Quantitative assessment of telomerase components in cancer cell lines

Semih Can Akınclılar a,b, Kee Chung Low c, Chia Yi Liu a, Ting Dong Yan c, Asami Oji d,e, Masahito Ikawa d,e, Shang Li c, Vinay Tergaonkar a,b,*

a Laboratory of NF-κB Signaling, Institute of Molecular and Cell Biology (IMCB), 61 Biopolis Drive, Proteos, Singapore 138673, Singapore
b Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore (NUS), Singapore 117597, Singapore
c Program in Cancer and Stem Cell Biology, Duke-NUS Graduate Medical School, Singapore 169857, Singapore
d Division of Microbiology, Osaka University, Suita, Osaka 565-0871, Japan
e Graduate School of Medicine, Osaka University, Suita, Osaka 565-0871, Japan

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A B S T R A C T

Besides its canonical function of catalyzing the formation of telomeric repeats, many groups have recently reported non-canonical functions of hTERT in particular, and telomerase in general. Regulating transcription is the central basis of non-canonical functions of telomerase. However, unlike reverse transcriptase activity of telomerase that requires only a few molecules of enzymatically active hTERT, non-canonical functions of hTERT or other telomerase components are typically required for several hundred copies. Here, we provide the first direct quantification of all the telomerase components in human cancer cell lines. We demonstrate that telomerase components do not exist in a 1:1 stoichiometric ratio, and there are several hundred copies of hTERT in cells. This provides the molecular basis of hTERT to function in other signaling cascades, including transcription.

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1. Introduction

Eukaryotic chromosomes are in a linear structure and the ends of these chromosomes consist of conserved tandem repeats of DNA known as telomeres. In these structures reside binding sites for factors collectively termed as the “shelterin complex” which protects the chromosomal ends [1,2]. The length of telomeric repeats shortens after each cell division due to the end replication problem. Cells with critically short telomeres undergo replicative senescence, unless an enzyme specialized to add telomeric repeats called telomerase is functionally active. Therefore, maintenance of the telomeres is crucial for the growth of cells [3]. It is known that the reverse transcriptase activity of telomerase holoenzyme complex is critical for maintaining telomere length. Apart from the reverse transcriptase subunit human telomerase reverse transcriptase (hTERT), the telomerase holoenzyme complex consists of a long non-coding RNA called human telomerase RNA (hTR) (human) or mouse telomerase RNA (Terc) (mouse) which serves as a primer for telomere elongation; Dyskerin (DKC1) and Nucleolar Protein 10 (NOP10) [4]. In addition, other proteins like Reptin, Pontin, GAR1 ribonucleoprotein (GAR1), NHP2 ribonucleoprotein (NHP2) and Telomerase Cajal body protein 1 (TCAB1) have been identified to be associated with the telomerase complex transiently and it is assumed that they play a role in its maturation and trafficking [5,6]. Unlike hTERT, expression of which is transcriptionally shut off in all somatic cells, the other telomerase associated components are constitutively expressed in most mammalian cell types.

Overcoming senescence and replicative immortality is a major hallmark of cancer, and this requires the reactivation of hTERT and hence reconstitution of telomerase activity. Indeed, mutations of the hTERT promoter are known to occur in over 80% of glioblastomas and to a lesser degree in other types of cancer.
and these mutations are responsible for hTERT reactivation. It is believed that the hTERT promoter mutations are necessary in cancers that arise in tissues that do not proliferate very much and probably do not arise from the stem cell compartment [7]. Nevertheless, it is clear that 90% of human cancers show reactivation of hTERT through the activation of the hTERT promoter, either through mutations or through other oncogenic signaling pathways [9–14]. While it could be speculated that maintenance of telomere ends is the primary function of the reactivated hTERT through its function in the telomerase, several leading groups have reported the acquisition of all other hallmarks of cancer in cells that express hTERT [15,16]. We and others have found that besides its canonical role in telomere length extension, hTERT could also regulate transcription, most notably that of the oncogenic Wnt and NFκB signaling pathways [17–19] which are well known to regulate various aspects of cellular physiology and oncogenesis [20,21]. This telomere independent ability of hTERT could explain why expression of hTERT, even in its catalytically inactive form, confers properties to cells that resemble those of the hallmarks of cancers [22–24]. The search for the molecular basis of these non-canonical functions of hTERT in particular and telomerase components in general has led to several unexpected findings which run contradictory to what is believed by people in the field. hTERT has been found to localize to the mitochondria, to inhibit apoptosis and reduce oxidative stress [25]. It has been shown that hTERT can also associate with the RNA component of mitochondrial RNA processing endoribonuclease (RMRP) and function as a RNA dependent RNA polymerase [26]. Various domains of telomerase can be separated into those required for its canonical functions as well as its non-canonical functions of DNA damage, cell proliferation, Wnt signaling and RMRP modulation [27]. It is beginning to be accepted that various canonical and non-canonical functions of hTERT (and other telomerase components) function in parallel and contribute in oncogenic transformation.

Despite the experimental evidence that the non-canonical functions of hTERT could contribute toward oncogenic transformation, there is scepticism about the molecular basis of this action. The key reason why these additional roles of telomerase have been received with scepticism is because Shay and Wright have estimated that there are only 2 molecules of hTert mRNA in most cells and thereby extrapolated that there could only be a few molecules of hTERT in a cell [28]. Furthermore, even to date there is a dearth of good reagents to directly quantify hTERT levels in cells. The presence of a few molecules of hTERT is not a problem if the sole function of this protein is to function as a non-metabolic enzyme, which typically has very few molecules in a cell [29]. However, to function as a co-transcription factor or to possess other non-canonical roles, hTERT levels in cells ought to be much higher than that required of an enzyme. Typical numbers of transcription factors range from a few hundred to more than 10000 [30,31]. However no direct and rigorous measurement of levels of hTERT along with other components of telomerase has been carried out largely due to technical reasons. Indeed it is critical to know the molar amounts of hTERT and other telomerase components to understand if there are sufficient numbers of these to function in macromolecular complexes and signaling pathways, besides the ones that they have known to function in. In this manuscript we report the first direct quantification of all telomerase components and most importantly that of hTERT. We show that there are at least several hundred copies of hTERT and thousands/millions of molecules of other telomerase components in cells. These results provide the molecular basis for non-canonical properties of telomerase.

2. Materials and methods

2.1. Cell culture

A549, Hela, 293T, MCF7, HepG2 and U2OS cells were grown in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin and fungizone. VA13 cells were grown in DMEM, supplemented with 10% FBS, penicillin, streptomycin and fungizone and 0.1 mM non-essential amino acids (NEAA). Cells are grown in 37 °C incubator with 5% CO2.

2.2. Plasmids and cloning for bacterial expression

pGEX-KG was used for the cloning of Reptin, Pontin, GAR1, TCAB1, and NHP2. For the cloning of DKC1 and NOP10 together, pETDuet-1 (Novagen) was used. C-terminal of hTERT was cloned into pETDuet-1. All the fragments encoding above mentioned proteins were amplified from cDNA prepared from 293T cells. Primer sequences were indicated in Table 2.

2.3. Plasmid, shRNA/siRNA transfection

Plasmids overexpressing the hTert and hTERT were reported earlier [32]. siRNA against hTERT (ON-TARGET plus SMARTpool—Human TERT) was purchased from Thermo Scientific (L-003547-00-0050). Following sequences were used 5’-GAACGGGGCC UGAAACCAGA-3’, 5’-CGCCUGAGCUCAUCUUUGU-3’, 5’-GGAUG CCGUGUCUCCAGA-3’, 5’-GAGGAGCGUCGUGUCCA-3’ for siRNA silencing. Control siRNA was purchased from Qiagen (All Star Negative Control). siRNA (ON-TARGET plus SMARTpool) for Reptin, pontin, DKC1, Nop10, NHP2, TCAB1 and Gar1 were purchased from Thermo Scientific. Cells were transfected using either Lipofectamine LTX (plasmid) or Lipofectamine RNAi-Max (siRNA) according to the manufacturer’s instructions. hTert deletion constructs (1–600, 1–925, 601–1127 and 350–925 amino acids) were cloned into pBobi vector [33] at AgeI – SfiI site. 293T cells were transfected by deletion constructs by using Lipofectamine LTX.

2.4. Protein purification

Sequence confirmed constructs were transformed into BL21 competent cells. After IPTG induction, cells were harvested (5000 rpm for 30 min) and lysed with lysis buffer (150 mM NaCl, 50 mM Tris and 0.5% NP40, pH7.5) with addition of protease inhibitor tablet (Roche) for 30 min on ice. After incubation, cells were sonicated for 5 min with 30 s on- 30 s off periods and centrifuged at 18000g for 20 min at 4 °C. The supernatants were incubated with Ni-beads (Qiagen) or Glutathione Sepharose 4B beads (GE Healthcare) for 1 h in a rotator incubator at 4 °C. Recombinant proteins were eluted with elution buffer containing 500 mM imidazole for His-tagged proteins (150 mM NaCl, 50 mM Tris, pH 8) or with 10 mM glutathione for GST-tagged proteins. Eluted samples were electro-eluted to remove impurities.

2.5. Western blotting

Cell numbers were quantified by hemocytometer and pelleted and stored at −80 as 106 cells. Total protein was extracted with Totex buffer (20 mM HEPES, pH 7.9, 0.35 M NaCl, 20% glycerol, 1% NP-40, 1 mM MgCl2, 0.5 mM EDTA, 0.1 mM EGTA) supplemented with protease inhibitor cocktail (Pierce). Purified proteins were loaded on gel with different amounts to be used as a standard for quantitative Western blot analysis. PVDF membrane (BioRAD) was
probed with hTERT, Pontin, Reptin, NOP10, NHP2 (Abcam), DKC1, GAR1, TCAB1 (Santa Cruz), antibodies followed by secondary mouse or rabbit HRP antibodies (Santa Cruz). Band intensities were quantitated by using Image J software for densitometry analysis.

2.6. Real time telomeric repeat amplification protocol (TRAP)

RT-TRAP assay was carried out using cell extracts equivalent to 1000 cells, 0.1 μg of telomerase primer TS supplemented with dNTPs for 30 min at 30 °C followed by PCR reaction with 50 ng ACX primers 40 PCR cycles with 30 s at 95 °C and 90 s at 60 °C. Primer sequences were indicated by Kim and Wu [34].

2.7. Immunoprecipitation and quantitative real-time PCR

Cells were lysed on ice for 20 min with lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.5% NP40, 0.5% sodium deoxycholate, 0.005% SDS supplemented with protease inhibitor cocktail and 200 u/m RNase inhibitor). Subsequently cells were sonicated for 4 min with 30 s on/off cycles. The homogenate was centrifugated at 13000g for 20 min at 4 °C. Supernatants were incubated with TERT, ELKS, Chk1, CD154 and Igg (Santa Cruz) antibodies and magnetic protein A beads (Dynabeads-Invitrogen) for 6 h at 4 °C on a rotator. TERT bound RNAs eluted by adding Trizol reagent on beads. Protein was eluted by boiling beads with 2 × SDS sample buffer for 10 min.

After IP reaction, cDNA reaction was performed from the elution, flow-through and input fractions by Superscript cDNA VILO kit (Invitrogen) according to manufacturer’s instructions. Real time PCR reaction was performed by Syber Green kit (Invitrogen) for hTR mRNA quantitation. Cq values were compared between input, flow-through and elution fractions. Percentage hTR was calculated in elution and flow-through fractions as compared to input amount. Primers for hTR were indicated in Table 2.

2.8. Competitive PCR

Competitive hTR RNA was cloned from hTR overexpressing plasmid [35]. Oligo sequences (5’GGATATATAGGGGCCTGCCATGGTGACATG3’ and reverse 5’CGCCCGCCCTGGTACCATGCGCCCGCCCTGGTACCATG3’) were annealed and added after position 71, following NarI (NEB) restriction enzyme digestion. Sequence confirmed construct was in vitro transcribed by T3 RNA polymerase (Promega) and purified by phenol:chloroform extraction. Purified competitor RNA was mixed with template RNA which was extracted from different cell types (~20000 cells). Cells were counted using hemocytometer. RNA was extracted using TRIZOL (Qiagen) according to the manufacturer’s instructions. Complementary DNA was prepared using Superscript Vilo reverse transcriptase (Invitrogen) together with cell RNA using different concentrations of competitive RNA amounts. PCR reactions were performed in duplicate using Dreamtaq polymerase (Thermo Fisher Scientific). Following PCR reaction, endogenous hTR and competitive hTR products were separated on a 2% agarose gel. Band intensities were measured by Image J software. Equal band intensities represent equal competitive and endogenous hTR. Primer sequences are available on request.

2.9. Generation of V5-TERT 293T cells

293T cells expressing N-terminal V5-TERT were created using the CRISPR/Cas9 method [36]. The DNA sequence of Tert (−350 to +350) was amplified and cloned into pCAG-EFp to generate pCAG-Egfp vector. Different guide RNAs (gRNAs) used for guiding Cas9 to the specific target sequence were cloned into pX330/Cas9 vector individually. pCAG-Egfp and pX330/Cas9-gRNA were co-transfected into 293T cells to verify the gRNA target efficiency. The gRNA resulting in the highest GFP+ cells was selected as a successful targeted gRNAs. To repair genomic DNA nick created by Cas9 nuclease and insert the V5 tag in the N-terminal of hTERT, 140 bp of oligonucleotides covering the nick region and V5 sequence was synthesized as the repair template. The selected pX330/Cas9-gRNA was co-transfected with the repair template V5-TERT DNA oligonucleotide into 293T cells. 48 h after transfection, cells were seeded into 96-well plates by limited dilution. Genomic DNA was extracted and sequenced for verification. DNA complementary gRNA sequence for positive colony was (5’CCGCCCCTCGGCCGCGCGATGGG3’). Primers and repair template sequences were indicated in Table 2.

3. Results

3.1. Telomerase activity status of various cell lines used in the study

Before embarking on quantifying the various components of the telomerase complex (Fig. 1A) we decided to identify cells that can serve as controls in this analysis. We chose U2OS and VA13 cells which have been reported to be negative for telomerase activity [37,38] by using a TRAP assay. These cell lines are predicted to utilize alternative lengthening of telomeres (ALT) for telomere elongation. In addition, cancer cell lines from various origins that possess telomerase activity and non-canonical functions of telomerase such as Hela (cervical cancer), 293T (kidney), MCF7 (breast), HepG2 (liver), A2780 (ovarian) and A549 (lung cancer) were chosen [18]. Real time PCR was used to assess gene expression levels of hTR-RNA component of the telomerase enzyme- in our selected cell lines. Unlike U2OS and VA13, all other cell lines express hTR in a moderate level (Fig. 1B). TRAP assay showed that both VA13 and U2OS cells have no measurable telomerase activity (Fig. 1C). To find out if this was due to lack of hTERT or hTR, these cells were reconstituted with either hTR alone or with hTERT; we found that reconstitution with both components is essential to reconstitute telomerase activity in both these cells (Fig. 1C). These experiments set the stage for further analysis as they suggested that (a) these 2 cells lines have no hTR or hTERT, (b) that hTR and hTERT together are sufficient to reconstitute telomerase activity and (c) that the levels of other telomerase components are non-limiting for reconstitution of telomerase activity.

3.2. Quantification of telomerase complex components

A number of proteins have been identified as part of telomerase complex either permanently or transiently associated with it [6,39]. However the functionality of most of these proteins and their roles, if any, in telomerase complex and in processes away from telomerase complex are not yet fully understood. To decipher the stoichiometric ratio of these components under steady state conditions, which may shed further light on their biological functions. Major proteins associated with telomerase (Fig. 1A) complex were quantitated using immuno-blotting with purified proteins as standards [40]. A schematic of the process employed is shown in Fig. 2A. The formula for calculation of the number of molecules is shown in Fig. 2B. Coomassie staining of the purified proteins and the linear curves are shown in Sup. Figs. 1 and 2, respectively. Specificity of the antibodies against telomerase components were shown by si-RNA knockdown strategy (Sup. Fig. 1). As a positive control, purified beta-actin was used and the amounts of beta-actin per cell were quantitated (Fig. 2C). Based on our analysis, 2–4 × 10⁶ molecules of Actin were estimated in the cells and this number is close to the value reported in literature [41], suggesting that our approach is correct.
Pontin and Reptin belong to AAA + ATPases family and function as co-factors for transcriptional regulators due to cooperation with chromatin remodeling proteins [42]. Furthermore they act together for telomerase assembly [6]. Initial reports showed that Pontin and Reptin interact and function collectively, but recently there is evidence that these proteins can also work independently of each other [43,44]. Based on the results presented in Fig. 3A, it was estimated that there are about 4–6 million Pontin molecules per cell, whereas there are about 3–5 million molecules of Reptin per cell (Fig. 3B). NHP2, GAR1, NOP10 and DKC1 are members of small nucleolar-ribonucleoprotein family and these proteins are known to have role in rRNA processing and modification. They are localized in Cajal bodies and nucleoli. Cajal bodies are sub-organelles which are found in the nucleus of proliferative cells.
They are responsible in RNA-related metabolic processes like histone mRNA processing, snRNP biogenesis and recycling. They recruit RNA to be extended by telomerase at the end of telomeres [45]. Calculations employing the mentioned technique showed that there are 400000 molecules of NHP2 per cell (Fig. 3C) whereas there are around 50000 molecules of GAR1 (Fig. 3D) per cell. The levels of NOP10 were estimated to be about 100000 molecules per cell (Fig. 3E). DKC1 levels range between 0.5 and 1 million molecules per cell (Fig. 3F). TCAB1 is a Cajal body RNA chaperone which is known to bind to hTR. This assembly results in recruitment of telomerase to the telomeres. Recent reports show TCAB1 also has Cajal body independent recruitment roles for telomerase to the telomeres [46]. The numbers of TCAB1 molecules in cancer cells are estimated to be about 3 to 4 million per cell (Fig. 3G). Non specificiy of the Telomerase Cajal body protein 1 (TCAB1) antibody tested, makes it difficult to perform these calculations. It must be noted that depending on the quality of the available antibodies and their affinities and avidities, different amounts of standards was used to obtain standard curves. The estimated numbers of these molecules in various cancer cell lines and

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**Fig. 2.** Flowchart and calculation formula for the quantification of telomerase associated proteins. (A) Flowchart showing purification and quantification strategy for various telomerase components. Telomerase complex proteins were cloned from cDNA in pETDuet1 (His-Tag) and pGEX-KG (GST-Tag) vectors. These vectors were transformed into BL21 strain and optimized for expression conditions. (NOP10 was cloned to pETDuet1 vector with DKC1 to increase folding capabilities.) Cells were cultured on large scale and tagged proteins were purified using affinity purifications as discussed in methods section. Purity profile was controlled by Coomassie blue staining and Western blots (WB). Impurities were eliminated by electro-eluting desired protein from SDS-PAGE gels. Purified proteins were used as a standard for quantitative Western blots after titration optimization with the respective antibodies. Quantitative WB was performed by running standards and cell lysates together. We used fixed number of cells for each immunoblot which were determined by hemocytometer counting. Cell numbers vary according to expression levels and efficiency of antibodies. Intensities of Western blot bands were measured by Image J software. (B) Calculation formula for quantitative Western blot. Western blot band intensities and protein amounts of standards were used to obtain linear curve and function to compare with the bands of cell lines. Obtained values were applied to the formula to obtain number of molecules per cell. (C) Quantitation of Actin in human cancer cell lines. Purified Actin was titrated for immunoblotting experiment along with cell lysates as indicated.
telomerase negative cell lines are shown in Table 1. The relatively similar level of these proteins in telomerase positive and negative cells indicates that these proteins do most likely have other functions apart from those of that required in the telomerase complex. Furthermore, given that there is a huge variation in the number of these proteins in all the cells irrespective of their telomerase activity status, it adds to the notion that only a few molecules of telomerase complex have all these proteins incorporated exist in cells, and the rest of the molecules of these proteins incorporated, whereas the rest of the molecules of these proteins most likely have telomerase independent roles in other macromolecular complexes.

3.3. Quantification of hTERT

Quantification of hTERT has been an important unsolved problem due to two reasons, firstly, being the lack of good antibodies that recognizes the endogenous protein coupled with seemingly

Fig. 3. Quantification of telomerase components in various cell lines. Purified proteins were used as a standard for quantitation by immunoblotting. Standards were titrated according to the antibody detection limit and expression levels in cell lines. Amount of protein standards used for Western blots have been indicated in Sup. Fig. 2. Protein molecule numbers per cell showed by bar graphs for different cell lines. Intensities of Western blot bands measured by Image J software. Quantitation of Pontin (A), Reptin (B), DKC1 (C), GAR1 (D), NHP2 (E), TCAB1 (F) and NOP10 (G) by immunoblotting was represented. Lysates from 10000 cells for Pontin, Reptin, DKC1 and GAR1, 200000 cells for NHP2 and TCAB1 and 400000 cells for NOP10 were loaded equally for each cell line. These data are representative of two independent experiments. FL: full length.
Table 1
Molecule numbers per cell for each protein in 8 different cancer cell line. Numbers were rounded to the nearest thousand.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Actin</th>
<th>Hela</th>
<th>MCF7</th>
<th>HepG2</th>
<th>A549</th>
<th>A2780</th>
<th>UZOS</th>
<th>VA13</th>
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<tbody>
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<td>36848000</td>
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<td>171875000</td>
<td>138341000</td>
<td>260684000</td>
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<tr>
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<td>4350000</td>
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</table>

3.4. Quantification of hTR and back calculation of hTERT

Since the number of hTERT quantified based on our antibody approach was far greater than that of the previous estimates or reports, and since all other possible approaches failed as indicated above, another approach was needed to verify this finding. Based on the fact that TERT associates 1:1 with hTR [4,49], a novel method to immunoprecipitate (IP) hTERT from cells and quantify the amount of hTR bound to it was carried out. In this approach, cells were counted prior to IP reaction to equalize input and output (elution + flow-through). RNA isolation was carried out from the input (10%), flow-through, wash and elution fractions and quantitative real time PCR performed for hTR. The input was set to 100% and the elution and flow-through fractions were normalized to it. From this result, the percentage distribution of hTR in elution (hTERT bound hTR) and flow-through (hTERT free hTR) could be calculated and hence back calculation of the amount of hTERT from the amount of hTR pulled down by the hTERT antibody (Fig. 4A) could be carried out. The amount of endogenous hTR molecules was estimated using competitive semi-quantitative PCR (Fig. 4B). Competitive PCR allows for more precise quantification of RNA as it relies on competitor RNA which is subjected to similar RT and PCR conditions as the template and it will not be influenced by the quality of the initial template and tube-to-tube variation during amplification [50]. Based on the calculations presented, the estimated amount of hTR is 11478(±1230) for Hela and 17218(±1570) for 293T and 0 molecules per cell for UZOS cell line (Fig. 4B). To evaluate the specificity of the TERT antibody in native IP conditions, irrelevant antibodies including those to ELKS, Chk1 and CD154 were used besides the IgG control antibody on control Hela cells and TERT overexpressing Hela-TERT cells. As compared to irrelevant antibodies which did not pull down either TERT or
hTR; this antibody showed good enrichment of these components (Fig. 4C), suggesting that the hTERT antibody works very well without non-specific interactions (unlike in the Western blots) in native IP conditions (Fig. 4C). IP result indicated that 5.79% (±0.5%) of total endogenous hTR molecules were detected in the elution fraction which equates to around 665 hTR and by equivalence, 665 hTERT molecules in a cell (Fig. 4D). Calculation of this number comes from the number of total hTR molecule in Hela cells which is 11,478 (Fig. 4B). This number obtained is solely RNA bound hTERT numbers, and as there are possibly hTR unassociated hTERT molecules in the cell, it can be surmised that at least 665 molecules of hTERT exist in these cells while the actual number could be far greater. Additionally, the IP results showed that 5–6 times more hTR and hTERT molecules in hTERT overexpressing Hela cells were trapped compared to control Hela cells (Fig. 4D). Western blot and SILAC experiments showed that the overexpression results in 6 fold higher expression of TERT compared to control Hela cells (data not shown). Taken together, these two results suggest that our methodology is working in the linear range and the levels of hTR and hence hTERT quantitated this way is accurate. As stated above,
the specificity of hTERT antibody in Western blots is a big problem and since the quantification of hTERT is dependent on the specificity of the hTERT IP, a TERT IP was performed using U2OS cells which have no detectable hTERT or hTR (Fig. 1B and C). As shown in Sup. Fig. 6, the anti-hTERT antibody could only IP hTR from U2OS cells that are reconstituted with both TERT and hTR (Sup. Fig. 6). These results showed that the TERT antibody of choice is excellent for IP and it specifically brings down hTR thus raising the level of confidence in our quantification of hTERT described in Fig. 4.

4. Discussion

There have been a few previous attempts to quantitate the amount of telomerase molecules in cells. Yi et al. [28] quantitated +α+β hTERT mRNA as an extrapolation to calculate the number of catalytically active telomerase molecules in a cell to be about 600 molecules. This +α+β form has no mutation on its reverse transcriptase (RT) site and therefore is catalytically active, whereas the alternatively spliced −α or −β containing forms have mutations on the α or β sites of the RT motif respectively, rendering hTERT catalytically inactive. However, it was noted that only 5% of hTert mRNA is in the +α+β form, and 95% of hTert mRNA does not produce active telomerase (+α−β, −α+β, −α−β forms). Cohen et al. [49] used an affinity purification to pull down hTERT followed by a telomere repeat primer to pull down active telomerase, finally arriving at an estimate of around 20–40 molecules of active telomerase per HEK293T cell. These methods are mainly used to provide an estimate of catalytically active telomerase molecules in a cell. Xi et al. quantitated hTERT using IP and western by two different hTERT antibodies [51]. Although they arrive at a similar number of hTERT molecules as us, we predict that the number of hTERT molecules is at least 2–5 times higher than that predicted by both studies as our method only traps hTR bound TERT. The limitation of the antibody used in that study could also be a reason why the estimates vary.

However, a few groups have noted that catalytically inactive telomerase can have other functions in a cell. This makes sense, as producing 95% of inactive telomerase is a waste and natural selection would have eliminated alternative splicing of hTERT if it does not serve any useful function. Supporting this, Hrdlicková et al. [52] found that alternative spliced telomerase can stimulate cell proliferation. Park et al. [17] reported that inactive telomerase can stimulate the Wnt signaling pathway whereas Ding et al. [53] showed that inactive telomerase can still bind p65 and regulate NFκB signaling. Also, a HA-tagged hTERT that could not elongate telomeres was found to protect against apoptosis [54]. Lastly, a 16 amino acid sequence of the catalytically active site of hTERT, GV1001, was found to be sufficient to confer extra-telomeric functions, in the absence of canonical telomerase activity in cells [55].

As the anti-hTERT antibodies in the field against hTERT are not very specific, we circumvented this problem by estimating the amount of hTR in a cell and calculating the amount of hTR pulled down by anti-hTERT antibody. From the first part of the study, we estimated the amount of hTR in a cell to be from 0 in the telomerase negative cell lines U2OS and VA13 to about 11 000–17 000 in the telomerase active cell lines. From our immune-precipitation experiments, we established that about 6% of hTR is pulled down together with hTERT and from this we back calculated the amount of hTERT to be about 665 per cell. It is important to note that the technical aspects of hTR quantification make the quantification of TERT very difficult as a large proportion of hTR is degraded during the IP procedure. The number of hTR and TERT found in these studies is a gross underestimation from the actual numbers that exist in cancer cells. However these numbers, since they are derived from direct methods and not from extrapolations tell us that at least several hundred molecules of TERT do exist in cells, which could easily be sufficient for them to function in pathways apart from telomere elongation. With this large number of hTERT molecules present in the cell; we believe that telomerase can possess many other roles, independent of its enzymatic activity. Thus, hTERT could possibly act as a transcriptional co-factor in oncogenesis. Compared to previous methods of hTERT quantitation [28,49], our method of telomerase estimates total hTERT protein amounts, regardless of whether it is catalytically active or not. Whereas Yi et al. and Cohen et al. extrapolated the number of catalytically active hTERT molecules based on mRNA quantitation of hTERT and affinity purification of active hTERT respectively and this will not reflect the true total number of inactive hTERT molecules that could serve other non-canonical functions of telomerase.

This study also suggest that the number of the various telomerase components in a given cell type varies significantly (Table 1), suggesting that besides association with the telomerase complex, these components have telomerase independent roles. Dyskerin possesses a role in p53 biogenesis as well as ribosome function [56]. The amount of DKC1 was 2 times higher than that of NHP2 and 10 times higher than that of GAR1, showing that besides being associated with NHP2, GAR1 and NOP10 to form the telomerase complex, DKC1 could possibly have other roles in the cell. Besides telomerase assembly, Reptin and Pontin exhibits diverse functions as components of protein complexes, with roles in chromatin remodeling, transcriptional regulation, DNA damage repair, etc [57,58]. Pontin and Reptin are involved in chromatin remodeling complexes [58], and it was found that they can form hetero-dodecamers [59], homo-oligomers [60,61]. Although they are commonly associated with the same chromatin remodeling complexes likeINO80, SRCAP, Tip60, they may have functions independently of each other [62] and may also repress each other’s role in transcriptional regulation. In conclusion, this study shows that the various components of telomerase do not exist in stoichiometric ratios of 1:1. It also shows that there are enough hTERT molecules to carry out non-canonical roles of telomerase in cells, probably in transcription.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.02.035.

References


