Telomerase RNA level limits telomere maintenance in X-linked dyskeratosis congenita

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Dyskeratosis congenita (DC) patients suffer a progressive and ultimately fatal loss of hematopoietic renewal correlating with critically short telomeres. The predominant X-linked form of DC results from substitutions in dyskerin, a protein required both for ribosomal RNA [rRNA] pseudouridine modification and for cellular accumulation of telomerase RNA [TER]. Accordingly, alternative models have posited that the exhaustion of cellular renewal in X-linked DC arises as a primary consequence of ribosome deficiency or telomerase deficiency. Here we test, for the first time, whether X-linked DC patient cells are compromised for telomerase function at telomeres. We show that telomerase activation in family-matched control cells allows telomere elongation and telomere length maintenance, while telomerase activation in X-linked DC patient cells fails to prevent telomere erosion with proliferation. Furthermore, we demonstrate by phenotypic rescue that telomere defects in X-linked DC patient cells arise solely from reduced accumulation of TER. We also show that X-linked DC patient cells averted from premature senescence support normal levels of rRNA pseudouridine modification and normal kinetics of rRNA precursor processing, in contrast with phenotypes reported for a proposed mouse model of the human disease. These findings support the significance of telomerase deficiency in the pathology of X-linked DC.

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Limits on the renewal capacity of the human hematopoietic system can have negative consequences for health and longevity (Chen 2005). Despite recent improvements in the success of stem cell transplantation as a bone marrow failure therapy, attaining clinical rescue of hematopoietic proliferative capacity is far from routine [MacMillan and Wagner 2005; Steward and Jarisch 2005]. Some insights about the requirements for long-term cellular renewal have come from studies of inherited bone marrow failure syndromes such as dyskeratosis congenita (DC) and Fanconi anemia (Federman and Sakamoto 2005). Early studies revealed that cultured cells from Fanconi anemia patients have enhanced sensitivity to DNA damaging agents, foreshadowing roles for the proteins altered in Fanconi anemia in DNA damage repair [Niedernhofer et al. 2005; Kennedy and D’Andrea 2005]. In comparison, despite their elevated frequency of chromosome aberrations, cultured cells from DC patients have apparently normal capacity for repair of induced DNA damage [Dokal 2000]. This distinct cellular phenotype of DC implied a novel molecular defect associated with hematopoietic failure.

DC has X-linked, autosomal dominant [AD], and autosomal recessive patterns of inheritance [Mason et al. 2005; Walne et al. 2005]. X-linked DC arises from amino acid substitutions in a protein termed dyskerin [Heiss et al. 1998], while AD DC arises from mutations in the locus encoding telomerase RNA [Vulliamy et al. 2001a]. Dyskerin, also known as Chi5p and NAP57, is one of several enzymes responsible for post-transcriptional modification of uridine to pseudouridine in noncoding RNA [Ferre-D’Amare 2003]. DC-linked dyskerin isoforms typically have single amino acid substitutions not predicted to influence catalysis [Mason et al. 2005; Walne et al. 2005]. Target sites of dyskerin-mediated pseudouridine modification are determined by its assembly as a larger ribonucleoprotein complex [RNP] with three other proteins, NOP10, NHP2, and GAR1, and one member of a family of small RNAs harboring the hairpin/Hinge/hairpin/ACA (H/ACA) motif [Henras et al. 2004, Meier 2005]. In RNP form, H/ACA-motif small
nucleolar RNAs (snoRNAs) hybridize to ~100 sites on ribosomal RNAs (rRNAs), and other H/ACA-motif RNAs hybridize to some sites on spliceosomal small nuclear RNAs (Ofengand et al. 2001; Kiss et al. 2004). Dyskerin has another function evolved only in vertebrate species. The telomerase RNP elongates chromosome ends by addition of telomeric simple sequence repeats, counteracting the repeat loss inherent in genome replication (Chan and Blackburn 2004; Autexier and Lue 2006; Collins 2006). Vertebrate telomerase RNAs (TERs) incorporate an H/ACA motif that is crucial for processing and accumulation of mature TER from a precursor transcript (Mitchell et al. 1999; Chen et al. 2000). The human TER H/ACA motif assembles with dyskerin, the other H/ACA-motif-binding proteins, and unknown factor[s] not shared with other H/ACA-motif RNPs (Mitchell et al. 1999; Dragon et al. 2000; Mitchell and Collins 2000; Pogacic et al. 2000; Fu and Collins 2003). Regions of TER flanking the H/ACA motif then recruit telomerase reverse transcriptase (TERT) to generate an active enzyme (Mitchell and Collins 2000; Chen and Greider 2004). Most human somatic cells accumulate TER but not TERT and correspondingly lose telomere length with proliferation, until a defect in telomere structure triggers apoptosis or cellular senescence. Only a few human cell types are thought to activate telomerase for telomere elongation, including cells in the germ-line, rapidly dividing epithelial and lymphoid progenitor cells and most cancers (Collins and Mitchell 2002; Forsyth et al. 2002; Wong and Collins 2003). Normal human somatic cells only transiently activate telomerase, but studies in cultured cells indicate that even transient telomerase activation can dramatically extend proliferative capacity (Steinert et al. 2000).

The defective cellular process underlying bone marrow failure in X-linked DC remains controversial. X-linked DC patient lymphoblasts and fibroblasts harbor much shorter telomeres and lower TER levels than the same cell types isolated from unaffected carriers of disease (Mitchell et al. 1999). Additional studies using peripheral blood mononuclear cells support the generality of short telomeres and reduced TER as hallmarks of X-linked DC (Vulliamy et al. 2001b; Wong et al. 2004). Also, heterozygous expression of truncated or altered TER can lead to AD inheritance of DC (Vulliamy et al. 2001a, 2006). These observations suggest that DC arises from a partial loss of telomerase function. However, previous studies stop short of addressing whether a modest reduction in TER actually compromises telomerase function on telomere substrates. Indeed, TERT rather than TER is generally considered to be limiting for telomerase activation in human somatic tissues. Even in human cancer cells, disruption of one copy of the TERT gene is sufficient to impose telomere shortening (Hauguel and Bunz 2003).

X-linked DC has also been attributed to a defect in ribosome biogenesis (Ruggero et al. 2003; Meier 2005; Yoon et al. 2006). Support for this hypothesis derives mainly from a proposed mouse model of X-linked DC (Ruggero et al. 2003), which shows some similarities and some disparities of phenotype with the human disease [Bessler et al. 2004]. The mouse model was created by integration of a targeting construct 9 kb downstream from Dkc1 in the neighboring Mpp1 gene, which reduced dyskerin mRNA by fourfold [males] or twofold [heterozygous females] in cultured embryonic fibroblasts. B-lymphocytes from the mouse model have reduced levels of TER and telomerase activity but unaltered telomere lengths. Instead of critically short telomeres, defects in ribosome biogenesis were detected. A substantial decrease in the pseudouridine content of mature 18S and 28S rRNAs was evident in six of eight independent lymphocyte pools, along with a dramatic kinetic delay in rRNA precursor processing (Ruggero et al. 2003). These biogenesis defects are proposed to underlie the impairment of IRES-mediated translation that has been reported for cells from the mouse model as well as human patients (Yoon et al. 2006). However, the premature telomere shortening and heterogeneous senescence characteristic of all commercially available X-linked DC patient primary cell cultures is a caveat to conclusions about ribosome biogenesis, because the altered growth properties can impact ribosomes independently of the DC genetic lesion. This caveat also applies to previous ribosome biogenesis studies that reported normal pseudouridine modification at specific residues of rRNA in X-linked DC patient cells [Mitchell et al. 1999].

Here, for the first time, we test whether X-linked DC patient cells and family-matched control cells differ in their capacity to support telomerase function at telomeres. We show that telomerase activation in X-linked DC patient cells does not allow telomere elongation or stable telomere length maintenance, in contrast to results from control cells cultured in parallel. Strikingly, we find that this X-linked DC telomere maintenance defect can be rescued by an increase in TER. Also, for the first time, we used long-term nonsenescent cultures of X-linked DC patient cells to investigate putative defects in ribosome biogenesis. We find that X-linked DC patient cells from two different families have normal levels of rRNA pseudouridine modification and no dramatic kinetic delay in rRNA precursor processing. These results support the significance of telomerase deficiency as a mechanism of X-linked DC, and suggest future evaluation of telomerase activation as a disease therapy.

**Results**

**TERT expression in DC patient cells produces catalytically active telomerase**

Primary dermal fibroblast cultures from an X-linked DC patient and his asymptomatic maternal grandmother were obtained with minimal prior passage in culture (see Materials and Methods). The DC patient cells express a dyskerin variant lacking leucine at position 37, designated ΔL37 dyskerin, and the family-matched control cells express only wild-type dyskerin despite carrying a mutant allele [Mitchell et al. 1999]. The DC patient pri-
mary fibroblast culture became completely senescent within 3–4 wk of continuous culture, consistent with the presence of short telomeres, while the control cell culture proliferated for at least 2 mo before reaching senescence, consistent with initial telomere lengths longer than those of the DC patient cells (see below; Mitchell et al. 1999).

Primary human cells ubiquitously and constitutively express TER but rarely express TERT. To activate telomerase in bulk cultures of presenescent primary cells, we infected them with a retrovirus encoding TERT. TERT expression was placed under the control of a relatively weak promoter to recapitulate the physiologically non-abundant level of TERT protein accumulation (see Materials and Methods). Cultures of DC patient and control cells transduced with TERT expression retrovirus both acquired a greatly extended proliferative lifespan. DC patient and control cell cultures with TERT reproducibly maintained robust growth for several months of continuous culture. In contrast, retroviral infections using vectors lacking TERT failed to delay replicative senescence (data not shown). This observation is consistent with the expected requirement for exogenous TERT to activate telomerase in primary human fibroblasts.

We monitored the level of telomerase activation in the DC patient and control cell cultures using whole-cell extracts for in vitro activity assays (Fig. 1A). Telomeric repeat amplification protocol (TRAP) assays (Kim et al. 1994) were performed using a titration of each extract normalized for total protein concentration. The parental primary fibroblast cultures produced undetectable telomerase activity (Fig. 1A, lanes 1–10). In comparison, telomerase activity was readily detected in both cell cultures expressing TERT (Fig. 1A, lanes 11–20). However, control cell extracts contained more telomerase activity than DC patient cell extract at each amount of total protein (Fig. 1A, see extract titration key). The level of activity in control cell extract matched the level of activity in DC patient cell extract when the control extract was diluted by between one and two of the 2.5-fold steps in amount of total protein (5–0.13 µg). (B,C) Genomic DNA was isolated from successive harvests of the parental primary cell cultures prior to their senescence (−TERT) and from the primary cells expressing TERT over ∼50 PDL of continuous growth (+TERT). Digested DNA was analyzed by in-gel hybridization with a telomeric-repeat probe. The migration of standards is indicated in kilobases.

Previous Northern blot hybridization data indicated that the DC patient primary cells expressing ΔL37 dyskerin had less TER than the control primary cells (Mitchell et al. 1999). We measured relative TER copy number in total RNA from different cells using quantitative RT–PCR (QRT–PCR), with the U64 H/ACA-motif snoRNA as the internal normalization control (Wong et al. 2004). QRT–PCR confirmed an approximately fivefold difference in TER accumulation between the DC patient and control cells (Fig. 1A; cf. lanes 12–14 or 13–15 and 16–18). These results were highly reproducible in independent controlled assays with different extracts (see Materials and Methods; data not shown).

We next investigated the impact of TERT expression on telomere length. As described above, control and DC pa-
tient primary cell cultures without TERT became senescent. In the brief interval of culture growth before senescence, telomeric restriction fragment lengths in both cultures had a broad distribution (Fig. 1B, C, left). In control cells expressing TERT, within the first ~50 post-selection population doublings (PDL) of continuous culture (Fig. 1B) and beyond (data not shown), telomere length increased and remained long in a stable manner. In DC patient cells expressing TERT, within the first ~50 post-selection PDL of continuous culture (Fig. 1C) and beyond (data not shown), telomeres instead continued to shorten. The average telomere length in DC patient cells expressing TERT became less than the average telomere length in the parental primary cell culture at senescence (Fig. 1C; cf. lanes 15 and 2). This observation adds to previous evidence that a level of telomerase activation insufficient for telomere length maintenance can nonetheless dramatically extend proliferative capacity [Zhu et al. 1999; Ouellette et al. 2000].

The series of results described above was reproduced using several independent retroviral infections of DC patient and control cell cultures. Among these repetitions, we compared in parallel the effect of expression of untagged TERT versus TERT tagged with a Flag epitope at its N terminus. Previous studies suggest that TERT N-terminal fusion proteins retain close to normal physiological function, while TERT C-terminal fusion proteins generate catalytically active enzymes that lack function on telomere substrates [Counter et al. 1998]. Control cell cultures selected for integration of retrovirus expressing either untagged TERT or N-terminally tagged TERT gained comparable levels of telomerase activity in extract, and both maintained long telomeres (Supplementary Figs. S1, S2A). DC patient cell cultures selected for integration of the retroviruses expressing untagged TERT and N-terminally tagged TERT gained comparable lengths of telomerase activity in extract, and both maintained long telomeres (Supplementary Figs. S1, S2A). DC patient cell cultures expressing either form of TERT gained proliferative capacity yet failed to accomplish telomere length maintenance (Supplementary Fig. S2B). This comparison demonstrates that a fully functional transgene-encoded TERT can be marked at its N terminus to discriminate recombinant from endogenous protein.

Extra TER expression rescues the DC telomere maintenance defect

We next addressed whether the inability of DC patient cells to maintain telomere length derives from the reduced steady-state level of TER alone. In previous transient transfection studies, optimal recombinant TER accumulation was obtained using the strong U3 snoRNA promoter to express a precursor transcript containing mature TER and ~500 base pairs of downstream genomic sequence [Fu and Collins 2003]. Even with an optimized expression context, mature TER accumulates to a copy number per cell that is several orders of magnitude lower than other small nuclear and small nucleolar RNPs, likely due to inefficient 3′ end processing of the TER precursor. The TER expression cassette was cloned into one of the long terminal repeats of the retroviral vector pBABE to create pBABE-U3-TER (Fig. 2A). At this location within the vector, the recombinant TER expression cassette becomes duplicated in the process of integration [De Angelis et al. 2002].

Bulk cultures of DC patient and control cells expressing TERT were selected for integration of the retroviral vector expressing TER or empty vector in parallel. TER accumulation levels were examined in the post-selection cultures by Northern blot hybridization and QRT–PCR. TER blot hybridization signal was barely detectable in DC patient cells selected for integration of empty vector, but it increased substantially in cells selected for integration of the vector expressing TER (Fig. 2B). The additional TER signal comigrates with mature TER, which typically resolves as a doublet due to partial folding during electrophoresis. QRT–PCR assays determined that recombinant TER expression in the DC patient cells led to an ~13-fold increase in TER accumulation, bringing the overall amount of TER in DC patient cells expressing both TERT and recombinant TER (TERT + TER) to 2.8-fold more than that in the control cells expressing TERT alone (Fig. 2C). Recombinant TER expression also increased the level of telomerase activity in DC patient cells to maintain telomere length.

**Figure 2.** Mature, functional TER can be produced from a retroviral expression vector. (A) Schematic showing the pBABE-U3-TER expression construct. An SV40 promoter (pSV40) drives expression of the selectable marker. The U3-TER cassette was cloned into the 3′ long terminal repeat (LTR) and becomes duplicated during retroviral integration. (B) Northern blot showing the accumulation of mature TER. Each lane contains nuclear RNA isolated from 3 × 10^6 patient cells expressing TERT and either TER expression vector or empty vector. The TER doublet derives from differential folding of mature RNA. LC denotes an internal control for RNA loading. (C) Whole-cell extracts were assayed for telomerase activity by TRAP. Extracts were prepared from DC patient or control cells expressing TERT, either with or without additional TER. Extracts were assayed using a series of 2.5-fold steps in amount of total protein [2–0.05 µg].
cell extracts by almost 15-fold [Fig. 2C; cf. lanes 1,2 and 9,10].

If the DC defect in telomere length maintenance derives exclusively from the reduced accumulation of TER, then DC patient cells expressing TERT + TER should have telomeres that are as long as or longer than DC patient cells expressing TERT. Indeed, DC patient cells expressing TERT + TER rapidly gained and then maintained long telomeres [Fig. 3A, left] in striking contrast with DC patient cells expressing TERT and empty TER vector [Fig. 3A, right]. Telomere length in DC patient cells expressing TERT + TER increased relative to telomere length in DC patient cells expressing TERT and even appeared to exceed telomere length in control cells expressing TERT. Telomere length compared among these cell lines varies in correlation with TER copy number [Fig. 2C], which increases from ∼0.22 (DC patient cells expressing TERT) to ∼1.0 (control cells expressing TERT) to ∼2.8 (DC patient cells expressing TERT + TER). This correlation across DC patient and control cells suggests that the assembly of a DC isoform of dyskerin into telomerase RNP does not greatly alter the specific activity of telomerase on its chromosome substrates. Although recombinant TER expression increased telomere length in DC patient cells, it did not alter their growth. DC patient cells expressing TERT + TER maintained a rate of population doubling closely similar to DC patient cells expressing TERT alone [Fig. 3B].

Control cells expressing TERT were also selected for integration of the retroviral vector expressing TER or empty vector in parallel. In several trials, control cell cultures given the TER expression vector recovered poorly from selection, while control cell cultures given the empty vector recovered normally [data not shown]. We expected control cells expressing TERT + TER to accumulate even more TER than the DC patient cells expressing TERT + TER. However, following outgrowth from selection, control cells with integrated TER expression vector showed little change in TER accumulation or in the level of telomerase activity in cell extract [Fig. 2C; cf. lanes 11–15 and 16–20]. These cells appeared to lose rather than gain telomere length, and the culture showed reduced growth compared with the control cells with integrated empty vector [Fig. 3C,D]. Most of the control cell culture with integrated TER expression vector lost viability if returned to the initial selection conditions at post-selection PDL25, while most of the control cell culture with integrated empty vector did not [data not shown]. These results suggest that above some level of TER overexpression, excess TER becomes deleterious for cell growth. This would promote preferential outgrowth of cells that have transcriptionally silenced the integrated expression vector, accounting for loss of expression of both the additional TER and the drug resistance marker.

**Telomere maintenance can be rescued in DC patient cells using a single retrovirus**

In the experiments described above, DC patient cells were selected for integration of the TERT retrovirus and then selected again for integration of the TER retrovirus. To evaluate the potential for telomere length rescue in DC patient cells using a single retrovirus, we designed the dual expression vector pBABE-TERT/TER [Fig. 4A]. Starting from the vector for TER expression, we added the TERT ORF in replacement of the ORF of the selectable marker cassette. We confirmed that functional TERT and TER were expressed from this vector by transient transfection of human VA13 cells. VA13 cells lack endogenous TERT and TER [Bryan et al. 1997], but when transfected with pBABE-TERT/TER they gained robust telomerase activity in cell extract [data not shown].

The DC patient primary cell culture was infected with dual expression retrovirus. The infected cell culture continued to proliferate past the senescence point of untreated primary cells passaged in parallel, which we designated as the end of selection. QRT–PCR revealed an ∼25-fold increase in TER accumulation in DC patient cells with pBABE-TERT/TER compared with DC patient cells with TERT expression vector and TER empty vector control [Fig. 4B]. A corresponding increase occurred in telomerase activity in cell extract [Fig. 4B; cf. lanes 2,3 and 9,10; note the fivefold steps of extract dilution]. The DC patient primary cell cultures infected with pBABE-TERT/TER gained substantial telomere length while cells expressing TERT alone did not [Fig. 4C]. These results demonstrate that expression of TERT and TER from a single retrovirus can induce telomere elongation and promote telomere length maintenance in DC patient cells.
Rescue of telomere length in DC patient cells expressing a different dyskerin isoform

To examine the generality of our findings, we used a primary cell culture established from an X-linked DC patient unrelated to the ΔL37 dyskerin family (see Materials and Methods). Sequencing of the expressed dyskerin cDNA revealed a threonine substitution for alanine at residue 386 (A386T) (data not shown). Using QRT–PCR, we determined that DC patient cells with A386T dyskerin have ∼20% the level of TER present in cells with wild-type dyskerin, comparable with the TER level determined for DC patient cells with ΔL37 dyskerin. The DC patient primary cells with A386T dyskerin were infected with retrovirus expressing TERT alone or with the dual-expression retrovirus. Compared with cells expressing TERT alone, the cells expressing TERT + TER had ∼13-fold more TER and correspondingly more telomerase activity in extract (Fig. 5A; cf. lanes 2, 3 and 9, 10). DC patient cells expressing TERT + TER gained and maintained long telomeres, while DC patient cells expressing TERT alone did not (Fig. 5B). These results obtained for DC patient cells with A386T dyskerin closely parallel the results obtained for DC patient cells with ΔL37 dyskerin described above.

DC patient cells do not have anticipated defects in ribosome biogenesis

The cell lines generated in the studies above provide a new opportunity to rigorously assay putative DC-linked defects in ribosome biogenesis. Because ribosome biogenesis is regulated by many factors including cell proliferation (Rudra and Warner 2004), comparisons made between patient and control primary cell populations of mixed proliferative status are subject to many caveats. The TERT-expressing DC patient and control cell lines generated above can be maintained in continuous growth without senescence for highly parallel, repeated assays of ribosome biogenesis. We used standard methods for determining mature rRNA pseudouridine content and monitoring the kinetics of rRNA precursor processing [see Materials and Methods] that were also used previously in studies of the proposed mouse model (Ruggiero et al. 2003).

To quantify the ratio of pseudouridine to uridine in mature 18S and 28S rRNAs, total RNA was prepared from cells radiolabeled with 32P-orthophosphate. We compared control cells expressing TERT, DC patient cells with ΔL37 dyskerin expressing TERT, DC patient cells with ΔL37 dyskerin expressing TERT + TER, and DC patient cells with A386T dyskerin expressing TERT. The 18S rRNA has a slightly higher ratio of pseudouridine to uridine than the 28S rRNA (Ofengand et al. 2001), so each mature RNA species was analyzed separately. Mature 18S and 28S rRNAs were resolved by denaturing gel electrophoresis, gel-purified, and hydrolyzed Figure 5A. Whole-cell extracts were assayed for telomerase activity by TRAP. Extracts were prepared from DC patient cells expressing TERT with or without additional TER. Extracts were assayed using a series of fivefold steps in amount of total protein (0.008 µg). (C) Genomic DNA was isolated from DC patient cells expressing TERT with or without additional TER. Digested DNA from cells at the indicated PDL of continuous culture was analyzed by in-gel hybridization with a telomeric-repeat probe. The migration of standards is indicated in kilobases.

Figure 4. Rescue of telomere length can be obtained using a single retrovirus. (A) Schematic of the dual-expression vector, pBABE-TERT/TER, for expression of both TERT and TER. (B) Whole-cell extracts were assayed for telomerase activity by TRAP. Extracts were prepared from DC patient cells expressing TERT with or without additional TER. Extracts were assayed using a series of fivefold steps in amount of total protein (5–0.008 µg). (C) Genomic DNA was isolated from DC patient cells expressing TERT with or without additional TER. Digested DNA from cells at the indicated PDL of continuous culture was analyzed by in-gel hybridization with a telomeric-repeat probe. The migration of standards is indicated in kilobases.
to mononucleotides, which were then separated by two-dimensional thin-layer chromatography [Fig. 6A]. The intensity ratio of pseudouridine to uridine was determined internally within each sample. No change in the pseudouridine content of 18S or 28S rRNA was detected in any of the cell lines, in any of several independent trials [Fig. 6B].

Rates of rRNA precursor processing were monitored by pulse-chase assay using 3H-radiolabeled methionine (see Materials and Methods). We compared control cells, DC patient cells with ΔL37 dyskerin and DC patient cells with A386T dyskerin all expressing TERT. Total RNA was isolated from plates of each cell culture at 15-min intervals post-chase, and later resolved by denaturing gel electrophoresis (Fig. 7). To normalize for variable 3H-radiolabeled RNA recovery from each separate plate of cells, the kinetics of processing must be evaluated as the ratio in signal intensity of different rRNA species within a lane over time. In all three cell cultures, the ratio in signal intensity of 45S rRNA precursor and 18S mature rRNA decreased to <1:1 between 15 min and 30 min of chase [Fig. 7, cf. lanes 2,3 and 7,8 and 12,13]. In all three cell cultures, the mature 28S rRNA accumulated later, following its processing from the 32S intermediate. The quantified ratio in signal intensity of 28S rRNA and 18S rRNA increased to slightly <1:1 at 1 h in each sample [Fig. 7, cf. lanes 5 and 10 and 15]. Across several independent trials, we found no consistent trend in the slight variability of rRNA processing kinetics between samples [data not shown]. Together, our rRNA processing and modification studies show that X-linked DC patient cells from two families do not display the severe ribosome biogenesis defects that were anticipated from studies of a mouse model.

Discussion

TER accumulation can limit telomere maintenance
The heterogeneous growth that accompanies premature senescence of X-linked DC patient primary cells has been a caveat to previous conclusions about defects in ribosome or telomerase biogenesis, because the ribosome and telomerase both have growth-linked regulation. The starting point for the studies described here was to overcome this limitation, which we did by generating patient cell lines with robust long-term growth. Most human somatic cells produce inactive telomerase RNP, with TERT limiting for telomerase catalytic activation and telomere maintenance [Bodnar et al. 1998]. TERT expression in control cells and DC patient cells was sufficient to induce telomerase activation and to prevent the premature senescence of DC patient cells. Due to the limited number of telomeres in each human cell, in theory, even a few molecules of active telomerase could be sufficient to accomplish stable telomere length maintenance. However, our findings above establish that a fewfold reduction in TER can severely limit stable maintenance of human somatic cell telomeres.

X-linked DC is more severe than AD DC in its earlier onset and broader spectrum of affected tissues, with the notable exception of the increasing severity of AD DC disease with anticipation [Vulliamy et al. 2004]. This might be explained by the more severe TER deficiency in X-linked versus AD DC patient cells [Collins and Mitchell 2002; Wong and Collins 2003]. In the minority of human somatic cells that produce TERT in excess of TER, any extent of TER reduction would limit telomerase activation. Lymphocyte subpopulations are candidate cell types for this particularly high level of TERT expression, because they can activate enough telomerase to gain rather than lose telomere length with proliferation [Weng 2002; Hathcock et al. 2005]. In cell types that produce less TERT than TER, TER deficiency may not limit telomerase activation unless the level of TER drops below that of TERT. In these cell types, TER accumulation could become limiting in X-linked but not AD DC. Finally, in somatic cells with very low levels of TERT, even the most severe extent of TER deficiency observed in X-linked DC patient cells would not limit telomerase activation.

Figure 6. X-linked DC patient cells do not have altered rRNA pseudouridine content. (A) Radiolabeled 18S rRNA (shown) and 28S rRNA (not shown) were purified and hydrolyzed to mononucleotides. Mononucleotides were resolved by thin-layer chromatography, and their relative amounts were quantified using PhosphorImager analysis. Note that pseudouridine monophosphate (Ψp) is present at ~10% the level of uridine monophosphate (Up). (B) Values were averaged across repeated trials of the experiment described in A. Each calculation used ratios determined in the four to seven independent experiments that generated well-resolved spots of pseudouridine and uridine. Values for 18S rRNA from left to right were WT + TERT = 0.101 + 0.020, ΔL37DC + TERT = 0.098 + 0.016, ΔL37DC + TERT + TER = 0.096 + 0.016, and A386TDC + TERT = 0.092 + 0.018. Values for 28S rRNA from left to right were WT + TERT = 0.085 + 0.010, ΔL37DC + TERT = 0.077 + 0.017, ΔL37DC + TERT + TER = 0.089 + 0.020, and A386TDC + TERT = 0.093 + 0.022.
Mouse and human cells may differ in dyskerin requirements for telomerase function

Mouse and human TERs have surprisingly high divergence in primary sequence, secondary structure, and function [Chen et al. 2000; Chen and Greider 2004]. Human TER can effectively reconstitute active telomerase with mouse TERT, but mouse TER does not effectively reconstitute active telomerase with human TERT [Beattie et al. 1998; Martin-Rivera et al. 1998]. Mouse and human telomerase enzymes show strikingly different profiles of product synthesis in vitro, with an increased repeat addition processivity of the human enzyme that appears to be required for maintenance of telomeres in vivo (Morin 1989; Prowse et al. 1993; Pascolo et al. 2002). These findings indicate that TER interactions with other telomerase RNP proteins are not directly comparable in mouse and human cells, and so a particular dyskerin sequence change may have differential impact on mouse versus human TER. Curiously, two lines of mouse embryonic stem cells expressing different dyskerin alleles show distinct changes in the accumulation of H/ACA-motif RNAs, neither of which matches the more TER-specific defect of the human X-linked DC patient cells tested to date [Mochizuki et al. 2004]. In future studies it will be important to examine mouse and human cells expressing the same dyskerin variants to directly compare dyskerin sequence requirements for the biogenesis and stability of H/ACA-motif RNPs.

If a DC dyskerin variant can be found that alters the accumulation of mouse and human H/ACA-motif RNAs in a similar manner, it will be equally important to consider differences between mouse and human somatic tissues in telomerase regulation, telomere length homeostasis, and demand for somatic renewal [Forsyth et al. 2002]. Heterozygous inactivation of mouse TER does not limit telomere length maintenance in cultured embryonic stem cells [Niida et al. 1998], contrary to the expectation from AD DC. However, TER haploinsufficiency for telomere length maintenance has been detected in some contexts; for example, following germline transmission for several generations [Hathcock et al. 2005]. The similarities and differences in phenotypes of X-linked DC and late-generation telomerase-deficient mice [Marciniak et al. 2000] suggest that it may be possible although challenging to develop a faithful mouse model of DC.

Telomerase activation has potential utility for disease therapy

The adaptive immune system plays a vital role in protecting human health and longevity. Inherited disease, infections, environmental insults and aging can exhaust its proliferative capacity [Wong and Collins 2003]. For effective clinical therapy of X-linked DC, transplanted hematopoietic cells must be able to restore cell counts and then maintain them for the duration of an adult human lifespan. Clinical success in this endeavor has not been obtained [Bessler et al. 2004]. Telomere loss occurs throughout life in all human hematopoietic cell types examined, with the most pronounced rates of loss in childhood or following bone marrow transplantation [Greenwood and Lansdorp 2003]. It may be possible to enhance the proliferative capacity of transplanted cells by substantially increasing their telomere length, which for X-linked DC patient cells will require exogenously induced expression of both TERT and TER. This potential therapeutic use of telomerase activation must be balanced against the potential increase in risk of cancer.

In early studies we examined the impact of expressing wild-type dyskerin in X-linked DC patient cells. DC patient primary cell cultures selected for integration of any expression vector lacking TERT ceased to proliferate even before selection was complete. Therefore, without TERT, telomere length cannot be rescued by expression of recombinant TER or dyskerin alone. This outcome is expected based on the extremely short telomeres of the available DC patient primary cells. In DC patient primary cells expressing exogenous TERT, we found that the introduction of wild-type dyskerin did not rescue TER accumulation or telomerase activity (J.M.Y. Wong and K. Collins, unpubl.). However, the wild-type dyskerin may not predominate in accumulation over the endogenous dyskerin isoform. It is plausible to expect that wild-type dyskerin overexpression will rescue TER accumulation in DC patient cells, but this increase in dyskerin level has potential to be broadly disruptive for the biogenesis and function of H/ACA-motif RNPs.

Our studies suggest that rRNA pseudouridine modification and processing defects are not a universal feature of X-linked DC. However, some DC-linked mutations not characterized here or some conditions of cell growth could alter the ability of a DC dyskerin variant to modify rRNA. Importantly, altered dyskerin expression could influence cellular processes beyond ribosome biogenesis or telomere maintenance, perhaps including translation.
Materials and methods

Cell lines

Dermal fibroblast cultures GM01774, GM01787, and AG04645 were obtained from Coriell Cell Repository. Cells were grown in DMEM with 10% fetal calf serum and antibiotics. Retroviral infection was conducted by standard methods (Wong et al. 2002). Chemical selections began 48 h after infection using 50 μg/mL hygromycin for a TERT expression vector or 5 μg/mL puromycin for a TER expression vector. Surviving cells were pooled and grown in bulk culture, with the PDL count initiated at the first split of post-selection culture. Construction of retroviral expression vectors for TERT alone has been described (Wong et al. 2002). We used the Flag-tagged TERT expression vector unless indicated otherwise. Recombinant TERT accumulated at a low level that was undetectable by immunoblot of cell extract using antibodies against TERT or the epitope tag. For construction of the retroviral TER expression vector, the U3-TER-500 cassette (Fu and Collins 2003) was cloned into the Nhel site of pBABEpuro to generate pBABE-U3-TER. The dual-expression vector pBABE-TERT/TER was constructed by cloning untagged TERT into the HindIII and Clal sites of pBABE-U3-TER in exchange for the puromycin resistance ORF, which placed TERT expression under control of the SV40 promoter.

Assays of dyskerin, TER, telomerase activity, and telomere length

RNA samples were prepared using TRizol. Protocols for dyskerin genotyping and QRT–PCR have been described previously (Wong et al. 2004). Northern blot hybridization for TER and the loading control were performed as described (Fu and Collins 2003), using end-labeled oligonucleotide probes. To increase the sensitivity of Northern blot detection of TER, we isolated nuclei prior to RNA extraction [Jordan et al. 1996]. Telomerase activity assays used whole-cell extracts made by freeze–thaw lysis and TRAP conditions described previously [Mitchell and Collins 2000], including the competitive internal amplification control longer than the longest authentic TRAP products [data not shown]. Telomere length was assayed using genomic DNA digested with HindIII and RsaI. Following electrophoresis, in-gel hybridization was performed using the end-labeled oligonucleotide [T2AG3]3.

Determination of pseudouridine content

Cells were seeded at 1.5 × 106 cells per 15-cm plate and allowed to recover overnight. Cells were then incubated with methionine-free media (Gibco) for 60 min, pulse-labeled with 50 μCi/mL [methyl-3H]methionine (Perkin Elmer) for 35 min and chased in DMEM supplemented with 50 μg/mL unlabeled methionine (Sigma). Cells were harvested every 15 min following the removal of the radiolabel. Total RNA was isolated with TRizol, resuspended, and heat-denatured before separation on a 1% MOPS-formaldehyde agarose gel. Following gel electrophoresis, samples were transferred onto nylon membrane by capillary action overnight. Dried membrane was treated with ENHANCE Spray (Perkin Elmer) and exposed to X-ray film at −80°C for at least 7 d. Scanned images were quantified using ImageJ.

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References

Autexier, C. and Lue, N.F. 2006. The structure and function of telomerase reverse transcriptase. Annu. Rev. Biochem. 75: 493–517.

Beattie, T.L., Zhou, W., Robinson, M.O., and Harrington, L. 1998. Reconstitution of human telomerase activity in vitro. Curr. Biol. 8: 177–180.

Bessler, M., Wilson, D.B., and Mason, P.J. 2004. Dyskeratosis congenita and telomerase. Curr. Opin. Pediat. 16: 23–28.

Bodnar, A.G., Ouellette, M., Frolkis, M., Holt, S.E., Chiu, C., Morin, G.B., Harley, C.B., Shay, J.W., Lichtsteiner, S., and Wright, W.E. 1998. Extension of life-span by introduction of telomerase into normal human cells. Science 279: 349–352.

Bryan, T., Marusic, L., Bacchetti, S., Namba, M., and Reddel, R. 1997. The telomere lengthening mechanism in telomerase-negative immortal human cells does not involve the telomerase RNA subunit. Hum. Mol. Genet. 6: 921–926.

Chan, S.R. and Blackburn, E.H. 2004. Telomeres and telomerase. Philos. Trans. R. Soc. Lond. B Biol. Sci. 359: 109–121.

Chen, J. 2005. Senescence of hematopoietic stem cells and bone marrow failure. Int. J. Hematol. 82: 190–195.

Chen, J.L. and Greider, C.W. 2004. Telomerase RNA structure and function: Implications for dyskeratosis congenita. Trends Biochem. Sci. 29: 183–192.

Chen, J.L., Blasco, M.A., and Greider, C.W. 2000. Secondary structure of vertebrate telomerase RNA. Cell 100: 503–514.

Collins, K. and Mitchell, J.R. 2002. Telomerase in the human organism. Oncogene 21: 564–579.

Counter, C.M., Hahn, W.C., Wei, W., Cadle, S.D., Beijersbergen, R.L., Lansdorp, P.M., Sedivy, J.M., and Weinberg, R.A. 1998. Dissociation among in vitro telomerase activity, telo-

chromatography plates (EM Scientific). Two-dimensional analysis of each sample was performed with isobutyric acid/ammonium hydroxide/water (57:38:35 by volume) in the first dimension and isopropanol/hydrochloric acid/water (70:15:15 by volume) in the second dimension. Dried plates were exposed to storage phosphor screens and analyzed using the Typhoon system (GE Heathcare).

Kinetic studies of rRNA processing

Cells were seeded at 5 × 105 cells per 10-cm plate and allowed to recover overnight. Cells were then incubated with methionine-free media (Gibco) for 60 min, pulse-labeled with 50 μCi/mL [methyl-3H]methionine (Perkin Elmer) for 35 min and chased in DMEM supplemented with 50 μg/mL unlabeled methionine (Sigma). Cells were harvested every 15 min following the removal of the radiolabel. Total RNA was isolated with TRizol, resuspended, and heat-denatured before separation on a 1% MOPS-formaldehyde agarose gel. Following gel electrophoresis, samples were transferred onto nylon membrane by capillary action overnight. Dried membrane was treated with ENHANCE Spray (Perkin Elmer) and exposed to X-ray film at −80°C for at least 7 d. Scanned images were quantified using ImageJ.
Dokal, I. 2000. Dyskeratosis congenita in all its forms. Br. J. Haematol. 110: 768–779.

Dragon, F., Pogacic, V., and Filipowicz, W. 2000. In vitro assembly of human H/ACA small nucleolar RNP reveal unique features of U17 and telomerase RNAs. Mol. Cell. Biol. 20: 3037–3048.

Federman, N. and Sakamoto, K.M. 2005. The genetic basis of bone marrow failure syndromes in children. Mol. Genet. Metab. 86: 100–109.

Ferre-D’Amare, A.R. 2003. RNA-modifying enzymes. Curr. Opin. Struct. Biol. 13: 49–55.

Forsyth, N.R., Wright, W.E., and Shay, J.W. 2002. Telomerase and differentiation in multicellular organisms: Turn it off, turn it on, and turn it off again. Differentiation 69: 188–197.

Fu, D. and Collins, K. 2003. Distinct biogenesis pathways for human telomerase RNA and H/ACA small nucleolar RNAs. Mol. Cell 11: 1361–1372.

Greenwood, M.J. and Lansdorp, P.M. 2003. Telomerases, telomerase, and hematopoietic stem cell biology. Arch. Med. Res. 34: 489–495.

Hathcock, K.S., Chiang, Y.J., and Hodes, R.J. 2005. In vivo regulation of telomerase activity and telomere length. Immunol. Rev. 205: 104–113.

Hauguel, T. and Bunz, F. 2003. Haploinsufficiency of hTERT leads to telomere dysfunction and radiosensitivity in human cancer cells. Cancer Biol. Ther. 2: 679–684.

Heiss, N.S., Knight, S.W., Vulliamy, T.J., Krauss, S.M., Wiemann, S., Mason, P.J., Pousta, K., and Dokal, I. 1998. X-linked dyskeratosis congenita is caused by mutations in a highly conserved gene with putative nucleolar functions. Nat. Genet. 19: 32–38.

Henras, A.K., Dec, C., and Henry, Y. 2004. RNA structure and function in C/D and H/ACA snoRNPs. Curr. Opin. Struct. Biol. 14: 335–343.

Jordan, P., Mannervik, M., Tora, L., and Carmo-Fonseca, M. 1996. In vivo evidence that TATA-binding protein/S1 co-localizes with UBF and RNA polymerase I when rRNA synthesis is either active or inactive. J. Cell Biol. 133: 225–234.

Kennedy, R.D. and D’Andrea, A.D. 2005. The Fanconi anemia/BRCAn pathway: New faces in the crowd. Genes & Dev. 19: 2925–2940.

Kim, N.W., Piatszczk, M.A., Prowse, K.R., Harley, C.B., West, M.D., Ho, P.L.C., Coviello, G.M., Wright, W.E., Weinrich, S.L., and Shay, J.W. 1994. Specific association of human telomerase activity with immortalized cells and cancer. Science 266: 2011–2015.

Kiss, A.M., Jaé, B.E., Bertrand, E., and Kiss, T. 2004. Human box H/ACA pseudouridylation guide RNA machinery. Mol. Cell. Biol. 24: 5797–5807.

MacMillan, M.L. and Wagner, J.E. 2005. Hematopoietic cell transplantation for congenital bone marrow failure. Curr. Opin. Oncol. 17: 106–113.

Marciniak, R.A., Johnson, F.B., and Guarente, L. 2000. Dyskeratosis congenita, telomeres and human aging. Trends Genet. 16: 193–195.

Martín-Rivera, L., Herrera, H., Albar, J.P., and Blasco, M.A. 1998. Expression of mouse telomerase catalytic subunit in embryos and adult tissues. Proc. Natl. Acad. Sci. 95: 10471–10476.

Mason, P.J., Wilson, D.B., and Bessler, M. 2005. Dyskeratosis congenita—A disease of dysfunctional telomere maintenance. Curr. Mol. Med. 5: 159–170.

Meier, U.T. 2005. The many facets of H/ACA ribonucleoproteins. Chromosoma 114: 1–14.

Mitchell, J.R. and Collins, K. 2000. Human telomerase activation requires two independent interactions between telomerase RNA and telomerase reverse transcriptase in vivo and in vitro. Mol. Cell 6: 361–371.

Mitchell, J.R., Wood, E., and Collins, K. 1999. A telomerase component is defective in the human disease dyskeratosis congenita. Nature 402: 551–555.

Mochizuki, Y., He, J., Kulkarni, S., Bessler, M., and Mason, P.J. 2004. Mouse dyskerin mutations affect accumulation of telomerase RNA and small nucleolar RNA, telomerase activity, and ribosomal RNA processing. Proc. Natl. Acad. Sci. 101: 10756–10761.

Morin, C.B. 1989. The human telomerase terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. Cell 59: 521–529.

Niedernhofer, L.J., Lalai, A.S., and Hoceima, J.H. 2005. Fanconi anemia (cross)linked to DNA repair. Cell 123: 1191–1198.

Niida, H., Matsumoto, T., Satoh, H., Shiwa, M., Tokutake, Y., Furuichi, Y., and Shinkai, Y. 1998. Severe growth defect in mouse cells lacking the telomerase RNA component. Nat. Genet. 19: 203–206.

Ofengand, J., Malhotra, A., Remme, J., Gutgsell, N.S., Del Campo, M., Jean-Charles, S., Peil, L., and Kaya, Y. 2001. Pseudouridinases and pseudouridine synthases of the ribosome. Cold Spring Harb. Symp. Quant. Biol. 66: 147–159.

Ouettel, M.M., Liao, M., Herbert, B., Johnson, M., Holt, S.E., Liss, H.S., Shay, J.W., and Wright, W.E. 2000. Subsclerotic telomere lengths in fibroblasts immortalized by limiting amounts of telomerase. J. Biol. Chem. 275: 10072–10076.

Pascolo, E., Wenz, C., Liniger, J., Hauel, N., Priepke, H., Kaufmann, I., Garin-Chesa, P., Rettig, W.J., Damm, K., and Schnapp, A. 2002. Mechanism of human telomerase inhibition by BIBR1532, a synthetic, non-nucleosidic drug candidate. J. Biol. Chem. 277: 15566–15572.

Pogacic, V., Dragon, F., and Filipowicz, W. 2000. Human H/ACA small nucleolar RNPs and telomerase share evolutionarily conserved proteins NHP2 and NOP10. Mol. Cell Biol. 20: 9028–9040.

Prowse, K.R., Avilion, A.A., and Greider, C.W. 1993. Identification of a nonprocessive telomerase activity from mouse cells. Proc. Natl. Acad. Sci. 90: 1493–1497.

Rudra, D. and Warner, J.R. 2004. What better measure than ribosome synthesis? Genes & Dev. 18: 2431–2436.

Ruggiero, D., Grisendi, S., Piazza, F., Rego, E., Mari, F., Rao, P.H., Cordon-Cardo, C., and Pandolfi, P.P. 2003. Dyskeratosis congenita and cancer in mice deficient in ribosomal RNA modification. Science 299: 259–262.

Steinert, S., Shay, J.W., and Wright, W.E. 2000. Transient expression of human telomerase extends the life span of normal human fibroblasts. Biochem. Biophys. Res. Commun. 273: 1095–1098.

Steward, C.G. and Jarisch, A. 2005. Haemopoietic stem cell transplantation for genetic disorders. Arch. Dis. Child. 90: 1259–1263.

Vulliamy, T. and Bessler, M., Mason, P.J., and Dokal, I. 2001a. The RNA component of human telomerase RNA and telomerase reverse transcriptase in vivo and in vitro. Mol. Cell 6: 361–371.

Vulliamy, T., Marrone, A., Goldman, F., Dearlove, A., Bessler, M., Mason, P.J., and Dokal, I. 2001a. The RNA component of telomerase is mutated in autosomal dominant dyskeratosis congenita. Nature 413: 432–435.
Vulliamy, T.J., Knight, S.W., Mason, P.J., and Dokal, I. 2001b. Very short telomeres in the peripheral blood of patients with X-linked and autosomal dyskeratosis congenita. Blood Cells Mol. Dis. 2001: 353–357.
Vulliamy, T., Marrone, A., Szydlo, R., Walne, A., Mason, P.J., and Dokal, I. 2004. Disease anticipation is associated with progressive telomere shortening in families with dyskeratosis congenita due to mutations in TERC. Nat. Genet. 36: 447–449.
Vulliamy, T.J., Marrone, A., Knight, S.W., Walne, A., Mason, P.J., and Dokal, I. 2006. Mutations in dyskeratosis congenita: Their impact on telomere length and the diversity of clinical presentation. Blood 107: 2660–2665.
Walne, A.J., Marrone, A., and Dokal, I. 2005. Dyskeratosis congenita: A disorder of defective telomere maintenance? Int. J. Hematol. 82: 184–189.

Weng, N.-P. 2002. Regulation of telomerase expression in human lymphocytes. Springer Semin. Immunopathol. 24: 23–33.
Wong, J.M.Y. and Collins, K. 2003. Telomere maintenance and disease. Lancet 362: 983–988.
Wong, J.M., Kusdra, L., and Collins, K. 2002. Subnuclear shuttling of human telomerase induced by transformation and DNA damage. Nat. Cell Biol. 4: 731–736.
Wong, J.M., Kyasa, M.J., Hutchins, L., and Collins, K. 2004. Telomerase RNA deficiency in peripheral blood mononuclear cells in X-linked dyskeratosis congenita. Hum. Genet. 115: 448–455.

Yi, X., Tesmer, V.M., Savre-Train, I., Shay, J.W., and Wright, W.E. 1999. Both transcriptional and posttranscriptional mechanisms regulate human telomerase template RNA levels. Mol. Cell. Biol. 19: 3989–3997.
Yoon, A., Peng, G., Brandenburg, Y., Zollo, O., Xu, W., Rego, E., and Ruggero, D. 2006. Impaired control of IRES-mediated translation in X-linked dyskeratosis congenita. Science 312: 902–906.

Zhu, J., Wang, H., Bishop, J.M., and Blackburn, E.H. 1999. Telomerase extends the lifespan of virus-transformed human cells without net telomere lengthening. Proc. Natl. Acad. Sci. 96: 3723–3728.
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