Short Telomeres in ESCs Lead to Unstable Differentiation

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SUMMARY

Functional telomeres are critical for stem cell proliferation; however, whether they are equally important for the stability of stem cell differentiation is not known. We found that mouse embryonic stem cells (ESCs) with critically short telomeres \((Tert^{-/-})\) initiated normal differentiation after leukemia inhibitory factor (LIF) withdrawal but, unlike control ESCs, failed to maintain stable differentiation when LIF was reintroduced to the growth medium. \(Tert^{-/-}\) ESCs expressed higher levels of Nanog and, overall, had decreased genomic CpG methylation levels, which included the promoters of Oct4 and Nanog. This unstable differentiation phenotype could be rescued by telomere elongation via reintroduction of \(Tert\), via suppression of Nanog by small hairpin RNA (shRNA) knockdown, or via enforced expression of the de novo DNA methyltransferase 3b. These results demonstrate an unexpected role of functional telomeres in the genome-wide epigenetic regulation of cell differentiation and suggest a potentially important role of telomere instability in cell fate during development or disease.

RESULTS

Critically Short Telomeres in ESCs Lead to Elevated Basal Levels of Nanog

We sought to address the impact of telomere dysfunction not only upon the capacity for cell differentiation but also upon the maintenance of a differentiated state. Late-passage \(Tert^{-/-}\) ESCs (\(Tert^{-/-}\)) (Liu et al., 2000) that possessed shorter telomeres and a significant accumulation of telomere signal-free ends relative to wild-type (WT) ESCs or \(Tert^{-/-}\) cells at earlier passages (\(Tert^{-/-}\)) (Figures S1A–S1C available online; \(p < 0.0001\); Fisher’s exact test) were nonetheless proliferation-competent and did not exhibit an altered doubling time, cell morphology, or cell-cycle distribution (Figures S1D and S1E; data not shown). However, Nanog messenger RNA (mRNA) and protein levels were significantly elevated (Figures 1A–1C and S1F, S1G). To test whether the difference in Nanog expression was related to telomere dysfunction, we reintroduced WT \(Tert\) into late-passage \(Tert^{-/-}\) ESCs (\(Tert^{-/-}\)), and, after the propagation of clonal lines expressing \(Tert\), we observed the reparation of telomere signal-free ends and a restoration of Nanog levels closer to the levels observed in WT ESCs and \(Tert^{-/-}\) ESCs at early passage (Figures 1A–1D, S1A–S1C, S1F, and S1G). Transient expression of \(Tert\) for 72 hr, a period of time insufficient to permit telomere extension, failed to restore Nanog to levels comparable to WT ESCs (data not shown). These data suggest that the dysregulation of Nanog in \(Tert^{-/-}\) ESCs is a consequence of critically short telomeres.

ESCs that express high levels of Nanog tend to self-renew, whereas cells that express low levels of this factor tend to differentiate (Chambers et al., 2007; Savarese et al., 2009; Singh et al., 2007). Immunofluorescence analysis of \(Tert^{-/-}\) ESCs cultured on gelatin in leukemia inhibitory factor (LIF)-containing media revealed a significant increase in the percentage of Nanog\(^{hi}\) cells in comparison to WT and \(Tert^{-/-}\) ESCs (Figures 1A and S1G) (Savarese et al., 2009). We confirmed elevated Nanog expression in \(Tert^{-/-}\) ESCs via fluorescence-activated cell sorting (FACS) analysis (Figure 1B). We also measured the...
expression of other factors involved in the pluripotency regulatory network (Rex1, Esrrb, and Tbx3) (Festuccia et al., 2012; Ivanova et al., 2006; Shi et al., 2006), including pluripotency factors that negatively regulate Nanog expression (Zfp281) (Fidalgo et al., 2011) and lineage differentiation markers (Cdx2) and the endoderm markers (Gata6 and Gata4) that are negatively regulated by Nanog (Singh et al., 2007). As anticipated, Rex1, Esrrb, and Tbx3 mRNA levels were increased in Tert−/− ESCs, whereas Zfp281 and Cdx2 levels were unaffected (Figure 1C).

However, Gata6 and Gata4 were also increased (Figure 1C). Consistent with these observations, chromatin immunoprecipitation (ChIP) analysis revealed lower levels of Nanog occupancy on the Gata6 promoter (Figure S1I). Nevertheless, the recruitment of Nanog to its own promoter, which represses its own expression (Fidalgo et al., 2011), increased in Tert−/− ESCs (Figure S1I). Thus, the increased expression of Nanog is not a consequence of the impaired occupancy of Nanog on its own promoter.

**Figure 1. Analysis of Pluripotency Factors in WT and Tert−/− ESCs**

(A) Quantification of Nanog levels normalized over DAPI (see Figure S1G for corresponding immunofluorescence images). Note a significant shift (p < 0.0001) from Nanog-low (DAPI to Nanog-488 ≥ 1.8) to Nanog-high (DAPI to Nanog-488 < 1.5) cells in Tert−/− in comparison to WT and Tert−/− ESCs (n ≥ 100 per cell population).

(B) FACS analysis of the Nanog expression profile in the same genotypes as in (A). Note the rightward shift and increase in average Nanog signal intensity in Tert−/− ESCs.

(C) Relative gene expression analyzed by qRT-PCR, normalized to GAPDH (n = 4). Data are represented as mean ± SD.

(D) (Top) Nanog protein expression with LI-COR quantification below (n = 3). Data are represented as mean ± SD; L, long telomeres (passage 30); S, short telomeres (passage 70); R, Tert rescue (70 passages, followed by clonal selection and an additional 4 passages after Tert reintroduction). The superscripts 1 and 2 indicate two independently generated Tert−/− colonies.

(E) ChIP analysis using an antibody to H3K27me3 and H3K4me3. Relative enrichment was quantified with the use of region-specific qPCR primers for Nanog, Oct4, and Gata6 promoters. Generic IgG was used as a control (n = 3). Data are represented as mean ± SD. *, p < 0.05; **, p < 0.01; ***, p < 0.0001.

See also Figure S1 and Table S1.
Perturbations in H3K27me3 Are Associated with Critically Short Telomeres

Telomere attrition is associated with the loss of certain heterochromatin markers and DNA hypomethylation at telomeric and subtelomeric regions (Benetti et al., 2007). We postulated that the increase in Nanog expression could be linked to a general dysregulation of epigenetic repression, given that low levels of trimethylation on histone H3 lysine 27 (H3K27me3) promote Nanog and Gata6 expression (Lu et al., 2011; Shen et al., 2008; Villasante et al., 2011). H3K27me3 was reduced at Nanog and Gata6 promoters in Tert−/−S ESCs, whereas H3K4me3 levels at the Nanog promoter were unaffected (Figure 1E). H3K27me3 and H3K4me3 enrichment on the Oct4 promoter was unaffected (Figure 1E). These perturbations, including a slightly increased level of global H3K27me3 in Tert−/−S ESCs, were restored upon telomere elongation (Figures 1E and S1H). These changes were not accompanied by a significant alteration in the three-dimensional localization of telomere DNA or chromatin in interphase nuclei (Figure S1J). Thus, the altered expression of Nanog and Gata6 reflects changes in heterochromatin at their respective promoters independent of Nanog occupancy. Moreover, these results demonstrate that critically short telomeres also affect chromatin organization at loci distal to telomeres.

Critically Short Telomeres Perturb the Ability of ESCs to Remain Stably Differentiated

The impact of Nanog misregulation upon differentiation was tested by treating ESCs with 5 μM all-trans retinoic acid (ATRA), which was followed by the removal of ATRA and the readdition of LIF-containing media (Figure 2). Although longer ATRA treatment times were required to achieve suppression of Oct4, Nanog, and Sox2 mRNA and protein to levels comparable to WT or Tert+/−S ESCs with longer telomeres (Figures 2A–2D and S2), Tert−/−S ESCs nevertheless exhibited a lower proliferative activity after ATRA treatment, which was consistent with a differentiated state (Figure 2E). However, after the readdition of LIF-containing media, Tert−/−S ESCs failed to maintain repression of Nanog and exhibited robust colony formation only 6 days after the readdition of LIF-containing media (Figures 2 and S2). As an independent marker of differentiation, WT and Tert−/−S cells were transduced with an Oct4 promoter-driven green fluorescent protein (GFP) construct, treated with ATRA for 12 days, and then sorted to allow the selection of the GFP-negative population by FACS. Sorted GFP-negative cells were plated in the presence of LIF-containing media for 10 days, followed by an assessment of the percentage of GFP-positive cells. Tert−/−S cells exhibited a high percentage of GFP-positive cells after the readdition of LIF-containing media (Figure 2F). These results demonstrate that ESCs with telomere dysfunction were able to execute only an incomplete, transitory repression of pluripotency genes in response to differentiation cues.

ESCs with Short Telomeres Exhibit DNA Hypomethylation

Critically short telomeres are associated with DNA hypomethylation at subtelomeric DNA (Benetti et al., 2007). Given that we observed chromatin alterations at loci distal to telomeres, we tested whether Tert−/−S ESCs also exhibited altered DNA methylation throughout the genome. Bisulphite-sequencing analysis of the Nanog and Oct4 promoters revealed a significant reduction in the acquisition of methylated cytosine in Tert−/−S ESCs treated with ATRA relative to WT or Tert+/−R ESCs (p ≤ 0.01 and p < 0.0001, respectively; Fisher’s exact test) (Figure 3A). Furthermore, Tert−/−S ESCs failed to maintain even this level of cytosine methylation after the readdition of the LIF-containing media (p < 0.0001 and p = 0.03, respectively). At both promoters, this impairment was rescued in Tert+/−R ESCs (p > 0.05; Figure 3A). Genome-wide methylation measured by an ELISA-based detection system against methylcytosine was also significantly reduced in Tert−/−S ESCs (Figure 3B). Nonspecific epigenetic drift appeared improbable, given that WT and Tert−/−R ESCs did not exhibit these changes after a similar propagation period. Although ESCs can tolerate DNA hypomethylation without impairment of cell proliferation (Tsumura et al., 2006), hypomethylation nonetheless impairs the capability of ESCs to achieve, and maintain a differentiated state (Feldman et al., 2006; Jackson et al., 2004; Sinkkonen et al., 2008). Thus, DNA hypomethylation in Tert−/−S ESCs arose in response to critically short telomeres and impeded their stable differentiation.

Restoration of Dnmt3b or Depletion of Nanog Rescue the Stable Differentiation of ESCs with Short Telomeres

We tested whether the restoration of DNA methylation might restore the differentiation capability of Tert−/−S ESCs. In mammals, genomic DNA methylation is principally regulated by three DNA methyltransferases (Dnmts): Dnmt1 (methylation maintenance) and the de novo methyltransferases Dnmt3a and Dnmt3b (Li et al., 1992; Okano et al., 1999). Although Dnmt1 expression was unaffected in Tert−/−S ESCs, the expression of de novo methylases was reduced (Figure 3C). Enforced expression of Dnmt3b in Tert−/−S ESCs restored repression of Nanog (Figures 3D, 3E, and S3A) and restored the repression of Nanog, Oct4, and Sox2 mRNA upon ATRA treatment (Figures 4A and 4B). Dnmt3b expression also led to a significant reduction in the colony formation of Tert−/−S ESCs after the readdition of LIF-containing media (Figure 4C). The level of H3K27me3 at the Nanog promoter was also partially rescued in Tert−/−S ESCs that expressed elevated Dnmt3b (Figure 4D). Consistent with a direct role of Nanog suppression in the maintenance of stable differentiation, Nanog depletion by small hairpin RNA (shRNA) was sufficient to overcome the inability of Tert−/−S ESCs to remain differentiated (Figure 4C), and all genotypes transduced with Nanog shRNA exhibited a decrease in pluripotency gene expression (Figure S4). These results demonstrate that the mechanism of impaired ability to maintain stable differentiation in Tert−/−S ESCs acts via the perturbation of de novo DNA methylation, which, in turn, influences chromatin organization and the ability to repress pluripotency factors such as Nanog under differentiation conditions.

DISCUSSION

Here, we report that critically short telomeres led to genomewide DNA hypomethylation and that changes in H3K27 trimethylation occurred at loci distal to telomeres. The trimethylation of H3K27 is mediated by the polycomb repressive complex 2 (PRC2) and is associated with ESC identity (Shen et al., 2008). H3K27me3 is one of the principal histone repression markers,
and its diminished enrichment on Nanog and Gata6 promoters has been linked to the upregulation of these genes (Kim et al., 2008; Lu et al., 2011; Shen et al., 2008; Villasante et al., 2011). Although the global level of H3K27me3 increased in Tert\(^{-/-}\)ESCs similar to recent studies that associate H3K27me3 enrichment with unmethylated CpG islands, its presence at Nanog and Gata6 promoters was reduced (Lynch et al., 2012; Mendenhall et al., 2010). These data support the observation that DNA hypomethylation leads to overall increased levels of H3K27me3 in normally methylated regions but decreased levels of H3K27me3 in ordinarily unmethylated regions (Brinkman et al., 2012). Our data suggest a model whereby telomere-shortening-induced de novo Dnmt downregulation leads to DNA hypomethylation and altered H3K27me3 enrichment at promoters, which, in turn, affects the ability to repress pluripotency factors critical to stable differentiation in ESCs (Figure 4E).
The regulation of factors that affect pluripotency and differentiation are important not only to development but also to disease. For example, pluripotency factors such as Nanog tend to be highly expressed in undifferentiated tumors and in putative cancer stem cells (Tysnes, 2010). In addition, some cancer therapies employ differentiation-inducing agents such as retinoic acid in the treatment of acute promyelocytic leukemia (Petrie et al., 2009). Thus, it will be important to test whether critically short telomeres also influence cell fate in human cancer cells, particularly in the case of telomerase-inhibition strategies designed to instigate telomere instability.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**

All experiments employed two separately generated ESC lines containing a disruption of endogenous Tert, as previously described (Liu et al., 2000). ESC lines were cultured on gelatin-covered dishes and maintained in Glasgow’s Modified Eagle’s Medium (GMEM; GIBCO) supplemented with 15% v/v fetal bovine serum (FBS), 0.055 mM b-mercaptoethanol (Sigma-Aldrich), 2 mM L-glutamine, 0.1 mM GMEM nonessential amino acids, 5,000 units/ml penicillin and streptomycin, 1,000 units/ml of recombinant LIF (Chemicon), and 1 mg/ml doxycycline and maintained at 37°C with 5% v/v CO2. To restore Tert expression to Tert+/−/− SESESCs cells at passage, we cotransfected 70 ESCs with pTRE-Bi-Tert-IRES-EGFP-Hygro (or a similar vector lacking Tert) and CAG-rtTA advanced (pTET-ON advanced vector; Clontech). For constitutive expression of Tert, Tert+/−/− SESESCs were transfected with CAG-mTert-IRES-Puro or CAG-IRES-Puro. For expression of Dnmt3b, Tert+/−/− SESESCs were transfected with CAG-Dnmt3b-IRES-Puro or CAG-ires-Puro. All transfections employed Fugene 6 (Roche) in a 3:1 ratio to DNA according to the manufacturer’s instructions. For Tert rescue or Dnmt3b reintroduction, cells were propagated for four passages under selection with hygromycin (500 mg/ml) or puromycin (5 mg/ml), and individual colonies were isolated. For Nanog shRNA transduction, cells were infected with commercially available lentiviral particles (Santa Cruz Biotechnology) and selected with puromycin (5 mg/ml). Cell transduction with Oct4-promoter GFP was performed by infection with commercially available lentiviral particles (System Biosciences). All lentiviral infections were performed in the presence of Polybrene (5 μg/ml; Santa Cruz Biotechnology). All experiments were performed with more than one clonal isolate.

**Figure 3. Expression of DNA Methyltransferases in ESCs Lacking Tert**

(A) CpG methylation analysis of the Oct4 and Nanog promoters during ATRA treatment, followed by culture in LIF-containing media. Each column represents CpGs in a sequenced clone. Full dots symbolize methylated CpGs, and empty dots symbolize unmethylated CpGs. Percentage values indicate the proportion of methylated cytosine relative to total cytosine residues (n = 10).

(B) Relative quantification of global DNA methylation (n = 3) is shown. Data are represented as mean ± SD.

(C) Relative gene expression of Dnmt1, Dnmt3b, and Dnmt3a2 analyzed by qRT-PCR. Values were normalized to GAPDH (n = 4). Data are represented as mean ± SD.

(D) (Top) Dnmt3b protein detection by western blot and (bottom) after LI-COR quantification (n = 3). Data are represented as mean ± SD.

(E) Nanog protein detection by western blot. Tub, b-tubulin (n = 5); R, Tert rescue; 3b, Dnmt3b rescue. Passage numbers are as in Figure 1. *, p < 0.05; **, p < 0.01; ***, p < 0.0001.

See also Figure S3.

The regulation of factors that affect pluripotency and differentiation are important not only to development but also to disease. For example, pluripotency factors such as Nanog tend to be highly expressed in undifferentiated tumors and in putative cancer stem cells (Tysnes, 2010). In addition, some cancer therapies employ differentiation-inducing agents such as retinoic acid in the treatment of acute promyelocytic leukemia (Petrie et al., 2009). Thus, it will be important to test whether critically short telomeres also influence cell fate in human cancer cells, particularly in the case of telomerase-inhibition strategies designed to instigate telomere instability.
Differential Assay

Cell populations of the indicated genotype (1 × 10^5) were plated in non-gelatin-covered dishes in LIF-free media containing 5 μM ATRA (Sigma-Aldrich) for the indicated amount of time with ATRA-media replaced every 3 days. At the indicated time point, cells were replated in gelatin-covered dishes with LIF-containing media. For the single colony formation assay, a set of serial dilutions was performed, and the number of viable ES cell colonies was assessed with alkaline phosphatase (Millipore).

Quantitative Fluorescence In Situ Hybridization

The quantitative fluorescence in situ hybridization (Q-FISH) protocol was carried out as described previously (Liu et al., 2000). Metaphase spreads were captured with the use of Metafer 4 software and analyzed with Isis software. Statistical analysis of telomere intensity distribution was performed with Welch’s unpaired t test. The incidence of telomere signal-free ends was defined as the number of chromosome ends possessing a telomere signal (in arbitrary units) between 0 and 600, and statistical significance was assessed with Fisher’s exact test (InStat 3, GraphPad).

qRT-PCR

Total RNA was isolated from cells with the use of Triazol (Invitrogen) according to the manufacturer’s instructions. Reverse transcription was carried out with 0.5 μg of template RNA, random hexamer primers, and smart MMLV reverse transcriptase (Clontech). Diluted complementary DNA (20×) was subjected to real-time PCR analysis using a SYBR Green Master Mix (Roche) on a LightCycler 480 system (Roche). Background values (no reverse transcription control) were subtracted from the signal values to obtain the real signal values. Statistical significance was assessed with a t test (Prism software; GraphPad).

See also Figure S4.
transcriptase added) were subtracted and values were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (n > 3). The oligos employed are listed in Table S1. Statistical analysis was performed by ANOVA and a related Dunnett’s test comparing every group with WT values.

**ChIP Sequencing**

ChIP experiments were conducted as described in Bergmann et al., 2011, except phenol-chloroform was replaced with a Chexol, 100-based DNA isolation method described in Nelson et al., 2006. Recovered DNA was analyzed by qRT-PCR as described above. For each primer pair, triplicate measurements were taken and normalized to input DNA and the amount of DNA recovered from the GAPDH promoter (n > 3). Antibodies employed were as follows: rabbit anti-Nanog (Bethyl Laboratories); mouse anti-H3K27me3 and anti-H3K4me3 (Abcam); and murine IgG (Sigma-Aldrich). Oligos employed are listed in Table S1. Statistical analysis was performed by ANOVA and a related Dunnett’s test comparing every group with WT values. In each experiment, the signal present after immunoprecipitation with IgG was defined as background and subtracted prior to normalization to input DNA and GAPDH.

**Methylation Assay**

Relative genomic DNA methylation was assessed with the use of the ELISA-based Imprint Methylation DNA Quantification kit (Sigma-Aldrich) according to the manufacturer’s instructions, with the use of 100 ng of genomic DNA per sample (n > 3).

**Bisulphite Sequencing Analysis**

DNA methylation levels were determined by bisulphite sequencing analysis (Crouaire et al., 2010). After bisulphite conversion of unmethylated cytosines to uracil, samples were resuspended in 1 x Tris-EDTA for PCR amplification. PCR products were cloned into pcDNA3.1 Directional TOPO Expression (Invitrogen) vector and colony PCR was performed. Clones (at least ten per sample) were sequenced, and results were analyzed with BiQ Analyzer (http://biq-analyzer.bioinf.mpi-inf.mpg.de). Primers employed are listed in Table S1. Statistical analysis of samples employed Fisher’s exact test (two-sided) using GraphPad InStat3 (www.graphpad.com).

**SUPPLEMENTAL INFORMATION**

Supplemental Information contains Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2013.01.018.

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