Transcriptional Silencing of a Novel *hTERT* Reporter Locus during In Vitro Differentiation of Mouse Embryonic Stem Cells

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The human telomerase reverse transcriptase *hTERT* is highly expressed in undifferentiated embryonic cells and silenced in the majority of somatic cells. To investigate the mechanisms of *hTERT* silencing, we have developed a novel reporter using a bacterial artificial chromosome (BAC) that contained the entire *hTERT* gene and its neighboring loci, *hCRR9* and *hXtrp2*. *Firefly* and *Renilla* luciferases were used to monitor transcription from the *hTERT* and *hCRR9* promoters, respectively. In mouse embryonic stem cells stably integrated with the BAC reporter, both *hTERT* and *hCRR9* promoters were highly expressed. Upon differentiation into embryoid bodies and further into mineral-producing osteogenic cells, the *hTERT* promoter activity decreased progressively, whereas the *hCRR9* promoter remained highly active, both resembling their endogenous counterparts. In fully differentiated cells, the *hTERT* promoter was completely silenced and adopted a chromatin structure that was similar to its native counterpart in human cells. Inhibition of histone deacetylases led to the opening of the *hTERT* promoter and partially relieved repression, suggesting that histone deacetylation was necessary but not sufficient for *hTERT* silencing. Thus, our result demonstrated that developmental silencing of the human *TERT* locus could be recapitulated in a chromosomal position-independent manner during the differentiation of mouse embryonic stem cells.

INTRODUCTION

The differentiation and lineage commitment of stem cells are accompanied and determined by chromatin remodeling and establishment of epigenetic marks (Szutorisz and Dillon, 2005). The process of differentiation involves switching on or off genes that are expressed in specific types of cells and at specific times. This programming of gene transcription occurs in the context of their native chromatin environment and is often stably maintained throughout the life span of an organism. Many genes essential for proper development of the embryo have been discovered and most of them encode proteins that regulate transcription (Arney and Fisher, 2004; Chambers and Smith, 2004). However, the dynamic interactions between these proteins and chromatin are still areas of intensive investigation. Detailed studies of gene transcription regulatory mechanisms during cell differentiation are essential not only for understanding the fundamental mechanisms of development and differentiation, but also for har-

nessing the differentiation and proliferation capacity that make stem cells invaluable for regenerative medicine.

Telomerase, an enzyme synthesizing TTAGGG telomere repeats, is pertinent to self-renewal potential of stem cells and is required for long-term cellular proliferation and survival (Morrison *et al.*, 1996; Lee *et al.*, 1998). Telomerase is tightly regulated throughout development (Kim *et al.*, 1994; Prowse and Greider, 1995). In early embryonic tissues and stem cells, telomerase is highly expressed and telomeres are stably maintained (Wright *et al.*, 1996; Sharpless and DePinho, 2004). Although telomerase is detected in many adult stem and progenitor cells, its level in these cells is not sufficient to maintain telomere length (Roy *et al.*, 2004). Furthermore, telomerase is undetectable in the majority of human adult somatic cells, which are programmed for a limited number of cell divisions (Wright *et al.*, 1996). These cells undergo senescence and/or apoptosis after their telomeres are depleted (Bodnar *et al.*, 1998; Zhu *et al.*, 1999), which is likely a molecular basis for the replicative senescence checkpoint in human cells, known as the Hayflick limit (Verfaillie *et al.*, 2002).

Telomerase consists of a catalytic subunit of telomerase reverse transcriptase (TERT) and a template RNA subunit (TER). The TERT subunit is the limiting subunit of telomerase (Liu *et al.*, 2000) and its gene transcription correlates with telomerase activity in most cells and tissues that have been examined (Greenberg *et al.*, 1998; Günes *et al.*, 2000; Wang and Zhu, 2003). Ectopic expression of the *hTERT* gene is sufficient for cellular immortalization (Bodnar *et al.*, 1998; Zhu *et al.*, 1999); yet normal human cells rarely undergo spontaneous immortalization. It has therefore been suggested that repression of the *hTERT* gene is very tight and stable throughout development (Wright and Shay, 2000;

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Abbreviations used: BAC, bacterial artificial chromosome; *hTERT* and *mTERT*, human and mouse telomerase reverse transcriptase; *CRR9*, cisplatin-resistance related gene; *Xtrp2*, a gene encoding an orphan transporter protein; DHS, DNase I hypersensitive site; HDAC, histone deacetylase; TSA, trichostatin A; ESC, embryonic stem cell; EB, embryoid body; *Fluc*, *Firefly* luciferase; *Rluc*, *Renilla* luciferase.

Table 1. Primer sequences

	Sizes	Positions ^a	5'-primers (5'→3')	3'-primers (5'→3')
Primers for RT-PCR				
Fluc	495 bp	ORF	AGAACTGCCTGCGTGAGATT	ATCCAGATCCACAACCTTCG
Rluc	273 bp	ORF	TCTTCGTGAAAACCATGTTG	TGGCACCTTCAACAATAGCA
mTERT	358 bp	ORF	CTAAAGGCCTCTGGCTCCTT	ATCTCCCATACCAACCCATGA
mCRR9	454 bp	ORF	TGCAGCCTTTCACCTTCTTT	GCCAAAGGCATAGACACCAT
PCR primers for probes				
CpG1	1.1 kb	−1080 to +31 bp	CGCCCCCTTAGATCCAAACTT	CAGCGCTGCCTGAAACTC
mHS1	387 bp	−2797 to −2411 bp	GGTAGGCCTTCCTCTGCTCT	TTTGGATTTTGTAGCCCTTG
α-globin (Hba-a1)	995 bp	−2380 to −1386 bp	AGTCCTGCTTGACAGTCCCTA	TGGGTTACAGTTTTCACCA
β-globin (Hbb-b1)	447 bp	−1291 to −826 bp	CTTCCCAGGTCCTTCTCCTC	CCCAGTGAAGGGATCTTGAA
Primers for ChIP				
hTERT	235 bp	−179 to +56 bp	CAGGACCGCGCTTCCCACG	GGCTTCCCACGTGCGCAGC
hCRR9	234 bp	−463 to −230 bp	GACATGGCCCCCACTTG	CCCTGGGAAACGGAGTCT

ORF, open reading frame.

^a Relative to transcription start sites.

Rangarajan and Weinberg, 2003). However, molecular mechanisms of this transcriptional repression in somatic cells remain to be elucidated. We have recently shown that the endogenous *hTERT* gene is embedded in a condensed chromatin environment in several immortal human cell lines and normal human fibroblasts (Wang and Zhu, 2004), suggesting that epigenetic mechanisms play a key role in the developmental regulation of hTERT expression. Thus, repression of the *hTERT* gene may be a model to understand how transcriptional programming is established and maintained during cell differentiation and development.

Embryonic stem cells (ESCs) are pluripotent cells that have potential to develop into all somatic cell types, providing a powerful system for studying mechanisms involved in differentiation and development. To understand the molecular mechanisms involved in hTERT repression, we utilized the mouse ESC system and asked how the human genomic DNA containing all the regulatory regions of the *hTERT* gene was programmed during differentiation of ESCs. Here, we report the creation of a novel chromosomally integrated reporter of hTERT transcription using a bacterial artificial chromosome (BAC) containing three consecutive human loci, *hCRR9*, *hTERT*, and *hXtrp2*. Using *Firefly* and *Renilla* luciferases (*Fluc* and *Rluc*) to monitor the hTERT and hCRR9 promoters, respectively, we showed that the hTERT promoter in stably integrated reporter lines was progressively down-regulated during differentiation of the mouse ESCs into embryoid bodies, whereas the hCRR9 promoter was constitutively transcribed at a high level throughout the differentiation process. The hTERT promoter underwent complete silencing upon further differentiation into osteogenic cells and their derivatives, a process dependent on the deacetylation of histones H3 and H4 at the hTERT promoter. In addition, the human loci in the integrated BAC reporter in the differentiated cells adopted chromatin structures similar to their respective endogenous counterparts in human cells. Inhibition of histone deacetylases by trichostatin A (TSA) resulted in chromatin opening at the hTERT promoter and a partial activation of hTERT promoter in differentiated cells. Thus, our results demonstrated that the ectopic human *TERT* loci underwent their designated developmental programming during differentiation of the mouse ESCs, providing a model for studying genetic and epigenetic regulation of hTERT transcriptional silencing.

MATERIALS AND METHODS

Plasmid and BAC Construction

The BAC reporter construct 117B23-cRtFsP was derived from the BAC clone RPC111-117B23 (Invitrogen, Carlsbad, CA). This reporter was constructed through a novel two-step recombination procedure, involving a positive and a negative selection step based on a BAC recombineering protocol from the Copeland lab (Lee *et al.*, 2001 and manuscript in preparation). First, a 2.7-kb BstEII fragment from pYF10 plasmid (Wang and Zhu, 2003) was recombined with the original BAC, resulting in the insertion of the *Fluc* open reading frame (ORF) and a SV40 poly-A site into the translational initiation codon of the *hTERT* gene. Next, a 1.5-kb HindIII-BamHI *Rluc* cassette from pRL-SV40 (Promega, Madison, WI) was inserted into the *hCRR9* initiation codon. Finally, the BspHI-ClaI fragment containing PGK promoter/puromycin gene from pL-ZRSpBMN was inserted into the SfoI site of SacB gene in the BAC vector backbone sequence (pBACe3.6). The resulting products were confirmed by restriction enzyme digestions and sequencing the recombination junctions.

Cell Culture, Transfection, and ESC Differentiation

The mouse ESC line HM-1 was cultured on 0.1% gelatin-coated dishes in DMEM with 20% fetal bovine serum (FBS) and 1000 U/ml LIF (Chemicon, Temecula, CA). The modified BAC construct 117B23-cRtFsP was transfected into HM-1 ESCs using FuGene6 (Roche, Indianapolis, IN) and selected with 0.5 μg/ml puromycin for 10–14 d. The colonies exhibiting both *Fluc* and *Rluc* activities were then isolated. Data obtained from three clones, C1, F2-1, and F2-9, are presented here. ESC differentiation into EBs and osteogenic cultures were as described previously (Woll and Bronson, 2006).

Chromatin Analyses

DNase I sensitivity assays were performed as described previously (Wang and Zhu, 2003, 2004). Full-length genomic bands were quantified by phosphorimaging and generalized DNase I sensitivities were calculated by the equation $S = \log(X_d/X_i/\log(C_d/C_i)) \times T$, where X and C are test and control band intensities for the initial (i) or digested (d) samples and T is the size ratios of the control to test fragments (Saitoh *et al.*, 2000). All probes specific to human genomic DNA with the exception of CpG1 have been published (Wang and Zhu, 2004). The CpG1 and mouse probes were amplified by PCR from genomic DNA using primers listed in Table 1.

Chromatin immunoprecipitation was performed as recommended by Upstate Biotechnology (Lake Placid, NY). 10^7 cells were cross-linked with 1% formaldehyde and stopped by 0.125 M glycine. Cell pellets were lysed and sonicated to generate DNA fragments of 200–800 base pairs. All antibodies were purchased from Upstate Biotechnology. DNA fragments were subjected to PCR amplification for 42 cycles using primers specific for the hTERT (Liu *et al.*, 2004b) and hCRR9 promoters (Table 1).

Gene Expression Analyses

RT-PCR was performed as described previously (Wang and Zhu, 2004). Equal amount of total RNAs from various samples was used for RT-PCR reactions. All the experiments were repeated at least once. Primers for osteocalcin (OCN), type I collagen (COL1), and Runx2 were the same as previously published (Woll and Bronson, 2006). The amplifications were 28 cycles for

Figure 1. Analyses of the BAC reporter in transgenic ESCs and differentiated cells. (A) A schematic diagram of the BAC reporter construct. *Fluc* and a *Rluc* cassettes were inserted into the *hTERT* and *hCRR9* initiation codons, respectively. A puromycin-resistant marker was placed in the BAC vector. The thick horizontal bar represents the human genomic DNA sequence and the thin line stands for the vector sequence. The vertical bars correspond to exons and arrows indicate the directions of transcription. (B) mRNA expression during ESC differentiation. Stable ESC lines, C1, F2-1, and F2-9, were induced to differentiate into EBs. Total RNA was isolated from EB cultures at 0, 1, 2, 3, 4, 6, 9, and 14 d and analyzed by RT-PCR using primer pairs specific to the endogenous *mTERT* and *mCRR9* mRNAs (left panels) and *Fluc* and *Rluc* coding sequences (right panels). Equal amounts of total RNAs were used for RT-PCR analyses. (C) Luciferase activities in ESCs and differentiated cells. *Fluc* and *Rluc* activities were measured in EB cultures (a), individual EBs at day 14 (b: C1, 0.022 ± 0.009 ; F2-1, 0.10 ± 0.06 ; F2-9, 0.15 ± 0.14), and individual bone nodules at 3 wk in osteogenic cultures (c: C1, 0.027 ± 0.025 ; F2-1, 0.009 ± 0.008).

OCN and Col1 and 32 cycles for Runx2. Other transcripts were amplified for 29 cycles using primers listed in Table 1.

Luciferase activities were measured using the Dual Luciferase Reporter assay system (Promega). For imaging, *Fluc* substrate D-luciferin (0.312 mg/ml, Prolume, Pinetop, AZ) or *Rluc* substrate Coelenterazine (12 μ g/ml, Prolume) were added into culture media. Bioluminescent images were acquired using an IVIS 50 Imaging System and analyzed with Living Image software Version 2.5 (Xenogen Biosciences, Hopkinton, MA).

RESULTS

Generation of a Stably Integrated hTERT Reporter in Mouse ESCs

To investigate the mechanisms of hTERT regulation, a novel reporter for hTERT transcription was constructed from a BAC clone that contains the entire human *TERT* locus. The BAC DNA contains 158-kb human genomic DNA sequence that includes three complete loci: the *CRR9*, *TERT*, and *Xtrp2* (Figure 1A). In addition, a partial *Xtrp2*-like gene, missing its first exon, is also present in the BAC. Previous studies showed that the *hCRR9* gene was ubiquitously expressed in all the cell types examined, whereas the *hXtrp2* gene was expressed exclusively in kidney (Nash *et al.*, 1998; Wang and Zhu, 2003, 2004). Therefore, these two genes can serve as ideal internal controls for analyzing the hTERT transcriptional regulation. The BAC DNA was modified using a new recombination procedure that was based on a BAC recombination method published by the Copeland laboratory (Lee *et al.*, 2001). The procedure involved a two-step positive and negative selection scheme, which allowed precise modifications of the BAC sequences without leaving behind

any unwanted sequences (unpublished results). Figure 1A shows the schematic representation of the final BAC construct, in which *Fluc* and *Rluc* cassettes were inserted into the *hTERT* and *hCRR9* translational initiation codons, respectively. Transcription from the hTERT and hCRR9 promoter can thus be monitored by luciferase activities. In addition, a puromycin-resistant marker was inserted into the middle of the *SacB* gene within the vector backbone. The modified BAC construct was transfected into murine ESCs, HM-1. A total of 12 puromycin-resistant colonies that expressed both *Fluc* and *Rluc* activities were isolated. The majority of clones contained most, if not all, of the BAC sequences, as demonstrated by Southern blot analyses (see data in Figures 4 and 5). Three independent lines, C1, F2-1, and F2-9, were used in this report.

Reporter Expression during ESC Differentiation

To determine the regulation of hTERT reporter expression during in vitro differentiation of ESCs, the transgenic ESCs were allowed to differentiate in the absence of leukemia inhibitory factor (LIF). Embryoid bodies (EBs) formed in suspension cultures over a period of 2 wk. During this time, the endogenous *mTERT* mRNA gradually decreased, whereas the *mCRR9* message remained unchanged, as determined by RT-PCR analyses (Figure 1B, left panels). Accordingly, the level of *Fluc* mRNA also declined progressively, whereas the *Rluc* message did not change during this 2-wk period (Figure 1B, right panels). Luciferase activities were first measured in pools of EBs and the results indicated that the ratio of *Fluc* to *Rluc*

Figure 2. Osteogenic differentiation of mouse ESCs. (A) Bone colonies. Left, colonies stained with silver nitrate; right, an enlarged bone nodule. (B) RT-PCR analyses of mRNA expression in individual bone colonies derived from F2-1 ESCs. B, bone colonies; OCN, osteocalcin; COL1, type I collagen. Equal amounts of total RNAs were used for RT-PCR analyses. (C) Bioluminescent images of bone colonies. D-luciferin was added to the 6-cm plate and the *Fluc* image (3 min) was acquired. Coelenterazine was then added to the same plate and the *Rluc* image (20 s) was obtained.

reduced by 5–10-fold, compared with that in undifferentiated ESCs (Figure 1Ca). Luciferase activities were also measured in individual EBs 2 wk after LIF withdrawal and the ratio of *Flu* to *Rluc* activities decreased by more than 10-fold in most EBs at this stage (Figure 1Cb). These results indicated that the hTERT promoter in the transgenic BAC reporter was down-regulated during the development of EBs.

Although EBs contained a range of differentiated cells, there were also undifferentiated and partially differentiated cells in these structures (Keller, 1995). These less differentiated cells might still express significant levels of TERT. To study the repression of the hTERT promoter in fully differentiated cells, the developing 2-d EBs were dispersed and cultured under a previously published osteogenic culture condition (Woll and Bronson, 2006). Bone nodules formed after 3–4 wk in osteogenic cultures. Most of these colonies stained positive by silver nitrate, indicating that the differentiated cells secreted mineralized bone matrix, a characteristic of osteoblast differentiation (Figure 2A). Cells of the osteogenic lineage express a number of molecular markers. For example, Runx2/cbfa1 plays a critical role in osteoblastic differentiation and regulates the expression of osteoblast-specific genes, such as osteocalcin and type I collagen. To examine the expression of these osteogenic markers in bone colonies, total RNAs were extracted from individual colonies and analyzed by semiquantitative RT-PCR. Compared

Figure 3. Luciferase expression in bone colony-derived cells. (A) Morphology of the two differentiated cell populations, F2-1d and F2-9d. (B) Bioluminescent images of F2-1 and F2-9 and their differentiated derivatives F2-1d and F2-9d. D-luciferin (for *Fluc*) or coelenterazine (for *Rluc*) was added to undifferentiated and differentiated cells (10⁵ cells each) in 12-well plates, and images were acquired. The *Fluc* images were scanned for 1 min and the *Rluc* images for 20 s. (C) Quantified luciferase imaging signals in ESC and differentiated cells. Luciferase activities were averages calculated from bioluminescent images of three wells. **p* < 0.000001.

with the mCRR9 RNA levels, which were similar in ESCs and bone colonies, levels of osteocalcin, type I collagen, and Runx2/Cbfa1 mRNAs were all significantly increased in bone colonies (Figure 2B), confirming that the colonies indeed contained differentiated osteogenic cells (zur Nieden *et al.*, 2003; Woll and Bronson, 2006).

The hTERT gene is transcriptionally repressed in differentiated somatic tissues. To assess transcription from both endogenous mTERT and transgenic hTERT promoters in osteogenic cells, mTERT, mCRR9, and luciferase mRNAs in individual bone colonies were measured by RT-PCR (Figure 2B). mRNAs of endogenous mCRR9 and *Rluc* transcribed from the transgenic hCRR9 promoter remained at similar levels in bone colonies compared with those in ESCs. In contrast, the mRNA level of *Fluc* was much lower in bone nodules, compared with that in ESCs. Likewise, the endogenous mTERT mRNA level was also reduced. Consistent with the mRNA expression, *Fluc* activity was barely above background in bone nodules, as shown in Figure 1Cc. Furthermore, *Fluc* signal was undetectable in live bone colonies by bioluminescent imaging, whereas *Rluc* signal was readily detected (Figure 2C). Thus, the transgenic hTERT promoter

Figure 4. DNase I hypersensitivity of the endogenous mTERT promoter (A) and the transgenic hTERT promoter (B) in F2-1 and F2-1d cells. Nuclei treated with 0, 2, 4, 8, and 16 U/ml DNase I. Genomic DNAs were digested with DraI (A) or EcoRI + HincII (B) and analyzed on Southern blots using probes specific to the 5' ends of the genomic fragments: probe *a* for the hTERT promoter (Wang and Zhu, 2003) and mHS1 for the mTERT promoter. Schematic promoter structures are depicted on the left sides and sizes (kb) are on the right.

was progressively silenced during differentiation of ESCs into osteogenic cells.

Epigenetic Regulation of the Transgenic hTERT Promoter in Differentiated Mouse Cells

To obtain a large number of differentiated cells for epigenetic analyses, the bone colonies were trypsinized and passaged in MEM medium containing 10% FBS. Relatively homogenous populations of proliferative and morphologically differentiated cells, F2-1d and F2-9d, were derived from ESC clones F2-1 and F2-9, respectively, after passaging for 2 months (Figure 3A). Luciferase activities in F2-1d and F2-9d were determined by bioluminescent imaging and compared with parental ESCs (Figure 3B). The quantified *Rluc* imaging signal from the hCRR9 promoter did not change before or after differentiation. However, the *Fluc* signal dramatically decreased by up to five orders of magnitude in both F2-1d and F2-9d cells compared with their respective parental ESCs (Figure 3C). In fact, little *Fluc* signal above background was detected in F2-1d and F2-9d cells, indicating that the hTERT promoter in the transgenic BAC construct was completely silenced in these differentiated cells.

To dissect epigenetic mechanisms in regulating TERT transcription during mouse cell differentiation, we characterized the configuration of the TERT promoters by DNase I sensitivity assays in undifferentiated F2-1 ESCs and their differentiated derivative F2-1d cells. Genomic DNAs extracted from DNase I treated nuclei were digested with restriction enzymes and subjected to Southern analyses. The restriction fragments containing the TERT promoters were hybridized to radiolabeled probes specific to the 5' ends of

the genomic fragments. Figure 4A shows that the mTERT promoter was digested into a 6.7-kb band with DraI (from -3.6 to +3.1 kb, relative to the mTERT transcription start site). However, in undifferentiated F2-1 ESCs, an additional band of 3.5 kb appeared. Its intensity increased with DNase I treatment and only disappeared at high concentrations of the nuclease. The size of this band identified a major DNase I-hypersensitive site (DHS) around the transcription start site of the endogenous mTERT promoter (Figure 4A). This DHS was not present in differentiated F2-1d cells, suggesting that the appearance of this DHS correlated with mTERT transcription.

The genomic DNAs were also digested with EcoRI and HincII. In addition to the full-length 5.4-kb band (from -3.9 to +1.5 kb, relative to the hTERT transcription start site), a major hypersensitive band (~3.9 kb), corresponding to a DHS near the core hTERT promoter, was detected in undifferentiated F2-1 cells (Figure 4B). This DHS disappeared in differentiated F2-1d cells, correlating with silencing of the hTERT promoter in differentiated mouse cells. The same DHS was also detected in F2-9 cells but not in F2-9d cells (data not shown). Because this DHS was also present at the endogenous hTERT promoter in human cells expressing telomerase, the data supported our earlier conclusion that its appearance correlated with hTERT transcription (Wang and Zhu, 2004).

Previous studies suggested that the physical location of hTERT gene occurred within a condensed chromatin environment, which might contribute to the repression of *hTERT* gene in human somatic cells (Wang and Zhu, 2004). To determine whether the *hTERT* locus in the transgenic BAC DNA had a similar chromatin conformation upon integration in ESCs, generalized DNase I sensitivity of the three loci, *hCRR9*, *hTERT*, and *hXtrp2*, in the transgenic BAC DNA was measured. Genomic DNAs isolated from DNase I-treated nuclei of F2-1 and F2-1d cells were digested with various combinations of restriction enzymes and subjected to Southern analyses (Figure 5). In the absence of any DHSs within a chromatin fragment, generalized DNase I sensitivity can be measured as disappearing rate of the restriction fragment as a function of DNase I concentration (Saitoh *et al.*, 2000; Wang and Zhu, 2004). Thus, fragments with no detectable DHSs were chosen for experiments in Figure 5. Because the kinetics of digestion also depends on fragment sizes, restriction fragments with similar sizes were compared wherever possible, and the digestion rates were normalized to their length. In addition, fragment *a* at the 5' half of the *hCRR9* locus was used as an internal control because the *hCRR9* promoter was constitutively active and thus was likely to maintain an open chromatin conformation throughout cell differentiation.

As shown in Figure 5, chromosomal fragments *a* and *b*, located at the 5' half of the *hCRR9* gene, were susceptible to DNase I treatment in both F2-1 ESCs and differentiated F2-1d cells. At the opposite end, fragment *f*, located at the 5' half of *hXtrp2* locus, was more resistant to DNase I digestion, compared with the *hCRR9* gene. Generalized nuclease sensitivity of the *hXtrp2* locus was more similar to that of β -globin loci, which were associated with pericentric heterochromatin regions in nonerythroid cells (Brown *et al.*, 2001). There was also a trend that the *hTERT* locus was more nuclease-resistant than the upstream *hCRR9* locus in differentiated F2-1d cells. However, for most chromatin fragments, the difference between F2-1 ESCs and F2-1d cells were not significant in these experiments by statistical analyses. As a control, the α -globin loci were highly sensitive to DNase I treatment in differentiated F2-1d cells, as was in

Figure 5. Generalized DNase I sensitivity of the transgenic loci in F2-1 and F2-1d cells. (A) A schematic structure of the BAC reporter and chromosomal fragments subjected to analysis. Arcs indicate fragments (a–f) that were examined by DNase I digestion. (B) Relative generalized DNase I sensitivity. The sensitivity values are the averages of lanes treated with 4, 8, and 16 U/ml DNase I in C, relative to fragment a (1.0). * $p < 0.05$, relative to the fragment a in the same cells, by Student's t test. (C) Southern analyses of chromosomal fragments. Nuclei isolated from F2.1 and F2.1d cells were treated with 2, 4, 8, and 16 U/ml DNase I and genomic DNAs were digested with various combinations of restriction enzymes, followed by Southern blotting. Restriction fragments and sizes are indicated on the right. Endogenous α - and β -globin loci were used as internal controls.

most somatic cells (Vyas *et al.*, 1992). Therefore, in both undifferentiated and differentiated cells, the integrated BAC reporter formed a chromatin environment that was similar to the corresponding native loci in human cells (Wang and Zhu, 2004).

Transcriptional silencing is often accompanied by CpG methylation. In fact, a CpG island is present at the hTERT promoter (Wick *et al.*, 1999). It was reported that the hTERT promoter was often methylated in human cell lines and cancer cells; however, the extent of CpG methylation was not directly correlated with hTERT gene expression (Devereux *et al.*, 1999; Dessain *et al.*, 2000; Liu *et al.*, 2004a). To determine whether silencing of the transgenic hTERT promoter is associated with CpG methylation, genomic DNAs from F2-1 ESCs and F2-1d cells were subjected to digestion by methylation-sensitive enzyme HpaII or methylation-insensitive MspI. The digested DNAs were analyzed by Southern analysis using a probe (CpG1) hybridized to a human genomic sequence, –1080 to +31 base pairs relative to the hTERT transcription start site. Both HpaII and MspI recognize sequence CCGG, which represents 17 of 99 CpG sites within the region recognized by this probe. As a control, the same blot was also hybridized to the *Rluc* sequence, which was integrated at the hCRR9 promoter and constitutively ex-

Figure 6. The status of CpG methylation at the hTERT promoter in F2-1 ESCs and F2-1d differentiated cells. Genomic DNAs were digested by DraI in combination with either methylation-sensitive restriction enzyme HpaII or methylation-insensitive MspI, separated on a 1.4% agarose gel, and probed with a CpG island probe CpG1 (top) or a *Rluc* probe (bottom). A431 cells were included as a positive control for CpG methylation at the hTERT promoter.

pressed. As shown in Figure 6, the ratio of HpaII- to MspI-digested low-molecular-weight DNA in the CpG1 region was identical to that of the *Rluc* fragment in both F2-1 and F2-1d cells, as determined by phosphorimaging analysis. As a positive control, the HpaII-digested low-molecular-weight CpG1 signal was almost completely absent in human cancer line A431, in which the hTERT promoter was heavily methylated (Guilleret *et al.*, 2002). In addition, the same blot was also probed with another CpG-rich hTERT genomic sequence +78 to +1682, which included most of exon 1, intron 1, and most of exon 2, and no significant CpG methylation was detected either (data not shown). Furthermore, treatment with 5 μ M of 5-aza-2'-deoxycytidine or 5-aza-cytidine for 3–6 d did not induce *Fluc* expression in either F2-1d or F2-9d cells (data not shown). Therefore, our results indicated that the CpG methylation status of the hTERT promoter might not be required for hTERT silencing during in vitro differentiation of ESCs.

Histone Deacetylation at the hTERT Promoter in Differentiated Cells

Transcriptional repression often involves deacetylation of core histone proteins (Jenuwein and Allis, 2001). It has been demonstrated that the endogenous hTERT transcription in human cells could be induced by inhibition of histone deacetylases in several cell types (Cong and Bacchetti, 2000; Wang and Zhu, 2003). To determine the role of histone deacetylation in the repression of transgenic hTERT promoter, histone acetylation at the hTERT promoter was examined by chromatin immunoprecipitation (ChIP; Figure 7A, top panel). In F2-9 ESCs, the N-terminal tails of histones H3 and H4 were acetylated (lanes 1 and 2). In contrast, such acetylation was undetectable in F2-9d differentiated cells. Likewise, binding of RNA polymerase II to the hTERT promoter was detected in F2-9 ESCs but not in F2-9d cells (lane 3). As a control, no significant changes in acetylation of histones H3 and H4 and PolII binding were detected at the hCRR9 promoter (Figure 7A, bottom panel). Similar results were obtained with F2-1 and F2-1d cells (data not shown).

Figure 7. Status of histone acetylation at the hTERT promoter in ESC and differentiated cells. (A) ChIP analysis of the transgenic promoters in F2-9 ESCs, F2-9d cells, and F2-9d cells treated with 5 μ M TSA for 24 h. Antibodies used in ChIP are as follows: 1, acetylated histone H3; 2, acetylated H4; 3, RNA polymerase II; 4, no antibody. Lanes 5-7 are 1/10, 1/20, and 1/40 of input chromatin fragments, respectively. Lane -, no template control. Precipitated DNA fragments were PCR-amplified using primers specific to the hTERT and hCRR9 promoters and analyzed on agarose gels. (B) TSA treatment on luciferase expression in differentiated cells. F2-1d and F2-9d cells were treated with 0, 2.5, or 5.0 μ M TSA for 12 or 24 h. The data are average ratios of *Fluc* to *Rluc* activities in triplicate wells of 24-well plates. * $p < 0.00003$.

These results demonstrated that silencing of the transgenic hTERT promoter was associated with histone deacetylation.

Next, we investigated whether inhibition of histone deacetylases could affect silencing of the transgenic hTERT promoter in differentiated cells. Differentiated cells F2-1d and F2-9d were treated with histone deacetylase inhibitor trichostatin A (TSA) for 12 or 24 h and this treatment resulted in acetylation of histones H3 and H4 at the hTERT promoter (Figure 7A). Although TSA treatment did not change *Rluc* levels in either F2-1d or F2-9d cells, *Fluc* activity was significantly induced. The ratio of *Fluc* to *Rluc* activities increased by 100-fold in both cell types (Figure 7B). Furthermore, the major DHS reappeared at the transgenic hTERT promoter in TSA-treated F2-1d cells (Figure 4B, right panel). These results indicated that histone deacetylation might be required for the repression of the hTERT promoter in differentiated cells. However, in spite of histone acetylation and opening of the hTERT promoter (as indicated by appearance of the DHS), the binding of RNA polymerase II to the hTERT promoter remained undetectable in TSA-treated cells (Figure 7A, F2-9d/TSA, lane 3). In fact, the 100-fold induction of transcription from the hTERT promoter in TSA-treated differentiated cells was still far below its activity in ESCs, which was higher than in differentiated cells by five orders of magnitude (Figures 3C). Thus, in addition to histone deacetylation and promoter opening, efficient PolII recruitment and transcription from the hTERT promoter might require *cis*-acting factors that were absent in differentiated cells.

DISCUSSION

Telomerase is highly regulated throughout human development and its regulation is crucial for cellular aging and tumorigenesis. To obtain a comprehensive view of developmental silencing of the *hTERT* gene, a chromosomally integrated reporter was created using a BAC genomic DNA clone containing the entire *hTERT* locus. Using in vitro differentiation of mouse ESCs into EBs and osteogenic cells as models, we demonstrated that expression of all three loci within the integrated human BAC genomic DNA mimicked their endogenous counterparts in human cells. hTERT-*Fluc* was expressed at a high level in undifferentiated ESCs and silenced upon cell differentiation. On the other hand, hCRR9-*Rluc* was constitutively expressed at the same level before and after differentiation, providing an ideal internal control for analysis of transcription from the hTERT promoter. The results demonstrated that developmental regulation of the *hTERT* gene could be recapitulated at ectopic chromosomal sites in mouse cells. Thus, the transgenic reporter could provide a model for studying both genetic and epigenetic factors involved in hTERT transcriptional repression in somatic cells. The wide range regulation of hTERT-*Fluc* activity during the entire differentiation process and hCRR9-*Rluc* as an internal control would allow easy detection of relatively small changes in the hTERT promoter activity.

Developmental silencing of hTERT gene is likely a multi-step process. Indeed, repression of hTERT promoter was progressive during differentiation of ESCs. The *Fluc* activity decreased about 10-fold during the 2-wk period of in vitro differentiation into EBs. In fully differentiated cells derived from bone colonies, the hTERT promoter was completely silenced. This was reminiscent of the endogenous *hTERT* gene in human cells, which has been shown to be under much tighter repression than the *mTERT* gene in mouse cells (Wright *et al.*, 1996). The native *hTERT* gene is located near the tip of human chromosome 5p. It was previously suggested that the regulation of hTERT transcription might be under influence of the telomere due to telomere positional effect (Shay and Wright, 2000; Baur *et al.*, 2001). Our data indicated that neither the transcriptional silencing nor the native chromatin conformation of *hTERT* locus required its physical position near telomere.

Regulation of hTERT transcription involves multiple *trans*-acting factors. Switch of promoter occupancy from c-Myc/Max to Mad1/Max complexes at the hTERT promoter was shown to correlate with hTERT down-regulation in differentiating HL60 and U937 cells (Günes *et al.*, 2000; Xu *et al.*, 2001). Other factors may also be involved in hTERT repression. For example, it has been reported that transcription factor E2F1, tumor suppressors Menin and Wilms' tumor 1 (WT1), and the TGF- β signaling pathway all mediated hTERT transcriptional repression in various cell types (Oh *et al.*, 1999; Crowe *et al.*, 2001; Lin and Elledge, 2003). The roles of these factors in hTERT repression during development remain to be elucidated.

Besides *trans*-acting factors, the *hTERT* locus may also be subject to epigenetic regulation (Liu *et al.*, 2004a). Recruitment of histone deacetylases by negative regulators and local histone deacetylation at the hTERT promoter were associated with hTERT repression (Xu *et al.*, 2001; Won *et al.*, 2002). Consistently, histones H3 and H4 at the transgenic hTERT promoter were acetylated in ESCs and deacetylated in differentiated cells. Inhibition of histone deacetylases by TSA led to a more than 100-fold induction of *Fluc* expression from the hTERT promoter, accompanied by the opening of

chromatin configuration at the hTERT promoter as demonstrated by DNase I assays. However, this TSA-induced expression was well below the level of *Fluc* expression in ESCs, suggesting that histone deacetylation and chromatin condensation at the hTERT promoter are necessary but not sufficient for the repression of hTERT promoter in differentiated cells. Additional mechanisms might also contribute to the silencing of hTERT promoter in these cells.

Methylation of the CpG island at the 5' end of the hTERT gene was proposed to play a role in hTERT repression (Devereux *et al.*, 1999; Dessain *et al.*, 2000). However, the transgenic hTERT promoter was not extensively methylated at CpG sites in either ESCs or differentiated cells (Figure 6). Apparently, extensive CpG methylation, as it occurs in many tumor-derived cell lines, was not required for the silencing of hTERT promoter in this ESC in vitro differentiation model. Although the role of CpG methylation in hTERT regulation may vary in different cell types, it is possible that most de novo CpG methylation at the hTERT promoter was acquired during tumor growth and/or in vitro passaging of oncogene-transformed cells (Dessain *et al.*, 2000). Thus, promoter methylation may not be directly involved in hTERT repression during normal development. Consistent with this notion, the CpG island at the hTERT promoter was more extensively methylated in SV40 transformed human fibroblasts than in parental normal human fibroblasts, even though neither cells expressed hTERT (data not shown).

Our recent data indicated that the native *hTERT* locus is located in a large condensed chromosomal environment in human cells (Wang and Zhu, 2004). In mouse ESCs and their differentiated derivatives, the integrated human sequence adopted a chromatin configuration that was similar to the native loci in human cells. A strong DHS appeared at the transcription start site in undifferentiated cells and disappeared upon differentiation, as was the case in many human somatic cells (Wang and Zhu, 2004). The generalized DNase I sensitivity of integrated human loci was also similar to their native counterparts in human cells: the *hCRR9* gene was sensitive to DNase I digestion, whereas the *hXtrp2* gene was resistant to the nuclease. A possible difference between the transgenic sequence in mouse cells and the native sequence in human cells was found in the *hCRR9/hTERT* intergenic region. In mouse F2-1 ESCs and F2-1d differentiated cells, this region was relatively sensitive to DNase I digestion, compared with the *hXtrp* locus. However, this region was highly resistant to nuclease digestion in most human somatic cells (Wang and Zhu, 2004). This difference might be attributable to the presence of abundant Alu-elements and other human-specific repetitive sequences, which accounted for about half of the 23-kb sequence in intergenic region. It would be reasonable to speculate that such human-specific repetitive elements are more prone to form repressive chromatin structure in human cells than in mouse cells. Studies on transgenic mouse lines using an 8-kb hTERT promoter fragment controlling a LacZ reporter revealed that the reporter expression was overall similar to hTERT expression, albeit with significant variations between transgenic lines (Ritz *et al.*, 2005). More recently, Horikawa *et al.* (2005) reported a transgenic mouse strain with a BAC fragment containing the *hTERT* gene as well as an 11-kb upstream sequence and a 1.2-kb downstream sequence. Although *hTERT* gene expression in this transgenic mouse line was similar to the endogenous hTERT expression in most human tissues, the transgene expression was also detected in a few tissues such as brain, in which native hTERT expression was not detectable. These results suggested either

that the 8- and 11-kb upstream sequences were not sufficient to maintain tight repression in some tissues or that the human sequence might behave differently in mouse cells. Although the role of repetitive sequences in regulating hTERT transcription remains to be determined, the overall resemblance between chromatin structures of the transgenic BAC DNA in mouse cells and the native counterparts in human cells rendered the transgenic human reporter in mouse ESCs a suitable model to study the developmental regulation of the *hTERT* gene in a relevant chromatin context.

It was previously reported that a 4.5-kb mTERT promoter fragment was able to control green fluorescence protein (GFP) expression in a way that was similar to the endogenous mTERT expression during the early differentiation of an ESC line (Armstrong *et al.*, 2000). The GFP expression decreased <5-fold in DMSO or all-*trans* retinoic acid induced differentiation of ESCs. Recently, GFP reporters controlled by shorter mTERT promoter fragments (1, 2, or 5 kb) were integrated into mouse ESCs. Down-regulation of GFP expression was also observed during differentiation of mESCs (Pericuesta *et al.*, 2006). These experiments suggested that short fragments of the mTERT promoter contained elements involved in repression of mTERT transcription. However, complete silencing of the mTERT promoter was not reported during in vitro differentiation of ESCs. One possibility is that the mTERT promoter is not as tightly repressed as the hTERT promoter. Alternatively, short mTERT promoter fragments might not contain all regulatory elements required for the complete silencing of the TERT promoter.

Although the established ESC lines contained randomly integrated BAC reporters and had multiple copies of BAC reporters (data not shown), regulation of luciferase expression was virtually identical in all three ESC lines that have been examined. Future studies using Cre-mediated recombination through the loxP and lox511 sites within the BAC vectors (pBACe3.6 or pTARBAC1) would allow targeted chromosomal integration of BAC constructs. Targeted single-copy integration, combined with mutagenesis of BAC reporters using BAC recombineering, would form the foundation of a genetically amendable system for dissecting *cis*-regulatory elements responsible for genetic and epigenetic regulation of hTERT silencing during differentiation and development. Furthermore, using dual luciferase measurements, the BAC reporter would be a suitable model to screen for potential therapeutic targets involved in telomerase regulation.

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