph et al., 1999). Increased telomere loss leads to the formation of complex cytogenetic rearrangements, including dicentric chromosomes and the generation of NRTs (reviewed in Maser and DePinho, 2002; Hande et al., 1999; Artandi et

al., 2000; Chang et al., 2003). These results further highlight the importance of telomerase in preserving genome stability by preventing telomere dysfunction, thus inhibiting the initiation of BFB cycles and their adverse effects.

Consequences of telomerase loss in vivo: the telomerase knockout mouse

Telomeric repeats in human cells are typically between 10 and 15 kb in length. Furthermore, telomerase activity is stringently regulated in human tissues, i.e. undetectable in many somatic tissues but present in germ cells, activated leukocytes and stem cells from a variety of organs, although recent evidence challenges this view (Harley et al., 1994; Wright et al., 1996; Newbold, 1997; Weng et al., 1997; Masutomi et al., 2003). This regulation is achieved almost entirely through stringent down-regulation of hTert (Meyerson et al., 1997). In contrast, the laboratory mouse species *Mus musculus* possesses much longer telomeres, ranging in size from 40 to 80 kb, although some chromosomes harbor significantly shorter telomeres (Blasco et al., 1997; Zijlmans et al., 1997). In addition, telomerase activity and gene expression of mouse Tert (mTert) appear to be less stringently regulated in murine somatic cells (Kipling and Cooke, 1990; Prowse et al., 1993; Prowse and Greider, 1995; Greenberg et al., 1998; Martin-Rivera et al., 1998). Despite these species-specific differences, mTert expression appears to be the critical determinant of telomerase activity in mouse cells, since mTert expression correlates well with the distribution of telomerase activity in mouse tissues and cells (Greenberg et al., 1998). Although murine telomerase is regulated less stringently, murine telomeres do exhibit telomere shortening during development and somatic growth in some tissues, implying that the low levels of telomerase present may be inadequate or that other factors allowing telomere access may be limiting (Prowse and Greider, 1995; Rudolph et al., 1999).

Work from a number of laboratories has shown that the mouse is an excellent system for studying telomere biology (reviewed in Chang et al., 2001). The telomerase deficient mouse lacks the critical RNA subunit mTerc, is viable, fertile and has no significant morphological abnormalities (Blasco et al., 1997). Careful telomere length measurements revealed that, as in yeast and human cells lacking telomerase, telomeres shortened by approximately 120 bp per cell division. Given the long telomeres in mice, successive generations of telomerase deficient mice were produced to achieve sufficient telomere attrition. By the sixth generation (G6), defects were revealed in multiple tissue compartments with high proliferative histories, including hematopoietic and reproductive systems. Defects in the hematopoietic system were manifested by a marked decline in hematopoietic precursor cell numbers, reduced proliferation of T and B lymphocytes upon mitogenic stimulation and splenic atrophy with a reduction in germinal center function (Lee et al., 1998; Herrera et al., 2000). Decreased fecundity was first noted in G4 mice, culminating in sterility in late generation (G6) animals. Both male and female reproductive function were severely compromised at this point, with the males displaying marked testicular atrophy accompanied by germ cell depletion, leading to a complete absence of spermatogenesis. In the females, a decrease in the number of oocytes upon ovulation was observed (Lee et al., 1998). Importantly, reconstitution of telomerase activity in late generation mTerc^{-/-} mice increased telomere lengths preferentially at the shortest telomeres and rescued many of the pathologies associated with telomere dysfunction (Hemann et al., 2001; Samper et al., 2001). Taken together, these results strongly support the view that the long-term renewal of germ and hematopoietic cells is dependent upon telomere function, and that telomerase expression is vital in long-term cellular survival and organ homeostasis.

Telomere dysfunction promotes tumorigenesis in the absence of p53

When cellular responses to telomere dysfunction were examined in the setting of *p53* deficiency, the outcome was strikingly different. In the late generation *mTerc*^{-/-} mice, loss of *p53* rescued many of the cellular defects despite the presence of critically short telomeres, including growth arrest, testicular atrophy, intestinal apoptosis and reduction in germ cell apoptosis (Chin et al., 1999). Cell culture transformation assays showed that, in contrast to *INK4a* deficiency, *p53* null cells with critically shortened telomeres exhibited increased susceptibility to transformation by *Myc* and *Ras*. Similar findings were observed *in vivo*, where the progressive decline in telomere function correlated with increased tumor incidence and decreased survival. Therefore, in the absence of *p53*, telomere dysfunction and the resultant genome destabilizing BFB cycle promotes tumorigenesis. These results again reinforce the importance of an intact *p53* pathway in tumor prevention. In human aging populations, cancer deaths are primarily due to carcinomas of the breast, lung and colon, which arise from the epithelial compartment. Loss of *p53* function characterizes most human carcinomas, where *p53* mutations are found in approximately 50% of human breast adenocarcinomas and 40-60% of colorectal adenocarcinomas (Hollestein et al., 1991; Greenblatt et al., 1994; Veloso et al., 2000). However, carcinomas are rarely observed in mice, which normally succumb to lymphomas and sarcomas. This species-specific difference in the tumor spectrum may be due to differences in the length of telomeres and how telomerase levels are regulated, since the long telomeres in mice and somatic telomerase expression would normally prevent the generation of dysfunctional telomeres. Intriguingly, a few carcinomas were observed in late generation *mTerc*^{-/-} mice, indicating a possible role for telomere dysfunction in promoting tumorigenesis in epithelial compartments. However, the rapid death of the *mTerc*^{-/-}*p53*^{-/-} mice from lymphomas and sarcomas masked the impact of telomere dysfunction and the ensuing genetic instability in renewing epithelial compartments. To uncover a potential link between telomere dysfunction and carcinoma development, late generation *mTerc*^{-/-}*p53*^{+/-} mice with much longer tumor latency were used to further evaluate the impact of age-related telomere shortening on the tumor spectrum of mice (Artandi et al., 2000). Strikingly, carcinomas of the skin, breast and colon emerged as the largest class of clinically apparent tumors, and their cytogenetic profiles resemble those of human carcinomas. Together, these findings demonstrate that telomere dysfunction promotes the development of carcinomas in the setting of *p53* deficiency, and may play a key role in driving genomic instability observed in human carcinomas lacking *p53*.

Telomere dysfunction inhibits tumorigenesis in the setting of intact p53

The data presented above suggest that dysfunctional telomeres promote cancer only when the *p53* pathway is abrogated. To test the hypothesis that dysfunctional telomeres limit neoplastic growth in the setting of a competent *p53* pathway *in vivo*, mouse models bearing deletions of other tumor suppressors were mated with telomerase knockout mice. The *INK4a*/*ARF* locus encodes the p16^{Ink4a} and p19^{ARF} tumor suppressors and both genes share a common second exon that is deleted in the *INK4a*^{-/-} mice (Serrano et al., 1996). These mice are highly prone to develop lymphomas and sarcomas. Importantly, the *p53*-dependent DDR is intact in the *INK4a*^{-/-} mouse (Kamijo et al., 1997). Telomere dysfunction in the setting of *INK4a*^{-/-} reduced tumor incidence from 64% to 31% (Greenberg et al., 1999). In addition, treatment of early generation *mTerc*^{-/-} *INK4a*^{-/-} mice with DMBA and UVB revealed that these mice are highly cancer prone. However, similar treatment of G6 *mTerc*^{-/-} *INK4a*^{-/-} mice with short dysfunctional telomeres yielded a dramatic reduction in tumor incidence and much longer survival. Primary fibroblasts isolated from these embryos exhibited a similar resistance to transformation by *Myc* and *Ras* (Greenberg et al., 1999). This result suggests that in the setting of an intact *p53* checkpoint,

tumor progression is inhibited by dysfunctional telomeres. A similar finding was observed in a skin carcinogenesis model, in which late generation *mTerc*^{-/-} mice produced 20-fold fewer skin tumors upon chemical carcinogenesis treatment of the skin compared to wild-type controls with long telomeres (Gonzalez-Suarez et al., 2000). Up regulation of p53 was detected in late generation *mTerc*^{-/-} papillomas, most likely due to dysfunctional telomeres being sensed as double strand DNA breaks, resulting in induction of p53 levels. Taken together, these results indicate that dysfunctional telomeres inhibit tumor initiation *in vivo* in the setting of an intact p53 DNA damage checkpoint, either by activating p53-dependent apoptosis or replicative senescence.

The link between telomere shortening and tumor suppression is further highlighted in the *mTerc*^{-/-}*Apc*^{Min} mouse (Rudolph et al., 2001). *Apc*^{Min} mice develop benign intestinal microadenomas and late stage macroadenomas upon loss of the wild type *Apc* allele (Dove et al., 1998). In early generation *mTerc*^{-/-}*Apc*^{Min} mice, early stage adenomas with low anaphase-bridge index predominated, presumably due to increased genomic instability, and many of these progressed into the more aggressive macroadenomas. In contrast, only microadenomas were present in G6 *mTerc*^{-/-}*Apc*^{Min} animals. These results suggest that initiation of microadenomas occurred predominantly at early generations, and these lesions were able to progress to macroadenomas due to robust telomere reserves. In contrast, progression from micro to macroadenomas was inhibited in G6 *mTerc*^{-/-}*Apc*^{Min} mice, presumably due to the activation of p53-dependent tumor suppressive pathways. Similar results were observed in p53-competent mouse models of hepatocellular carcinoma, in which dysfunctional telomeres served to initiate tumor growth, but also limited the size of these tumors (progression) by activating apoptosis (Farazi et al., 2003).

Dysfunctional telomeres activate a p53-dependent senescence program to suppress tumorigenesis

Given that the aforementioned mouse models all possess wild-type p53 capable of inducing both apoptosis and senescence, it remained unclear how dysfunctional telomeres limit neoplastic growth *in vivo*. While apoptosis clearly has a tumor suppressive role *in vivo*, it is not clear whether p53-dependent senescence plays a role in tumor suppression. Two recent studies specifically examined the role of telomere-induced replicative senescence as a mechanism of tumor suppression *in vivo*. The first study looked at lymphoma development in the context of telomere dysfunction (Feldser and Greider, 2007). Using an established model for Burkitt's lymphoma, *Eμ*-myc transgenic mouse was crossed with *mTerc*^{-/-} mouse. As in previous cancer models, tumorigenesis was dramatically reduced in late generation *mTerc*^{-/-}, *Eμ*-myc cohort. While wild type mice and early generation *mTerc*^{-/-}, *Eμ*-myc mice developed lymphoma by 200 days time, only 25% of mice with dysfunctional telomeres succumbed to this cancer. Examination of tumors from late generation mice revealed increased end-to-end chromosomal fusion and non-reciprocal translocations, hallmarks of genomic instability due to telomere dysfunction. To examine the role of p53-dependent apoptosis in mediating tumor suppression, the anti-apoptotic gene *Bcl-2* was overexpressed in hematopoietic stem cells harvested from *mTerc*^{-/-} mice with dysfunctional telomeres. If apoptosis is the main driver of tumor suppression in the setting of telomere dysfunction, its elimination should result in rapid development of lymphoma when transplanted into lethally irradiated recipients. Reconstitution of *Bcl2* expressing stem cells derived from wild type and G1 *mTerc*^{-/-}, *Eμ*-myc bone marrows resulted in palpable tumors 6 weeks post-transplantation. However, transplantation of G5/6 *mTerc*^{-/-}, *Eμ*-myc, *Bcl-2* bone marrow failed to produce any tumors 100 days post-transplantation. Examination of lymph nodes from these mice revealed the presence of small encapsulated tumors with a 3-fold decrease in mitotic index and positive staining for senescent markers SA-β-Gal, p16 and p15. These results suggest that dysfunctional telomeres could activate a senescent

pathway in the absence of apoptosis, and this pathway was not elicited in the tumors with competent telomeres.

A second approach to examining the role of senescence in tumorigenesis used a knock-in mouse with a single amino acid mutation (R172P) within the p53 protein (Cosme-Blanco et al., 2007). Cells harboring this mutation ($p53^{P/P}$) are incapable of activating apoptosis (Liu et al., 2004), but the senescence pathway is intact as induction of telomere damage through overexpression of TRF2-DN in these cells led to dramatic reduction of cellular proliferation and the appearance of senescent cells that stain positive for SA- β -Gal. To genetically dissect the contribution of p53-dependent apoptosis vs. cellular senescence to tumor suppression in the setting of telomere dysfunction *in vivo*, we utilized an intergenerational (iG) mating scheme to generate 4 cohorts of mice: $mTerc^{+/-} p53^{P/+}$, $mTerc^{+/-} p53^{P/P}$, $iG1 mTerc^{-/-} p53^{P/+}$ and $iG1 mTerc^{-/-} p53^{P/P}$ mice. Metaphase spreads of primary bone marrow and splenocyte cultures derived from telomerase competent, $p53^{P/+}$ or $p53^{P/P}$ mice showed minimal structural chromosome abnormalities. In contrast, $iG1 mTerc^{-/-} p53^{P/+}$ and $iG1 mTerc^{-/-} p53^{P/P}$ metaphase spreads revealed a 6-8 fold increase in p-p arm fusions characteristic of chromosomes possessing dysfunctional telomeres. In addition, compared to $mTerc^{+/-} p53^{P/+}$ and $mTerc^{+/-} p53^{P/P}$ intestines, a 2-3-fold increase in the formation of anaphase bridges, a hallmark of telomere dysfunction, was detected in $iG1 mTerc^{-/-} p53^{P/+}$ and $iG1 mTerc^{-/-} p53^{P/P}$ intestines. To determine whether p53-dependent apoptosis is required to suppress spontaneous tumorigenesis, tumor development was monitored in the 4 mouse cohorts over a 28-month period. While $mTerc^{+/-} p53^{P/+}$ and $mTerc^{+/-} p53^{P/P}$ cohorts readily developed lymphomas, the presence of dysfunctional telomeres was associated with a near complete suppression of tumor formation in both $iG1 mTerc^{-/-} p53^{P/+}$ (0/23 mice with tumors) and $iG1 mTerc^{-/-} p53^{P/P}$ mice (1/9 mice with tumors), comparable to the degree of tumor suppression observed in $iG1 mTerc^{-/-} p53^{+/+}$ controls (2/11 mice with tumors). Immunohistochemical staining with antibodies against p21 and p53 revealed abundant positive cells in intestinal epithelium from $iG1 mTerc^{-/-} p53^{P/+}$ and $iG1 mTerc^{-/-} p53^{P/P}$ mice, while minimal staining was observed for $mTerc^{+/-} p53^{P/+}$ and $mTerc^{+/-} p53^{P/P}$ intestines. Robust SA- β -galactosidase staining was also detected in multiple organs from $iG1 mTerc^{-/-} p53^{P/P}$ mice. Taken together, these results suggest that p53-dependent apoptosis is dispensable in mediating telomere-dependent spontaneous tumor suppression *in vivo*. Instead, the p53-p21-dependent cellular senescence pathway is potently activated throughout diverse cellular compartments of $p53^{P/+}$ and $p53^{P/P}$ mice bearing dysfunctional telomeres, and may be responsible for the tumor suppression observed in these animals.

One interesting result from this study is the failure of the $iG1 mTerc^{-/-} p53^{P/P}$ mouse to suppress tumorigenesis in a DMBA skin carcinogenesis model (Cosme-Blanco et al., 2007). This is in sharp contrast to the strong tumor suppression observed in late generation $mTerc^{-/-}$ null mice with intact p53 function (Gonzalez-Suarez et al., 2000). One explanation is that while telomere induced cellular senescence is capable of suppressing spontaneous tumorigenesis from mesenchymal and lymphoid cancers, it is insufficient to suppress tumorigenesis in epithelial compartments such as the skin. This hypothesis is supported by the observation that restoration of endogenous p53 function in $p53^{-/-}$ lymphomas activates primarily an apoptotic response to induce tumor regression, while in sarcomas activation of a cellular senescence program is observed (Ventura et al., 2007).

From the results presented above, one would hypothesize that telomere dysfunction in the absence of p21 would abrogate the senescence response *in vivo*, resulting in accelerated tumor formation. However, this was not the case: late generation $mTerc^{-/-}$, $p21^{-/-}$ mice do not show elevated chromosomal instability nor do they succumb to increased tumorigenesis (Choudhury et al., 2007). Instead, loss of p21 extended the lifespan and rescued the defect in proliferative capacity characteristic of the telomerase null mouse, but age-dependent

increases in apoptosis in these organs were not altered. Taken together, these studies suggest that activation of either the apoptotic or cellular senescence pathway is sufficient to block tumorigenesis in most tissues. However, when one pathway is abrogated, the other can serve as a back-up. It is also possible that different pathways dominate in different tissues, as the senescence pathway was able to suppress spontaneous lymphoma formation, but was inadequate in the skin.

Conclusion

The telomerase-deficient mouse has been invaluable in providing insights into basic questions pertaining to consequences of telomere dysfunction during aging and cancer in the context of the mammalian organism. Studies in this mouse model have demonstrated that cellular responses to telomere dysfunction are fundamentally conserved in both humans and mice, and that p53 plays a key role in sensing and mediating responses to dysfunctional telomeres. Telomere dysfunction leads to genomic instability and activation of the DNA damage response checkpoints. In the setting of an intact p53 pathway, telomere dysfunction in late-generation *mTerc*^{-/-} mice activates p53, leading to tumor suppression as a result of increased cellular senescence (Cosme-Blanco et al. 2007). However, inappropriate activation of p53 also promotes premature organismal aging (Rudolph et al. 1999, Herrera et al. 2000). On the other hand, loss of p53 function cooperates with dysfunctional telomeres to promote carcinogenesis at the expense of premature aging (Chin et al. 1999). The conclusion suggested by these contrasting results argue that how telomere length is sensed by p53 in an organism may be one important factor in mediating the balance between predisposition to cellular senescence or progression to cancer.

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