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Citation for published version:

Digital Object Identifier (DOI):
10.1016/j.celrep.2011.12.004

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Cell Reports

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Long Telomeres Bypass the Requirement for Telomere Maintenance in Human Tumorigenesis

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DOI 10.1016/j.celrep.2011.12.004

SUMMARY

Despite the importance of telomere maintenance in cancer cell survival via the elongation of telomeres by telomerase reverse transcriptase (TERT) or alternative lengthening of telomeres (ALT), it had not been tested directly whether telomere maintenance is dispensable for human tumorigenesis. We engineered human tumor cells containing loxP-flanked hTERT to enable extensive telomere elongation prior to complete hTERT excision. Despite unabated telomere erosion, hTERT-excised cells formed tumors in mice and proliferated in vitro for up to 1 year. Telomerase reactivation or ALT was not observed, and the eventual loss of telomeric signal coincided with loss of tumorigenic potential and cell viability. Crisis was averted via the reintroduction of active but not inactive hTERT. Thus, telomere maintenance is dispensable for human tumorigenesis when telomere reserves are long. Yet, despite telomere instability and the presence of oncogenic RAS, human tumors remain susceptible to crisis induced by critically short telomeres.

INTRODUCTION

The limited in vitro life span of normal human cells, referred to as the Hayflick limit, cellular senescence, or mortality stage 1 (M1), was first described in 1961 (Hayflick, 1973). The temporal onset of senescence is correlated tightly to telomere length (Allsopp et al., 1992; Harley et al., 1990), and is bypassed by expression of the telomerase reverse transcriptase hTERT (Bodnar et al., 1998; Vaziri and Benchimol, 1998). Transformation via factors such as SV40 T antigen lead to life span extension beyond M1; however, cells acquire genetic instability and eventually undergo apoptosis, referred to as M2 or crisis (Wright et al., 1989). Further, the discovery that tumor cells possessed shorter telomeres compared with normal tissues suggested that telomere maintenance was required to avert crisis during tumorigenesis (de Lange et al., 1990; Hastie et al., 1990). This hypothesis was borne out in SV40-transformed human cells, in which rare clones that acquired telomerase activity survived the genetic instability and cell death that accompany crisis (Counter et al., 1992). In fact, enforced expression of TERT in combination with oncogenic RAS and the SV40 early region (ER) elicits tumorigenic conversion of fibroblast, kidney epithelial, and mammary epithelial cells (Elenbaas et al., 2001; Hahn et al., 1999a, 2002). Thus, the acquisition of telomerase activity appears essential for immortality in many normal and cancer cell types.

While mice have proven a useful model system in which to study cancer, the response to a critically short telomere differs markedly between mice and humans (for review, see Smogorzewska and de Lange, 2002). Another critical difference between mice and humans is that many human tumor cell types possess a subset of telomeres that are already critically short (Capper et al., 2007; Xu and Blackburn, 2007), whereas laboratory murine strains typically possess much longer average telomere lengths (Hemann and Greider, 2000). For example, inhibition of telomerase reverse transcriptase (TERT) in human tumor lines induces cell death almost immediately, confounding the ability to distinguish the role of TERT in cell viability independent of telomere maintenance (Hahn et al., 1999b; Zhang et al., 1999). Thus, an important unresolved question is whether TERT, or indeed any mechanism of telomere maintenance, is essential for human tumorigenesis.

To address this question, we engineered a human tumor line in which telomere length and hTERT expression could be controlled genetically and temporally. We employed the
The Cre-loxP system, which enables stringent and reversible control of hTERT in primary human cells to generate human tumor cells with long telomeres from which hTERT could be excised (Cascio, 2001; Jaiswal et al., 2007; Steinert et al., 2000; Ungrin and Harrington, 2006). The results demonstrated unequivocally that TERT is dispensable for human tumorigenesis and cell viability when telomeres are long. However, despite the continuous presence of RAS and SV40, induction of endogenous telomerase or other telomere maintenance mechanisms (e.g., ALT) was not observed, and the cells eventually succumbed to telomere-induced crisis.

RESULTS

Establishment of hTERT-Excisable Human Tumor Cells

The human TERT cDNA (Harrington et al., 1997) and an Escherichia coli phosphotransferase gene encoding resistance to hygromycin B (hph) (Gritz and Davies, 1983) were flanked by loxP sites and introduced into human HA5 embryonic kidney cells (HA5) containing the SV40 early region (ER) (Stewart and Bacchetti, 1991; Figure 1). Upon hTERT introduction, HT (HA5 + hTERT) cells became telomerase-positive and immortal but could not support anchorage-independent growth in 0.6% w/v agar. However, after infection with a retrovirus encoding HRASG12V (HT + RAS = HTR) (Hahn et al., 1999a), HTR cells formed colonies in 0.6% w/v agar and gave rise to tumors in immunocompromised mice when injected subcutaneously or beneath the kidney capsule epithelium (Figures 1 and 2H). In this tumor cell model, we chose to use an SV40-transformed cell line (HA5) that cannot escape crisis spontaneously (Counter et al., 1992), and hTERT was introduced as the second (rather than first) step in the tumorigenic conversion process (Elenbaas et al., 2001; Hahn et al., 1999a, 2002). Thus, immortalization is not an obligate first step for human tumorigenesis.

TERT-Excised Tumor Cells with Short Telomeres Capable of Transient Tumor Formation

After a short period of propagation in culture (population-doubling level [PDL] 12, mean TRF <6 kbp, e.g., Figure 2F, lane 11), Cre recombinase or the appropriate empty vector control encoding zeocin resistance (Sh Ble) was queried via RT-PCR analysis (Figure 2B, lanes 4–8). Cell crisis ensued in hTERT-excised populations soon thereafter (Figure 2C); however, the two longest-lived cell lines supported anchorage-independent growth immediately after hTERT excision (Figure 2D, HTR EP Cre-3 and HTR EP Cre-4). HTR EP Cre-4 cells, although hTERT negative (Figure 2E, lanes 6, 7), formed tumors in mice at an incidence indistinguishable from hTERT-positive HTR cells (HTR EP Vec) (Figure 2H). This controlled hTERT genetic excision is consistent with the transient survival observed upon telomerase suppression in human cancer lines with short telomeres (Hahn et al., 1999b; Zhang et al., 1999).

(G) Schematic of elements introduced into HA5 cells, at indicated PDL.

Error bars illustrate the standard deviation of the mean, using the number of replicates as indicated.
TERT-Excised Tumor Cells Exhibit Robust Tumor Formation until Telomere Crisis

To create hTERT-negative human tumor populations with long telomeres, the HTR population was propagated in culture for more than 240 days (PDL 146) until average telomere length reached 12 kbp (Figure 3E, lane 11) prior to hTERT excision. Control cell clones in which an empty vector (HTR Vec) was introduced retained hTERT and hph expression, and exhibited telomere elongation and colony forming potential in 0.6% w/v agar (Figures 3A–3E). In clones selected for Cre recombinase expression, hTERT was excised (Figure 3F), and telomere length was similar to that of parental HTR cells (Figure 3G). Tumor formation was observed in immunodeficient mice (Figure 3H).
expression (HTRCre), loss of hTERT expression was confirmed by RT-PCR and measurement of telomerase activity (Figures 3A and 3B, lanes 1–12). The maximum life span of these hTERT-excised clones exceeded 250 days, and one clone survived for 1 year (Figure 3C). Telomerase activity remained absent, and telomere attrition continued unabated with no evidence of the telomere length heterogeneity typical of telomerase-negative tumor cells that undergo telomere recombination (ALT) (Figures 3A, 3E, and 3G). Even in the complete absence of hTERT, HTRCre lines retained a significant initial capacity for anchorage-independent growth (Figure 3D). Upon injection into the subrenal capsule, which in some instances is more permissive for tumor
formation than subcutaneous injection (Liang et al., 2008; Sun et al., 2005), HTR<sup>C<sup>re</sup></sup> lines exhibited a tumor incidence of 100% even after more than 1 month in culture (PDL 27) (Figure 2H). This incidence was indistinguishable from the 100% tumor incidence of telomerase-positive HTR<sup>Vec</sup> cells, and exhibited statistical significance at high probability (\(\alpha\) actual = 0.05, two tailed). Analysis of HTR<sup>C<sup>re</sup></sup> tumor explants confirmed the absence of <sup>hTERT</sup> and retention of <sup>Sh Ble</sup> expression specific to HTR<sup>C<sup>re</sup></sup> cells (Figure 3H, lane 2). The eventual loss of tumor-forming capability and anchorage-independent growth at later passages was coincident with the appearance of chromosome ends with no detectable telomeric DNA (Figures 2H, 3D, 3F, and 3G). These results demonstrate that longer telomere reserves permit human tumor formation for prolonged periods in the absence of telomere maintenance and <sup>hTERT</sup>, but that the eventual loss of telomeric DNA leads to crisis and an inability to support tumor formation.

**Crisis in TERT-Excised Cells Is Rescued by Catalytically Active TERT**

To confirm that crisis was induced by critically short telomeres and not via unrelated genetic events, wild-type <sup>hTERT</sup> or inactive <sup>hTERT</sup> mutants Q169A (Sealey et al., 2010; Wyatt et al., 2009) or D868A/D869A (Harrington et al., 1997) were introduced into HTR<sup>Cre<sup>-<sup>Δ</sup></sup></sup> cells at PDL 6 (Figure 4). Only wild-type <sup>hTERT</sup> restored telomerase activity (Figure 4A), extended cellular life span (Figure 4B), and conferred anchorage-independent growth (Figure 4C). HA5 cells without <sup>HRAS<sup>G12V</sup></sup> also depend on the catalytic activity of <sup>hTERT</sup> to avoid crisis (Sealey et al., 2010). The fact that telomerase catalytic activity was essential to avert crisis and promote anchorage-independent growth supports the critical role of <sup>hTERT</sup>-mediated telomere extension activity in tumor cell survival when telomeres are short.

**DISCUSSION**

In the presence of sufficiently long telomeres, telomere erosion or the absence of <sup>hTERT</sup> did not impede human tumorigenesis. Only when telomeric DNA was lost from chromosome ends did cells resume dependence upon the telomere elongation activity of <sup>hTERT</sup>. Other examples of tumor-forming capability in cells that do not express <sup>hTERT</sup> are known, for example, in ALT cells or primary tissues transformed with oncogenic <sup>RAS</sup> (Liang et al., 2008; Sun et al., 2005), but these examples did not permit the ability to test the compatibility of ongoing telomere erosion with cell survival. Examples of tumors that lack in vitro telomerase activity have been correlated with clinical regression (e.g., retinoblastoma or neuroblastoma) (Gupta et al., 1996; Hiyama et al., 1995); however, these studies preceded the cloning of <sup>hTERT</sup> or identification of ALT and in many cases these tumor types are now known to exhibit ALT-like characteristics or low <sup>hTERT</sup> expression (reviewed in Cesare and Reddel, 2010). Here, we showed in a defined genetic system that telomerase-negative human tumor cells are capable of tumor formation and cell viability in the absence of endogenous <sup>hTERT</sup> expression or ALT.

Although tumorigenic potential has not been examined in mice lacking <sup>Tert</sup>, its absence has no phenotypic consequences in normal murine tissues while telomere reserves remain intact (Erdmann and Harrington, 2009; Meznikova et al., 2009; Strong et al., 2011; Vidal-Cardenas and Greider, 2010). The fact that <sup>hTERT</sup> is dispensable in telomerase-positive tumor cells was not foreseen. For example, deletion of one subunit of the Ku heterodimer, a complex important in maintaining telomere integrity, is lethal in human tumor cells but is dispensable in other organisms (Fattah et al., 2008; Li et al., 2002). Once telomeres became critically short, however, aversion of tumor cell crisis depended upon active <sup>TERT</sup>. In contrast, when <sup>TERT</sup> is overexpressed, its ability to stimulate proliferation does not always depend on catalytic activity, e.g., in ALT cells (Stewart et al., 2010; Cattanach et al., 2010; Cesare and Reddel, 2010).

**Figure 4. Ability of hTERT to Rescue Crisis in hTERT-Excised Cells**

(A) Wild-type (WT) or mutant (Q169A; D868A, D869A) <sup>hTERT</sup> or empty vector (Vec) were introduced into HTR<sup>Cre<sup>-<sup>Δ</sup></sup></sup> cells and analyzed for telomerase activity (200, 100, 50 ng lysate).

(B) Replicative life span of cell lines as indicated above. hTERT WT cells remained immortal.

(C) Anchorage-independent growth of cell lines as indicated (n = 4).

Error bars illustrate the standard deviation of the mean, using the number of replicates as indicated.

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were stained with 0.01% w/v crystal violet and images acquired with a Bio-Rad

CTT-3

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Equal cell numbers (5

Anchorage-Independent Growth Assay

were anti-SV40 T Ag (Pab-108) (Santa Cruz), anti-HRAS (C-20) (Santa Cruz),

2002) or murine hair follicles (Flores et al., 2005; reviewed in Parkinson et al., 2008; Sarin et al., 2005).

Human tumor cells retained their susceptibility to telomere-induced crisis even after prolonged growth periods. This delayed dependence upon telomerase function differs from the “addiction” to oncogenic factors such as MYC or RAS, in which cell survival remains reliant on these factors (Greider, 1999; Weinstein and Joe, 2008). Thus, human tumor cells are reliant upon telomere integrity rather than hTERT or telomerase activity. Although such dependence was well established for normal cell growth, it was not possible to predict whether tumor cells might somehow subvert telomere-induced crisis via induction of endogenous telomerase, ALT, or another mechanism. For example, Saccharomyces cerevisiae lacking telomerase and the recombination factor RAD52 can escape senescence indefinitely via activation of RAD52-independent telomere maintenance mechanisms, provided the strain possesses long telomeres initially (Grandin and Charbonneau, 2009; Lebel et al., 2009). In contrast, our results suggest that human tumor cells with initially long telomeres can only temporarily avert the requirement for telomere maintenance.

These results have implications for telomerase inhibition in cancer therapy. Telomerase-negative pediatric cancers such as ependymoma possess a better long-term prognosis than telomerase-positive cancers (reviewed in Tabori and Dome, 2007), and low telomerase expression or ALT correlates with a better outcome in histiocytoma and colorectal cancer (Matsuo et al., 2009; Tatsumoto et al., 2000). Our finding that telomerase-negative tumors do not invoke ALT and remain mortal may provide a mechanism to explain the more favorable prognosis for a subset of telomerase-negative tumor types in vivo. Thus, even in telomerase-positive tumors with long telomeres, telomerase inhibition combined with adjunct treatments that limit tumor progression could prove effective as an anticancer therapy.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**

Cell culture and PDL determination was performed as described (Hayflick, 1973; Sealey et al., 2010). hTERT was introduced via electroporation and clonal populations selected in 200 μg/ml hygromycin (Invitrogen), followed by retroviral infection with pBABE- puro-HRASG12V (Addgene) (Hahn et al., 1999a) and selection in 2 μg/ml puromycin (Invitrogen). Transfection with pcDNA3.1-zeo-Cre (Cre recombinase cDNA provided by Dr. Michael Reth) or pcDNA3.1-zeo (Invitrogen) was performed using Fugene6 (Roche) with transient selection in 200 μg/ml zeocin (Invitrogen). Inactive hTERT variants were introduced as described (Sealey et al., 2010).

**Protein and RNA Analysis**

Western blots, RT-PCR mRNA analysis, and the telomere repeat amplification protocol (TRAPeze, Millipore) were performed as described (Sealey et al., 2010). RT-PCR analysis of mRNA encoding zeocin resistance (Sh Ble) was conducted using the following DNA primers: 5′-GACCTCGTGAGGACGCA CTT-3′ and 5′-GACAGCAGCTCCTGGACACT-3′. Primary antibodies employed were anti-SV40 T Ag (Pab-108) (Santa Cruz), anti-HRAS (C-20) (Santa Cruz), and anti-actin (Sigma).

**Anchorage-Independent Growth Assay**

Equal cell numbers (5 x 10⁵) were plated onto 0.6% w/v agar and incubated at 37°C (5% v/v CO₂) for 21 days as described (Cifone and Fidler, 1980). Colonies were stained with 0.01% w/v crystal violet and images acquired with a Bio-Rad Molecular Imager Gel Doc XR System. Colonies were counted using Imagequant TL (GE Healthcare).

**Cell Line Injections In Vivo**

A suspension of 5 x 10⁶ cells was injected subcutaneously or under the sub-renal capsule space of Rag2−/− γ-chain−/− immunodeficient mice (Mazurier et al., 1999). After 21–22 days, the mice were sacrificed and examined. Explanted tissues were extracted for RNA and analyzed by RT-PCR as described above. Experiments were performed in accordance with protocols approved by the Animal Care Committee at the University of Guelph, as outlined in the animal utilization protocol AUP08R007 (issued to D.H.B).

**Telomere Terminal Restriction Fragment and Q-FISH Analysis**

Telomere length was analyzed via telomeric restriction fragment (TRF) analysis (Sealey et al., 2010), and average length determined after Southern blotting using Imagequant TL and UTSWTELORUN software first developed by H. Vaziri and C. Harley (Ouellette et al., 2000). Q-FISH was performed as described (Erdmann and Harrington, 2009) on ten separate metaphases for each PDL indicated.

**Statistical Analysis**

Differences in average colony number were assessed via analysis of variance (ANOVA), assuming unequal variance and using a Tukey post-test (Instat3, GraphPad). Statistical significance of tumor incidence was assessed using Fisher’s exact test (Prism5, GraphPad). Q’power was used to determine power and alpha values where indicated (Faul et al., 2009). Quantification of telomere-signal free ends (SFE) after Q-FISH was compared using ANOVA with a Tukey post-test (Instat3, GraphPad).

**LICENSING INFORMATION**

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**ACKNOWLEDGMENTS**

The authors wish to dedicate this publication to our late colleague and friend Dr. Michael A.S. Taboski, whose passionate commitment to science will continue to inspire us. Supported by the BCFC-Ontario Chapter to the late M.A.S.T., the CIHR (MOP-86453) to D.H.B., and the NCIC (15072), NIH (RO1-AG024398), and Wellcome Trust UK (84637) to L.H.

Received: August 23, 2011

Revised: December 6, 2011

Accepted: December 15, 2011

Published online: February 2, 2012

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