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Super-telomeres in transformed human fibroblasts

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ABSTRACT

Telomere length maintenance is critical for organisms' long-term survival and cancer cell proliferation. Telomeres are kept within species-specific length ranges by the interplay between telomerase activity and telomeric. chromatin organizationIn this paper, we exploited telomerase immortalized human fibroblasts (cen3tel) that gradually underwent neoplastic transformation during culture propagation to study telomere composition and length regulation during the transformation process. Just after telomerase catalytic subunit (hTERT) expression, cen3tel telomeres shortened despite the presence of telomerase activity. At a later stage and concomitantly with transformation, cells started elongating telomeres, which reached a mean length greater than 100 kb in about 900 population doublings. Super-telomeres were stable and compatible with cell growth and tumorigenesis. Telomere extension was associated with increasing levels of telomerase activity that were linked to the deregulation of endogenous telomerase RNA (hTERC) and exogenous telomerase reverse transcriptase (hTERT) expression. Notably, the increase in hTERC levels paralleled the increase in telomerase activity, suggesting that this subunit plays a role in regulating enzyme activity. Telomeres ranging in length between 10 and more than 100 kb were maintained in an extendible state although TRF1 and TRF2 binding increased with telomere length. Super-telomeres neither influenced subtelomeric region global methylation nor the expression of the subtelomeric gene *FRG1*, attesting the lack of a clear-cut relationship between telomere length, subtelomeric DNA methylation and expression in human cells. The cellular levels of the telomeric proteins hTERT, TRF1, TRF2 and Hsp90 rose with transformation and were independent of telomere length, pointing to a role of these proteins in tumorigenesis.

1. Introduction

Telomeres are specialized nucleo-protein structures that protect human chromosome ends from nucleolytic digestion and end-to-end fusions, thus being essential for chromosome stability [1]. Human telomeric DNA is composed of tandem repetitions of the TTAGGG hexanucleotide, it is double stranded, except at the 3′ region, where it forms a 100–250 nucleotide long single strand overhang [2]. Because the extension of the repetitions varies from chromosome to chromosome and from cell to cell, telomeric DNA length is heterogeneous both within cells and among chromosomes of a single cell, but it is generally maintained within a species-specific range [3]. In human embryonic cells, for example, the average telomere length is around 15 kb; in contrast, in mice, telomeres can extend over 50 kb. Thus, mechanisms exist that restrain telomere length within a definite extension range.

Telomeres are associated with several proteins, which play an essential role in telomere maintenance. A six subunit complex (TRF1, TRF2, RAP1, TIN2, TPP1, POT1), named shelterin [4], specifically binds to telomeres and controls telomere stability and length. TRF1 and TRF2 act as *in cis* negative regulators of telomere elongation [5–7]. Evidence has been reported that TRF1 controls telomerase-mediated telomere lengthening through its interaction with POT1, which binds the telomeric single strand overhang and transduces TRF1 signals to telomerase, the enzyme deputed to telomere elongation [8].

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Telomerase allows cells to overcome the end replication problem, which is due to the unidirectional and primer-dependent DNA polymerase synthetic activity. Telomerase is a ribonuclear-protein complex that elongates the 3′ telomeric ends through a reverse-transcription reaction, using the RNA moiety (TERC) as template and the catalytic subunit (TERT) as enzymatic activity [9]. In human cells, together with TERC and TERT, the regulatory protein dyskerin is required for the formation of a catalytically active telomerase [10] and other proteins participate in telomerase biogenesis and regulation. hTERT (human TERT) expression is tightly regulated during development and its expression correlates with the presence of telomerase activity; in contrast, hTERC (human TERC) is constitutively expressed, even when telomerase is absent. hTERT is expressed at very low levels in most adult somatic cells [11], while is present in stem cells, germ-line and embryonic cells. In somatic cells, the low telomerase activity is not sufficient to maintain telomeres, which shorten at each cell division. When their length falls below a threshold level, telomeres trigger cellular senescence [12]. Senescent cells remain metabolically active even for years, but are unable to divide, because short telomeres are recognized as DNA double strand breaks and activate the DNA damage response that arrest cells' proliferation [13]. Thus, telomere shortening has been considered as a "mitotic clock" [14].

In contrast to normal somatic cells, telomerase is active in the vast majority of tumors (about 85%), in agreement with the requirement of functional telomeres for an indefinite cellular proliferation [15]. The necessity of preserving functional telomeres in tumor cells is confirmed by the observation that tumor cells lacking telomerase activity maintain telomeres through a recombination based mechanism known as alternative lengthening of telomeres (ALT) [16]. Characteristics of cells adopting this mechanism are a high frequency of sister chromatid exchanges between telomeres, leading to telomeres highly heterogeneous in length, extrachromosomal telomeric DNA circles and PML (Promyelocytic Leukemia) nuclear bodies containing telomeric chromatin (ALT PML bodies) [17].

In several somatic cells, ectopic hTERT expression is sufficient to induce telomerase activity, allows overcoming cellular senescence and leads to cellular immortalization [18,19]. In different hTERT immortalized cell lines, telomeres can reach different extensions and telomerase activity levels, as well as hTERT and hTERC expression, play a role in the determination of their length [20,21]. Very long telomeres, longer than 50 kb, were found in different cell types in which both hTERT and hTERC were ectopically expressed; these high levels of telomere elongation correlated with high degrees of telomerase activity [22,23].

By ectopic hTERT expression, we have obtained a human fibroblast cell line (named cen3tel) that has become immortal and has undergone neoplastic transformation during *in vitro* propagation [24–27]. The acquisition of the neoplastic phenotype was a gradual phenomenon; in fact, initially cells maintained a phenotype similar to that of parental fibroblasts (represented in this work by cells around population doubling, PD, 30), then (around PD 100) became able to grow in the absence of solid support, a feature typical of the initial phases of transformation [25]. Subsequently (around PD 160), cen3tel cells acquired the capacity to induce tumors in about one month when injected subcutaneously into immunocompromised mice and, upon further propagation in culture, they increased their aggressiveness, forming tumors with shorter latencies (~8 days around PD 600 and ~2 days around PD 1000). Moreover, cells around PD 1000 were also able to induce lung metastases when injected into the tail vein of nude mice [24,27]. In cen3tel cells, anchorage independent growth was associated with downregulation of the *CDKN2* locus, while neoplastic transformation with p53 inactivation and c-MYC overexpression [27].

We also found that, after the transformation process, cen3tel cells lost the ability to regulate telomere length at a steady state level and reached an average telomere extension greater than 100 kb. In this work, we investigated the mechanisms leading to the loss of telomere length homeostasis.

2. Materials and method

2.1. Cells and cell culture

Cen3tel cells were obtained from primary cen3 fibroblasts, by infection with an hTERT-containing retrovirus [25]. MDA-MB-231 (breast cancer), U373 (glioblastoma), primary cen3 and cen3tel cells were grown in Dulbecco's modified Eagle's Medium (DMEM, Euroclone) supplemented with 10% fetal bovine serum (Lonza), 2 mM glutamine, and 1% non-essential amino acids (Euroclone), 0.1 mg/ml penicillin (Euroclone), 100 U/ml streptomycin (Euroclone) at 37 °C in an atmosphere containing 5% CO_2. Cen3tel cells were used at different PDs comprised between PD 30 and PD 1200. To establish clonal cen3tel populations, single cells at PD 262 were seeded at low density (50 cells/10 cm diameter dish) and clones derived from a single cell were isolated and propagated *in vitro*. PD numbering of the clones was restarted, counting all the divisions performed by the single cell that generated the clone.

2.2. Analysis of telomere length and telomerase activity

Telomere length was determined by analyzing the mean length of the terminal restriction fragments (TRFs) by Southern blotting. For the analysis of TRFs below 20 kb, DNA samples were processed and hybridized as described in Mondello et al. [25]. For the analysis of longer telomeres, DNA samples were prepared from 10^6 cells embedded in 2% InCert Agarose (Cambrex) and sequentially digested with 20 U of *Afa*I first and *Hinf*I then. DNA fragments were separated trough a 1% agarose gel in 0.5× TBE, using the CHEF-DR®II Pulsed Field Electrophoresis System (Bio-Rad). Separation was performed at 14 °C either for 13 h at 6 V/cm at a switch time of 0.5–2 s or for 16 h at 6 V/cm at a switch time of 5–20 s. Southern blot was then carried out as in [25]. The signal intensity along each lane was quantified by the Image-Quant software and the data were used to determine the mean length of TRFs as described by Harley et al. [28].

Telomeres were visualized on mitotic chromosome by performing fluorescence *in situ* hybridization (FISH) on metaphase spreads using a FITC-labeled (ccctaa)3 peptide nucleic acid probe (PNA, PerSeptive Biosystems) following the procedure set up by Lansdorp et al. [29], with minor modifications. Cells were denaturated in situ at 70 °C for 2 min in 70% formamide, 1% Blocking Reagent (Roche), 10 mM Tris–HCl pH 7.0, 2 μg/ml PNA, and then hybridized at room temperature for 2 h. Washes were performed at room temperature in 70% formamide in 10 mM Tris–HCl pH 7.2, and then in 150 mM NaCl, 50 mM Tris–HCl pH 7.5, 0.05% Tween 20. Chromosomes were counterstained with 0.2 μg/ml DAPI (4′,6-diamidino-2-phenylindole) in PBS for 10 min. Slides were analyzed using an optical microscope Olympus IX71 equipped with a 100× objective. Images were acquired with a digital camera Cool SNAPES (Photometrics) using the MetaMorph software. Figures were assembled using Adobe Photoshop and Adobe Illustrator.

Telomerase activity was analyzed using the TRAPeze kit (Chemicon) according to the manufacturer's instructions. DNA fragments were separated on polyacrylamide gels, which were stained for 15 min with 1× SYBR Gold Nucleic Acid Gel Stain (Molecular Probes, Life Technologies) in 0.5× TBE.

2.3. RNA extraction, reverse transcription-PCR (RT-PCR) and real-time RT-PCR

Total RNA was extracted from actively dividing cells using the Trizol reagent (Life Technologies). For RT-PCR, cDNA was generated from 1 μg of RNA using the QuantiTec Reverse Transcription Kit

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