Extra View

Soothing the Watchman: Telomerase Reduces the p53-dependent Cellular Stress Response

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Abbreviations: Abbreviations: EGF, epidermal growth factor; HMEC, human mammary epithelial cells

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Abstract

In addition to conferring an indefinite replicative life span, telomerase renders p16(-) human mammary epithelial cells (HMEC) resistant to growth arrest by TGFβ or by loss of EGF or insulin signaling. In contrast to earlier reports, we recently found that growth factor signaling was not directly affected by telomerase expression. Rather, short dysfunctional or near-dysfunctional telomeres in proliferating telomerase(-) HMEC sensitized the cells to p53-dependent signals for growth arrest. We showed that during serial passage and *before* any signs of replicative senescence, HMEC lacking telomerase experience enhanced p53 stability and DNA damage signaling, as determined by increased phosphorylation on p53-Ser15 and Chk2-Thr68, and formation of 53BP1/phosphorylated histone H2AX foci at chromosome ends. This heightened activity of the p53 pathway enhanced the efficiency with which cells arrested growth in response to TGFβ or to EGF or insulin withdrawal, and was abolished by ectopic expression of hTERT, the catalytic subunit of telomerase. Telomerase elongated short telomeres, thereby reducing the basal level of activated p53 and raising cellular tolerance for other p53-dependent signals, including those emanating from non-genotoxic sources. These findings explain a number of observed effects of telomerase expression on cell growth and survival without postulating additional functions for telomerase.

Telomeres, telomerase and cellular lifespan

Telomeres are nucleoprotein structures that protect chromosomal ends from being recognized and processed as double strand DNA breaks[1, 2]. Due in part to the "end-replication problem," most human somatic cells lose telomeric DNA by 70 to 200 base pairs after each round of replication[3]. When the telomeric DNA becomes critically short, telomere function is compromised, and the chromosome ends are recognized as damaged DNA[4]. Depending on the cell type and status of p53 and the DNA damage checkpoints, the result is either an irreversible arrest of cell proliferation (replicative senescence), cell death, or genomic instability[5]. Expression of telomerase, the cellular reverse transcriptase that replenishes telomeric DNA, can prevent telomere dysfunction and the subsequent cellular responses. Most normal human cells express little or no telomerase, and hence have a finite replicative life span that is limited by telomere length and function. By contrast, most malignant tumors and cancer cell lines express telomerase and have an indefinite capacity for proliferation (replicative immortality)[6-8].

The minimal components of mammalian telomerase are an RNA that provides the template for the telomeric repeat sequence (TTAGGG in vertebrates), a putative H/ACA pseudouridine synthase (dyskerin), and a catalytic component (hTERT in human cells) that adds the telomeric repeat to the chromosome ends[9]. Telomerase expression is limited in most human cells by failure to express hTERT. Ectopic expression of hTERT in several human somatic cell types appears to be sufficient to prevent replicative senescence without conferring other attributes of tumorigenic transformation[10-12]. However, several studies have suggested that hTERT has other functions, other than telomere maintenance, that might contribute to tumor cell survival and proliferation[13].

Unexplained cellular and organismal consequences of telomerase expression

In previously published studies, we observed that cultured human mammary epithelial cells (HMEC) immortalized after carcinogen and/or oncogene exposure, acquired endogenous telomerase activity accompanied by gradual acquisition of resistance to TGFβ-induced growth arrest[12, 14, 15]. Likewise, ectopic expression of hTERT in finite life span $p16^{INK4A}$(-) HMEC rapidly conferred resistance to TGFβ-induced growth arrest[12]. Similar links between telomerase expression and cell phenotypes have since been observed in human lens epithelial cells, HMEC and human fibroblasts[16-19]. In these cases, ectopic hTERT expression caused accelerated cell proliferation, reduced growth factor requirements for cell proliferation, altered gene expression profiles, enhanced DNA repair, and increased tumorigenesis.

A variety of studies in mice have also suggested that telomerase may have functions distinct from telomere maintenance. Telomerase is frequently expressed at high levels in mouse tumors, despite the fact that mice have very long mean telomere lengths, suggesting that telomerase may promote tumorigenesis by mechanisms unrelated to telomere length maintenance[20, 21]. In addition, wound healing was accelerated in transgenic mice that over-express mTERT in epidermal keratinocytes[22]. These mice, and mice in which mTERT was expressed from the beta-actin promoter, were more prone to develop spontaneous tumors at advanced ages, suggesting that high telomerase activity may cooperate with genetic alterations that occur with age to promote tumorigenesis[23]. Conversely, the first generation of TERC-/- mice, which lack telomerase activity but still have long mean telomere lengths, developed fewer skin tumors after chemical carcinogen treatment, indicating that lack of telomerase might protect cells, even those

with long telomeres, from transformation[24]. Other studies conducted in neuronal tissues suggested that mTERT expression protects against apoptosis. In one case, down-regulation of mTERT by anti-sense RNA in mouse embryonic neurons triggered immediate apoptosis despite the presence of long mean telomere lengths[25]. mTERT was also found to protect mouse embryonic neurons against apoptosis induced by amyloid β-peptide[26]. Collectively, these data have led to suggestions that telomerase may promote cell proliferation, survival and/or other aspects of tumorigenesis independently of its known role in telomere maintenance. Nevertheless, direct evidence that telomerase has functions other than telomere maintenance has been lacking.

Telomere dysfunction and cellular stress

In work recently published[27], we reported that introduction of hTERT into finite life span HMEC positively affects the ability of these cells to continue DNA synthesis in the short-term absence of EGF or insulin signaling. These findings were in agreement with a previous study showing that ectopically expressed hTERT provides a growth advantage to HMEC in medium lacking EGF and bovine pituitary extract[19]. However, in contrast to this latter study, we found no evidence that hTERT alters the expression of EGF receptors or downstream signaling components. We found instead that: a) actively growing HMEC contain low but detectable levels of the phosphorylated forms of DNA damage responsive proteins Chk2 and p53, and accumulate 53BP1/phosphorylated histone H2AX foci at chromosome ends, b) phosphorylated forms of Chk2 and p53, and 53BP1/phosphorylated histone H2AX foci are reduced in HMEC that ectopically express hTERT, c) interference with p53 function mimics the effect of ectopically expressed hTERT on growth, and d) transient hTERT expression causes long-term positive effects on DNA synthesis in cells deprived of growth factors. On the basis of these data, we

proposed that many or all the observed effects of hTERT on cell growth and survival could be explained through its direct effects on short telomeres and indirect effects on p53 signaling. Most importantly, our data suggested that in the presence of functional p53, cells with near-dysfunctional telomeres are primed to respond to externally generated growth arrest signals.

The small amounts of activated DNA damage response proteins found in growing pre-senescent HMEC are likely due to undamaged telomeres in compromised or near-dysfunctional states[28]. It is not clear how telomere erosion leads to a DNA damage response, however, there is mounting evidence that telomere structure, not length per se, determines functional status[29-31]. Moreover, the presence of one critically short telomere is apparently insufficient to cause replicative senescence[32]. As the number of telomere repeats on individual chromosome ends decrease, the ability of these ends to recruit and retain telomeric proteins may decrease, increasing the interval the ends spend in an "unprotected" state or alternative conformation that can be recognized by DNA damage pathways. Indeed, Verdun and Karlseder[33] have recently reported that an ATR-dependent damage response is initiated during every S-phase when single stranded DNA accumulates at telomeres as a result of uncoupling replicative unwinding and polymerization, due to stalled replication forks. After the DNA damage response is triggered at telomeres, DNA repair and replication proteins are recruited, and these proteins complete replication at the chromosome ends. Such proteins, which are required for the generation of D loops with telomeric sequences, are recruited through interactions with specialized proteins such as TRF1 and TRF2, which bind directly to telomere repeats in complex, highly dynamic manners[34]. The presence of one or a few transiently exposed chromosome ends may not be sufficient to signal growth arrest or replicative senescence. However, when combined with other stress-related

signals, the cumulative response may cross a threshold beyond which growth arrest occurs.

Integration of telomere and growth factor deprivation signals by p53

Although the role of p53 in growth arrest due to genotoxic stress is well characterized, its role in growth arrest by other causes has been less well documented. For example, p53 has been implicated in establishing growth arrest in response to ribonucleotide depletion[35], TGFβ administration[36, 37], and serum withdrawal[38]. In our studies, we directly demonstrated a role for p53 in growth arrest caused by blockage of EGF receptor signaling, insulin withdrawal, or TGFβ. Thus, p53 plays a more general role in cell cycle regulation, coordinating growth arrest signals with genome surveillance. Although the upstream factors that mediate p53 activation under non-genotoxic conditions remain to be elucidated, p53 is likely to be at the nexus of a variety of cell fate pathways. The p53 protein itself undergoes a variety of posttranslational modifications. TGFβ-mediated induction of p21 has recently been shown to require phosphorylation of p53 on Ser-6 and Ser-9, but not Ser-15, Thr-18, or Ser-20 more commonly associated with DNA damage responses[39]. Small qualitative and quantitative changes in the type/level of p53 modifications alter its ability to complex with other transcription factors, such as Smad2/3, and thus influence the threshold for cytostasis.

The levels of phospho-Ser-15 p53 ultimately decreased with time in both pre-senescent and hTERT-transduced HMEC after blockage of EGF receptor or insulin signaling. While these data might appear contradictory to the data implicating p53 in growth arrest under these conditions, two possible non-exclusive explanations may reconcile these findings. First, p53 and its downstream effector p21 may be important for the initiation, but not maintenance, of growth

arrest. Second, the ratio of these proteins to their binding partners, rather than their overall abundance, may be the critical determinant of growth arrest under the conditions examined. Gene disruption studies in human fibroblasts show that p21 contributes to cell cycle arrest after serum withdrawal, but cells lacking p21 eventually become quiescent, albeit less efficiently[40]. Likewise, human fibroblasts lacking p53 function (owing to expression of viral oncoproteins or genetic suppressor elements), resisted growth arrest due to serum withdrawal, but eventually became quiescent[38]. Similarly, we found that HMEC ectopically expressing hTERT or a dominant negative p53 genetic suppressor element eventually cease DNA synthesis in the absence of EGFR signaling, albeit less efficiently. Other cell cycle regulators, such as pRb and its regulators (e.g., the $p27^{Kip1}$ CDK inhibitor), very likely cooperate with p53 and p21 in establishing and maintaining quiescence, and may do so independently. For example, antisense studies have implicated p27 in growth arrest due to serum deprivation[41] or TGFβ[42].

Our experiments using mutated and modified hTERT proteins indicated that the effects of telomerase on growth arrest require the enzyme be catalytically active and capable of telomere maintenance *in vivo*, but do not require its continuous expression. The effects also do not appear to require large increases in mean telomere length, since for unknown reasons the hTERT cDNA flanked by *loxP* sites that we used in our experiments conferred less telomerase activity than an unmodified hTERT cDNA, and consequently, the mean terminal restriction fragment size (an indication of telomere length) was relatively unchanged. Under these conditions of limiting telomerase activity, the shortest telomeres are preferentially elongated[43]. Therefore we hypothesize that the presence of the shortest telomeres, rather than mean telomere length, contributes to the heightened p53 signaling in growing HMEC. Once the shortest telomeres are

sufficiently elongated during the transient expression of telomerase, the stimulus for increased p53 signaling is removed, and no further benefit is derived from the continued presence of telomerase.

Our finding that phosphorylated forms of p53 and Chk2 are present in actively growing finite life span HMEC prior to replicative senescence suggests that p53 is partially activated by short, but not completely dysfunctional, telomeres, and that additional signals from other sources further activate p53 to promote growth arrest (Fig.1). Thus, our results support the notion that p53 integrates different signals contributing to growth arrest by non-genotoxic as well as genotoxic stimuli. Telomerase down-regulates the levels of phosphorylated p53 and Chk2, most likely by extending critically short telomeres, thus reducing the DNA damage signal. Since it is the sum of activated p53 that determines whether cells arrest growth or continue to proliferate, the decreased activated p53 in cells that have been exposed to telomerase increases the threshold for growth arrest signaling by other stimuli. This model can explain a variety of observed effects of hTERT expression on cell growth and survival without postulating additional telomere-independent functions for the telomerase enzyme.

Indirect effects of telomere maintenance on p53-dependent functions may explain most, but not all consequences of TERT expression

Do the results of our studies in cultured cells rule out the possibility that TERT has other functions in addition to telomere maintenance? As in any scientific enterprise, the old dictum that "absence of proof does not constitute proof of absence" still applies. Nevertheless, the indirect effects of telomere maintenance on p53-dependent functions may explain observations

in other systems as well, negating the necessity for alternative telomerase functions. Although murine telomeres are, on average, much longer than human telomeres, there is considerable variability in the lengths of individual telomeres and the shortest telomeres in mouse cells are comparable to those in human cells[44]. Reduced p53 stress responses due to telomerase maintenance of short telomeres in murine cells may exert subtle changes in p53 mediated growth arrest and/or apoptotic responses, indirectly allowing accelerated wound repair[45] and continued proliferation of cells bearing pre-malignant defects. This would readily explain the additive effects on tumorigenicity of loss of one functional p53 allele and TERT over-expression[46]. Changes in the levels of p53 activation may also explain observed neuroprotective effects of TERT over-expression[47]. Notably, although p53 null mice are viable and fertile, some developmental abnormalities have been noted, including overgrowth of neural tissues[48]. Findings such as those showing that over-expression of mTERT in TERC-/- mice causes proliferation of hair follicle stem cells[49] and over-expression of catalytically inactive hTERT causes apoptosis resistance in Burkitt lymphoma cells[50] are, however, harder to explain. It is prudent to keep in mind in experiments such as these, that when expressed at physiological levels, TERT is an extremely low abundance protein. When artificially over-expressed using strong promoters, TERT levels may increase to the point where they bind cellular components that they would not normally bind, or they titrate cellular components required for other processes. A rigorous accounting of physiologically relevant telomere-independent functions of TERT should include demonstrations of phenotypic effects of its absence as well as of its presence.

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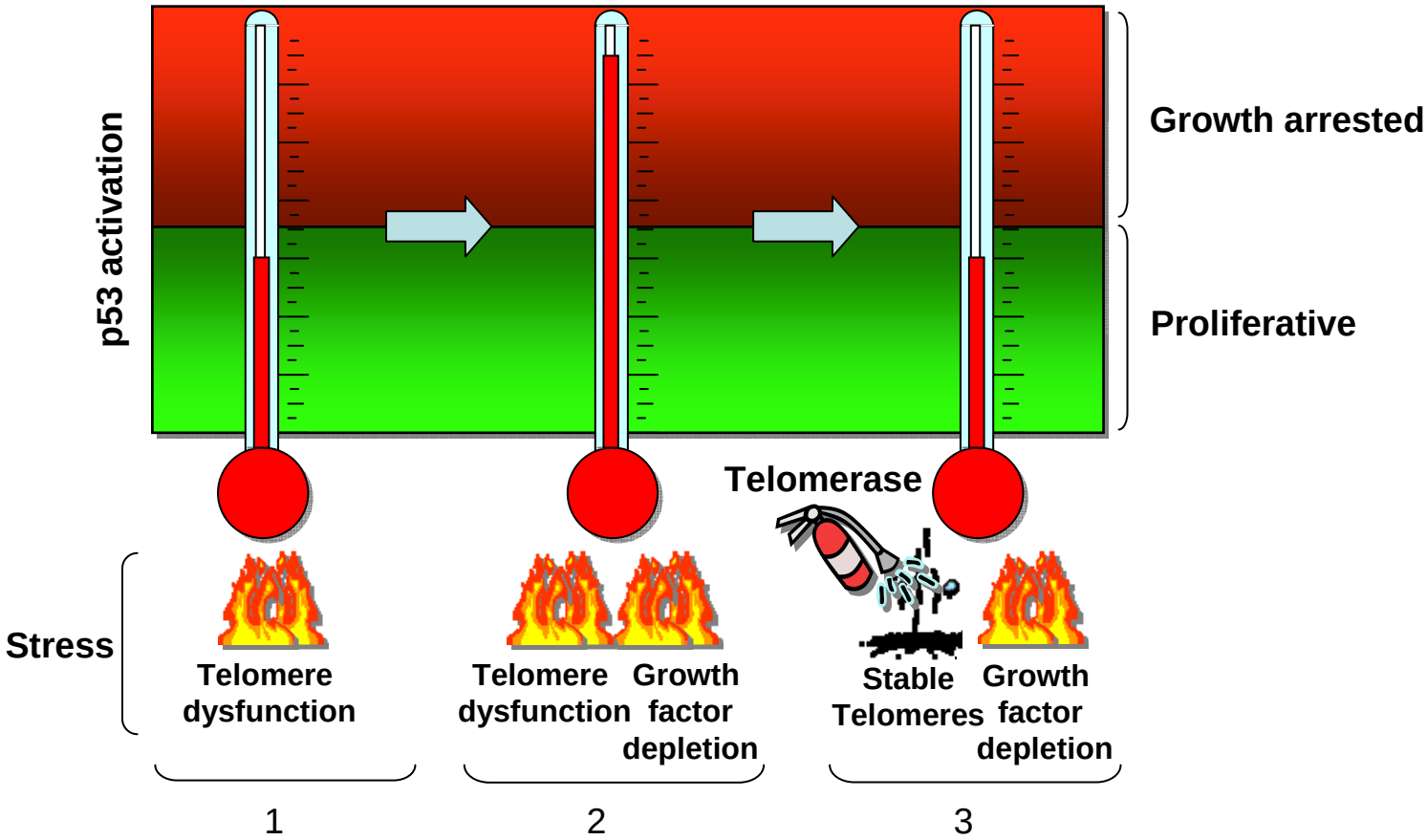
Figure legend

Figure 1. Model for how dysfunctional telomeres and growth arrest signals activate p53 pathway additively. Prior to the onset of replicative senescence, p53 is modestly activated by short telomeres. In the absence of other p53 activating stimuli (1), this low level of activation does not reach a threshold capable of triggering growth arrest until the number or length of short telomeres crosses a critical threshold. Modest activation of p53 by growth factor depletion adds to the low level of p53 activation due to short telomeres (2), crossing the threshold for growth arrest sooner than would occur due to short telomeres alone. Telomerase lowers the level of activated p53 by extending short telomeres (3). Since it is the sum of activated p53 that determines cell fate, the decrease in activated p53 in telomerase-positive cells increases the magnitude of other stimuli required for growth arrest.

Figure 1


p53 activation
Growth arrested
Proliferative
Telomerase
Stress
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