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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−/− ESCs) 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.

INTRODUCTION

Murine embryonic stem cells (ESCs) are self-renewing, pluripotent cells able to differentiate into cells of all three germ layers. Pluripotency and self-renewal are maintained primarily by the core transcriptional factors Nanog, Oct4, and Sox2 (Heng et al., 2010) but require both the cooperation of other factors and coregulators (Li, 2010) and an efficient telomere maintenance mechanism (Huang et al., 2011). In mammals, telomere maintenance is achieved via a telomerase reverse transcriptase (Tert) and an integral RNA component (Terc) that synthesize new telomeric DNA during cell proliferation. An appropriate telomere maintenance system is important for ESC replicative potential (Agarwal et al., 2010; Batista et al., 2011; Marion et al., 2009). During the reprogramming of differentiated cells into stem cells, an increase in telomerase activity leads to telomere elongation and the acquisition of epigenetic marks characteristic of longer telomeres (Marion et al., 2009). Notably, the teratoma-forming ability of ESCs derived from late generation (G3–G4) Terc−/− mice with critically short telomeres is greatly reduced (Huang et al., 2011).

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−/− S) (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−/− L) (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 1C and S1E; data not shown). However, Nanog messenger RNA (mRNA) and protein levels were significantly elevated (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 (Figures 1A and S1C). Transient expression of Nanog in Tert−/− ESCs (Tert−/− S), 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 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−/− S ESCs cultured on gelatin in leukemia inhibitory factor (LIF)-containing media revealed a significant increase in the percentage of Nanoghigh cells in comparison to WT and Tert−/− S ESCs (Figures 1A and S1G) (Savarese et al., 2009). We confirmed elevated Nanog expression in Tert−/− S 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.
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 dysregulation of epigenetic repression, given that low levels of H3K27me3 tested by treating ESCs with 5 \( \mu \)M to Remain Stably Differentiated

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 \( \mu \)M 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 \( \text{Tert}^{-/-} \) ESCs with longer telomeres (Figures 2A–2D and S2), \( \text{Tert}^{-/-} \) ESCs nevertheless exhibited a low proliferative capacity after ATRA treatment, which was consistent with a differentiated state (Figure 2E). However, after the readdition of LIF-containing media, \( \text{Tert}^{-/-} \) 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 \( \text{Tert}^{-/-} \) 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. \( \text{Tert}^{-/-} \) 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 \( \text{Tert}^{-/-} \) 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 \( \text{Tert}^{-/-} \) ESCs treated with ATRA relative to WT or \( \text{Tert}^{-/-} \) ESCs (\( p < 0.01 \) and \( p < 0.0001 \), respectively; Fisher’s exact test) (Figure 3A). Furthermore, \( \text{Tert}^{-/-} \) 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 \( \text{Tert}^{-/-} \) ESCs (\( p > 0.05 \); Figure 3A). Genome-wide methylation measured by an ELISA-based detection system against methylcytosine was also significantly reduced in \( \text{Tert}^{-/-} \) ESCs (Figure 3B). Nonspecific epigenetic drift appeared improbable, given that WT and \( \text{Tert}^{-/-} \) 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 \( \text{Tert}^{-/-} \) 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 \( \text{Tert}^{-/-} \) 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 \( \text{Tert}^{-/-} \) ESCs, the expression of de novo methylases was reduced (Figure 3C). Enforced expression of Dnmt3b in \( \text{Tert}^{-/-} \) ESCs restored repression of Nanog and Oct4, and Sox2 mRNA upon ATRA treatment (Figures 4A and 4B). Dnmt3b expression also led to a significant reduction in the colony formation of \( \text{Tert}^{-/-} \) ESCs after the readdition of LIF-containing media (Figure 4C). The level of H3K27me3 at the Nanog promoter was also partially rescued in \( \text{Tert}^{-/-} \) 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 \( \text{Tert}^{-/-} \) 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 \( \text{Tert}^{-/-} \) 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 genome-wide 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 β-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 μg/ml doxycycline and maintained at 37°C with 5% v/v CO₂. To restore Tert expression to Tert⁻/⁻/⁻ SESECs 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⁻/⁻/⁻ SESECs were transfected with CAG-mTert-IRES-Puro or CAG-IRES-Puro. For expression of Dnmt3b, Tert⁻/⁻/⁻ SESECs 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 μg/ml) or puromycin (5 μg/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 μg/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, β-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.
Differentiation Assay

Cell populations of the indicated genotype ($1 \times 10^5$) were plated in non-gelatin-covered dishes in LIF-free media containing 5 mM 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 the use of 0.5 μg of template RNA, random hexamer primers, and smart MMLV reverse transcriptase (Clontech). Diluted complementary DNA (20x) was subjected to real-time PCR analysis using a SYBR Green Master Mix (Roche) on a LightCycler 480 system (Roche). Background values (no reverse 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).

Figure 4. Differentiation Ability of Tert$^{-/-}$ ESCs after Enforced Expression of Dnmt3b

(A) Nanog protein detection by western blot. Tub, β-tubulin (n = 3). The first two panels on the left are reproduced from Figure 2C.

(B) qRT-PCR analysis of pluripotency genes upon ATRA-induced differentiation. Gene expression at day 0 was arbitrarily set as 100 and the expression through the time course was normalized to mRNA levels at day 0. Values were expressed as a ratio to GAPDH. The first two genotypes were reproduced from Figure 2D.

(C) Single-colony formation assay after the removal of ATRA and the readdition of LIF-containing media (n = 3). The difference in the incidence of colony formation between Tert$^{-/-}$ and all the other genotypes, apart from short hairpin control-transduced Tert$^{-/-}$ cells, was statistically significant (p < 0.0001; ANOVA and related Dunnett’s test comparing every group with Tert$^{-/-}$ values). The y axis indicates colony number. Data are represented as mean ± SD.

(D) Chromatin immunoprecipitation analysis of H3K27me3 enrichment at the Nanog promoter, as described in Supplemental Experimental Procedures. Data are represented as mean ± SD (n = 3). *, p < 0.05; **, p < 0.01; ***, p < 0.0001.

(E) A schematic showing that telomere shortening impairs the expression of Dnmt3 isoforms, leading to genome-wide DNA hypomethylation, which, in turn, affects H3K27me3 enrichment on specific loci (e.g., Nanog), thus impairing the ability of ESCs to sustain pluripotency factor repression after differentiation and growth restimulation.

See also Figure S4.
transcriptase added) were subtracted and values were normalized to glyceralde- 
hyde 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 performed as described in Bergmann et al., 2011, 
except phenol-chloroform was replaced with a Chelex, 100-based DNA isola-
tion method described in Nelson et al., 2006. Recovered DNA was analyzed by 
qRT-PCR as described above. For each pair of primers, triplicate measure-
ments were taken and normalized to input DNA and the amount of DNA recov-
ered 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-Alrich). 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 Methylated DNA Quantification kit (Sigma-Alrich) according 
to the manufacturer’s instructions, with the use of 100 ng of genomic DNA 
per sample (n > 3).

**Bisulphite Sequencing Analysis**

DNA methylation was performed as described previously (Clouaire 
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 (Invto-
gen) vector and colony PCR was performed. Clones (at least ten per sample) 
of the correct molecular mass were sequenced, and results were analyzed with 
BiQ Analyzer (http://biq-analyzer.bioinf.mpi-inf.mpg.de). Primers em-
ployed are listed in Table S1. Statistical analysis of samples employed Fisher’s 
extact 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:// 
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