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.

[**Keywords:** Telomerase RNA; telomere length; dyskeratosis congenita; dyskerin; ribosomal RNA; pseudouridine]

Supplemental material is available at <http://www.genesdev.org>.

Received July 31, 2006; revised version accepted August 28, 2006.

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 Cbf5p 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

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Article published online ahead of print. Article and publication date are at <http://www.genesdev.org/cgi/doi/10.1101/gad.1476206>.

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 germline, 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 embryo 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 $\Delta L37$ 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 extract 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 protein concentration (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).

Previous Northern blot hybridization data indicated that the DC patient primary cells expressing Δ L37 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 five-fold difference in TER accumulation between the DC patient and control cells (Fig. 1A, relative TER level determined by QRT-PCR is indicated). We found that TERT expression slightly increased the steady-state level of TER by up to approximately twofold, closely paralleling results from a previous study using other cell lines (Yi et al. 1999). DC patient cells expressing TERT possessed a level of TER $\sim$ 30% of the level of TER in

Figure 1. TERT expression activates telomerase in DC patient and control cell cultures. (A) Whole-cell extracts were assayed for telomerase activity by TRAP. Whole-cell extracts were prepared from control cells expressing wild-type dyskerin (WT) or DC patient cells expressing Δ L37 dyskerin, each with or without an integrated retrovirus expressing TERT. Extracts were assayed using a series of 2.5-fold steps in amount of total protein (5–0.13 μ g). (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 $\sim$ 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.

control cells expressing TERT. This difference in TER correlates with the difference in telomerase activity in cell extracts, suggesting that endogenously assembled telomerase enzymes harboring wild-type or Δ L37 dyskerin have a similar specific activity of product synthesis as assayed by TRAP in vitro. It remains possible that telomerase RNPs harboring wild-type or DC dyskerin differ in particular features of their catalytic activity in vitro that are not revealed by the TRAP assay.

TERT expression in DC patient cells does not confer telomere length maintenance

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 also had comparable telomerase activity in cell extract, which was less than the level of activity detected in the control cell extracts, as expected (Supplementary Fig. S1). The 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 termi-

nus 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

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 μ g).

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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 pBAGE-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 pBAGE-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 pBAGE-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 cells infected with pBAGE-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.

Figure 3. TER expression rescues the DC telomere maintenance defect. (A,C) Genomic DNA was isolated from DC patient or control cells expressing TERT, either 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. (B,D) Growth rates of the DC patient or control cell cultures expressing TERT with or without additional TER are compared by cumulative PDL with time.

Figure 4. Rescue of telomere length can be obtained using a single retrovirus. (A) Schematic of the dual-expression vector, pBAGE-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 μ g). (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.

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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 $\Delta L37$ 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 $\Delta L37$ 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 $\Delta L37$ 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 (Ruggero 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 32 P-orthophosphate. We compared control cells expressing TERT, DC patient cells with $\Delta L37$ dyskerin expressing TERT, DC patient cells with $\Delta L37$ 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 5. TERT and TER expression rescue telomere length in other X-linked DC patient cells. (A) Whole-cell extracts were assayed for telomerase activity by TRAP. Extracts were prepared from DC patient cells (with A386T dyskerin) expressing TERT with or without additional TER. Extracts were assayed using a series of fivefold steps of total protein (5–0.008 μ g). (B) Genomic DNA was isolated from DC patient cells (with A386T dyskerin) 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 (Vulliamey 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 $\sim 10\%$ 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 .

Figure 7. X-linked DC patient cells do not show altered kinetics of rRNA precursor processing. Pulse-chase analysis of rRNA precursor processing was performed by isolating total RNA from parallel plates of a cell culture starting at 0 min of chase and taking additional chase time points each 15 min. Total RNA was resolved by denaturing gel electrophoresis. The 45S precursor and 41S intermediate contain the 28S, 5.8S, and 18S rRNAs. The 32S processing intermediate contains the 28S and 5.8S rRNAs only. To normalize for differential recovery of RNA from each independently harvested plate of cells, the kinetics of processing must be evaluated as a change in ratio of the signal intensities in a lane over time.

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 expect-

tation 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

(Ruggero et al. 2003; Yoon et al. 2006). The stable X-linked DC patient cell lines generated in this work should be highly useful for additional studies of any putative X-linked DC impact on dyskerin function.

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 μ g/mL hygromycin for a TERT expression vector or 5 μ g/mL puromycin for a TERT 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 TERT expression vector, the U3-TERT-500 cassette (Fu and Collins 2003) was cloned into the NheI site of pBABEpuro to generate pBABE-U3-TERT. The dual-expression vector pBABE-TERT/TER was constructed by cloning untagged TERT into the HindIII and ClaI sites of pBABE-U3-TERT in exchange for the puromycin resistance ORF, which placed TERT expression under control of the SV40 promoter.

Assays of dyskerin, TERT, 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 TERT 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 TERT, 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 HinfI and RsaI. Following electrophoresis, in-gel hybridization was performed using the end-labeled oligonucleotide (T2AG3)3.

Determination of pseudouridine content

Cells were seeded at 1.5×10^6 cells per 15-cm plate and allowed to recover overnight. Cells were then incubated with phosphate-free media for 90 min before labeling with 100 μ Ci/mL 32 P-orthophosphate for 4 h. Following cell harvest, total RNA was isolated using TRIzol and separated on a 1% MOPS-formaldehyde agarose gel. The radiolabeled 28S and 18S mature rRNAs were recovered from excised gel slices by electroelution, and were ethanol precipitated, resuspended, and digested to completion with 0.5–1 U of RNase T2 (Sigma) in 50 mM ammonium acetate, 0.05% SDS, and 1 mM EDTA. The radioactivity in each digested RNA sample was determined by scintillation counting and aliquots equivalent to 50,000 counts per minute were spotted onto plastic-backed cellulose thin-layer

chromatography plates (EM Scientific). Two-dimensional analysis of each sample was performed with isobutyric acid/ammonium hydroxide/water (577:38:385 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 Healthcare).

Kinetic studies of rRNA processing

Cells were seeded at 5×10^5 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 μ Ci/mL [methyl- 3 H] methionine (Perkin Elmer) for 35 min and chased in DMEM supplemented with 50 μ g/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 EN 3 HANCE Spray (Perkin Elmer) and exposed to X-ray film at -80°C for at least 7 d. Scanned images were quantified using ImageJ.

Acknowledgments

We thank Dragony Fu for design and construction of pBABE-U3-TERT. This research was supported by funding from NIH grant HL079585 to K.C.

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

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Genes Dev. 2006, **20**: originally published online October 2, 2006
Access the most recent version at doi:[10.1101/gad.1476206](https://doi.org/10.1101/gad.1476206)

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