

DNA hypermethylation enhanced telomerase reverse transcriptase expression in human-induced pluripotent stem cells

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Abstract During reprogramming into human induced pluripotent stem cells (iPSCs), several stem cell marker genes are induced, such as *OCT-4*, *NANOG*, *SALL4*, and *TERT*. *OCT-4*, *NANOG*, and *SALL4* gene expression can be regulated by DNA methylation. Their promoters become hypomethylated in iPSCs during reprogramming, leading to their induced expression. However, epigenetic regulation of the *TERT* gene remains unclear. In this study, we focused on epigenetic regulation of the human *TERT* gene and identified a differentially methylated region (DMR) at a distal region in the *TERT* promoter between human iPSCs and their parental somatic cells. Interestingly, the *TERT*-DMR was highly methylated in iPSCs, but low-level methylation was observed in their parental somatic cells. Region-specific, methylated-promoter assays showed that the methylated *TERT*-DMR up-regulated the promoter activity in iPSCs. In addition, Lamin B1 accumulated at the *TERT*-DMR in iPSCs, but

not in their parent somatic cells. These results suggested that the *TERT* transcription was enhanced by DNA methylation at the *TERT*-DMR via binding to nuclear lamina during reprogramming. Our findings shed light on a new functional aspect of DNA methylation in gene expression.

Keywords Human *TERT* gene · Human iPSCs · DNA methylation · Epigenetics · Lamin B1 · Reprogramming

Introduction

Telomerase reverse transcriptase (TERT) is a key component of telomerase and an important molecule for stem cells. TERT elongates telomere sequences at the ends of chromosomes and defines the high proliferative capacity of stem cells. The human *TERT* gene is highly expressed in induced pluripotent stem cells (iPSCs), but not somatic cells. Therefore, activation of the *TERT* gene is essential for reprogramming human somatic cells into iPSCs [1].

Reprogramming is accompanied with genome-wide epigenetic modifications. Genomic DNA methylation, one mode of epigenetic regulation, serves as an important mechanism in development and cell differentiation [2–5] as well as reprogramming in iPSCs. Stem cell marker genes, such as octamer-binding transcription factor 4 (*OCT-4*) [1], homeobox transcription factor *NANOG* (*NANOG*) [1] and *SAL*-like 4 (*SALL4*) [6], show tissue-specific demethylation corresponding to their expression during reprogramming. For instance, the CpG sites in the *OCT-4* promoter are kept hypomethylated, which permits its expression in pluripotent stem cells. In contrast, hypermethylation of the *OCT-4* promoter silences its expression in somatic cells. Therefore, the DNA methylation profiles of the *OCT-4* promoter have been used as an epigenetic marker of iPSC reprogramming [1, 7].

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Although *TERT* is a pluripotency marker gene in humans, the epigenetic regulation of human *TERT* gene by DNA methylation remains unclear.

In this study, we identified a differentially methylated region in the distal promoter of the human *TERT* gene. Hypermethylation of the *TERT* promoter induced its expression. We show here a novel epigenetic regulatory mechanism wherein hypermethylation of the *TERT* promoter was activated through binding to the nuclear membrane.

Materials and methods

Human cell culture

Human endometrial (UtE1104) [8], amniotic (AM936EP) [9], menstrual blood cell (Edom22) [8], and fetal lung fibroblast (MRC-5) [10] cells were maintained in Poweredby10 medium (GlycoTechnica, Yokohama, Japan). Placental artery endothelial cells (PAE551) [9] were cultured in medium from the EGM-2MV BulletKit (Lonza, Walkersville, MD, USA) containing 5% fetal bovine serum. The human embryonal carcinoma NCR-G3 and NCR-G4 cell lines were cultured in G031101 medium, as previously described [11]. The human iPSC lines, MRC-iPS-25 [12], PAE-iPS-1 [13], AM-iPS-8 [9], UtE-iPS-6 [13], Edom-iPS-2 [13], and iPS-201B7 [1] were derived from the MRC-5, PAE551, AM963EP, UtE1104, Edom22 and HDF cell lines, respectively. iPSCs were maintained on irradiated mouse embryonic fibroblasts in KO-DMEM (Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 20% knockout-serum replacement (Thermo), supplemented with 1% GlutaMAX (Thermo), 1% nonessential amino acids (Thermo), 55 μ M 2-mercaptoethanol (Thermo), 1% penicillin and streptomycin (Thermo) and 10 ng/ml recombinant human basic fibroblast growth factor (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Frozen pellets of human embryonic stem cell lines (HUESCs) [14] were kindly gifted from Drs. C. Cowan and T. Tenzan (Harvard Stem Cell Institute, Harvard University, Cambridge, MA). For demethylation assays, MRC-iPSCs were cultured for 4 days in medium containing 0, 0.5, 1, or 5 μ M 5-aza-2'-deoxycytidine (5-aza-dC, Merck Millipore, Billerica, MA, USA). The human cells used in this study are summarized in Supplemental Table 1.

Gene expression analysis

For the reverse transcription-polymerase chain reaction (RT-PCR), first-strand cDNA was synthesized using total RNA (1 μ g) with random hexamers and ReverTra Ace reverse transcriptase (TOYOBO Co., Ltd., Osaka, Japan). The cDNA template was amplified using BIOTAQ™ HS DNA Polymerase (Bioline Ltd; London, UK) and specific primers

for *TERT*, *POU5F1*, *NANOG*, *SOX2*, *SALL4*, *EPHA1*, and *GAPDH*. All PCR experiments were performed under the following thermocycling conditions: 95 °C for 10 min; 30 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min; with a final extension at 72 °C for 10 min. Quantitative real-time PCR (qPCR) was performed using SYBR® Green PCR master mix (Applied Biosystems, Waltham, MA, USA). Data were normalized to *GAPDH* expression. Gene expression levels are presented as the fold-change in expression, which was calculated using the $2^{-\Delta\Delta CT}$ method [15]. The sequences of primers used in this study are summarized in Supplemental Table 2.

Gene expression array data were obtained from Gene Expression Omnibus microarray data archive (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) (accession numbers GSE20750, GSE42099, and GSE24677) [13, 16]. All expression data had been analyzed using the same platform, namely an Agilent-014850 Whole Human Genome Microarray 4x44K G4112F. The expression data were processed by median normalization, then used for our analysis.

Genome-wide DNA methylation analysis

Genome-wide DNA methylation analysis was performed using the Illumina Infinium HumanMethylation450K BeadChip Kit (Illumina Inc., San Diego, CA, USA) and the BeadChip was scanned on a BeadArray Reader (Illumina Inc.) according to the manufacturer's instructions. Methylated and unmethylated signals were used to compute a β value, which was a quantitative score of the DNA methylation levels, ranging from "0.00" (completely unmethylated) to "1.00" (completely methylated). All DNA methylation data were submitted to GEO under accession numbers GSE73938 and GSE99716.

Bisulfite sequencing

Sodium bisulfite treatment of genomic DNA was performed using the EZ DNA Methylation-Gold™ Kit (Zymo Research, Irvine, CA). PCR amplification was performed using BIOTAQ™ HS DNA Polymerase (Bioline Ltd) and specific primers for *TERT* promoter. All PCR experiments were performed under the following thermocycling conditions: 95 °C for 10 min; 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; with a final extension at 72 °C for 10 min. The sequences of primers used in this study are summarized in Supplemental Table 2. To determine the methylation states of individual CpG site at the *TERT* promoter, the PCR product was gel-extracted, subcloned into the pGEM-T Easy vector (Promega, Madison, WI), and sequenced. Methylation sites were visualized, and quality control was performed using the web-based tool, "QUMA" (<http://quma.cdb.riken.jp/>) [17].

Region-specific methylated *TERT* reporter assay

The 5'-flanking fragment of the human *TERT* gene was isolated from genomic DNA of MRC-iPSCs by genomic PCR. The position of the transcription start site (TSS) was designated as + 1. The 1.6-kbp, 5'-flanking region (from – 1560 to + 36) containing an *EcoRI* site at – 625 nucleotides (nt) was cloned into pGL4.10-Basic vector (Promega). This construct was designated as *TERT*-1560. The *TERT*-626 construct was generated by removing the region from – 1560 to – 625 from *TERT*-1560, following digestion with *Acc65I* [New England BioLabs (NEB), Beverly, MA] and *EcoRI* (NEB), and self-ligation. The *TERT*-1560_Me construct was generated by re-ligation of the fragment (from – 1560 to – 625) that was methylated in vitro with *SssI* methylase (NEB). MRC-5 cells and MRC-iPSCs were co-transfected with the reporter constructs and pGL4.74[hRluc/TK], at a 6: 1 molar ratio in each case. Luciferase activities were determined using the Dual-Luciferase Reporter Assay system (Promega) at 48 h post-transfection, according to the manufacturer's instructions. Promoter activity was normalized to Renilla luciferase activity. Assays were performed at least 3 times independently.

Chromatin immunoprecipitation (ChIP) assay

The cells were fixed with 1% formaldehyde for 10 min at room temperature and then incubated in 125 mM glycine for 10 min, to quench reactive aldehydes. The cells were collected after rinsing them with phosphate-buffered saline (PBS). The cells were lysed in lysis buffer [10 mM Tris-HCl (pH 8.0), 10 mM NaCl, and 0.2% Triton-X] and sonicated until the chromatin fragments became 200–1000 bp in size. The antibodies used for ChIP were as follows: anti-trimethylated histone H3 Lys 4 (H3K4me3) antibody (#MABI0004: MAB Institute Inc., Sapporo, Japan), anti-trimethylated histone H3 Lys 27 (H3K27me3) antibody (#17-622: Merck Millipore), anti-HP-1 α antibody (#2616: CST Inc., Danvers, MA, USA), and anti-Lamin B1 antibody (#ab16048: Abcam, Cambridge, UK). Rabbit IgG (#PP64B: Merck Millipore) and mouse IgG (#12-371B: Merck Millipore) were used as negative controls to test the specificity of the ChIP results. After the ChIP step, the recovered chromatin samples were subjected to qPCR (ChIP-qPCR) using specific primers (Supplemental Table 2). The percent-input values were calculated as described previously [18]. The qPCR data for each primer pair were represented as percent-input by calculating each specific DNA fragment in immunoprecipitates relative to that in input DNA. The final values were determined by subtraction of the percent-input value obtained for a negative control immunoprecipitation with rabbit IgG or mouse IgG from the percent-input value of interest.

Statistical analysis

Differences between 2 independent samples were evaluated by performing two-tailed Student's *t* test. All error bars represent the standard error of mean (SEM). Linear regression analysis was used to analyze correlations between gene expression and DNA methylation.

Results and discussion

Identification of a differentially methylated region of the human *TERT* gene

Initially, we confirmed *TERT* expression in human embryonic stem cells (ESCs), embryonic carcinoma cells (ECCs), and iPSCs and their parental somatic cells. The *TERT* gene was expressed in pluripotent stem cells including ESCs, ECCs, and iPSCs, but was not detected in any of the parental somatic cells (Fig. 1a).

Analysis of DNA methylation rates around the *TERT* promoter using DNA methylation array data appeared to indicate that the distal region (probe positions a–c in Fig. 1b) was more highly methylated in iPSCs than in somatic cells, although little difference was found at the proximal region (probe positions d–f in Fig. 1b) between iPSCs and somatic cells (Fig. 1b). Bisulfite sequencing analysis was performed to further investigate the DNA methylation state of individual CpG sites around the *TERT* promoter from positions – 2056 to + 46 nt, which contains 123 CpG sites. Regardless of *TERT* expression and the cell types, the CpG sites located between – 610 and + 46 nt were rarely methylated and those located between – 2056 and – 1566 nt were highly methylated. However, the CpG sites from – 1415 to – 638 nt showed cell type-specific differential methylation. The methylation rates at this region in somatic cells were 20.29, 29.71, 34.12, 40.59, and 36.18% in AM936EP, Edom22, MRC-5, PAE551, and UtE1104 cells, respectively. In contrast, the methylation rates in pluripotent stem cells were 88.24, 92.94, 89.71, 80.88, and 72.06% in MRC-iPS-25, PAE-iPS-1, UtE-iPS-6, HUES-8 and NCR-G3 cells, respectively (Fig. 2a). This region includes 34 CpG sites that was highly methylated in ESCs, iPSCs, and ECCs expressing the *TERT* gene, but was hypomethylated in somatic cells lacking *TERT* expression. We designated this region as *TERT*-DMR (*TERT* differentially methylated region).

To gain insight into the correlation between gene expression and DNA methylation, a scatter plot of gene expression versus DNA methylation with pluripotent marker genes including *POU5F1* (also known as *OCT-4*), *SALLA*, *DPPA4*, and *TERT* of 2 ESCs, 6 iPSCs, and the parental somatic cells were plotted using array data for gene expression and DNA methylation. As expected, *POU5F1*, *SALLA*, and

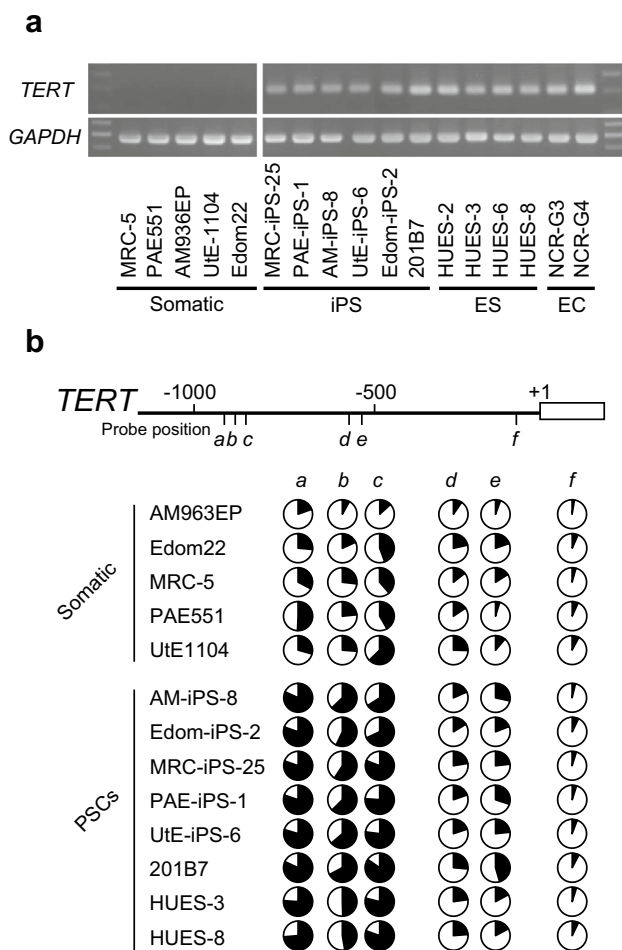


Fig. 1 Expression of the human *TERT* gene and DNA methylation of the *TERT* promoter region. **a** Expression of the *TERT* gene in 5 somatic, 6 iPSC, 4 ESC, and 2 ECC lines, as detected by RT-PCR. The upper and lower panels show *TERT* and *GAPDH* expression, respectively. **b** DNA methylation rate at the *TERT* promoter region in parental somatic cells and pluripotent stem cells. (Top) diagram of the *TERT* gene. The white box indicates the 1st exon of the *TERT* gene. The position of the transcription start site (TSS) was designated as +1. Black vertical lines (a–f) indicate the position of each probe. The probe identification numbers of a–f are cg07380026 (– 845), cg26006951 (– 826), cg17166338 (– 807), cg10767223 (– 637), cg11625005 (– 575), and cg10896616 (– 105), respectively. (Bottom) DNA methylation levels were determined with the Illumina Infinium HumanMethylation450K assay. The relative ratios of methylated and unmethylated DNA are indicated by the black and white areas, respectively, in the pie chart. PSCs, pluripotent stem cells

DPPA4 showed negative correlations (Fig. 2b; $R^2 = 0.8428$, 0.9745, 0.8039, respectively) between DNA methylation and gene expression by linear regression analysis. These results indicated that *POU5F1*, *SALL4*, and *DPPA4* with hypomethylated promoters were highly expressed in ESCs and iPSCs, whereas low expression of those genes with hypermethylated promoters occurred in the parental somatic cells. Interestingly, *TERT* showed positive correlation (Fig. 2b; $R^2 = 0.9117$) between gene expression and

DNA methylation at the distal promoter region, suggesting that in ESCs and iPSCs, *TERT* was highly expressed from hypermethylated promoters and that in the parent somatic cells, *TERT* were poorly expressed from hypomethylated promoters. These results suggested the *TERT* expression is restricted in pluripotent stem cells and that *TERT* expression might be regulated by DNA methylation in manner different than other pluripotent marker genes.

Demethylation led to suppression of the human *TERT* gene

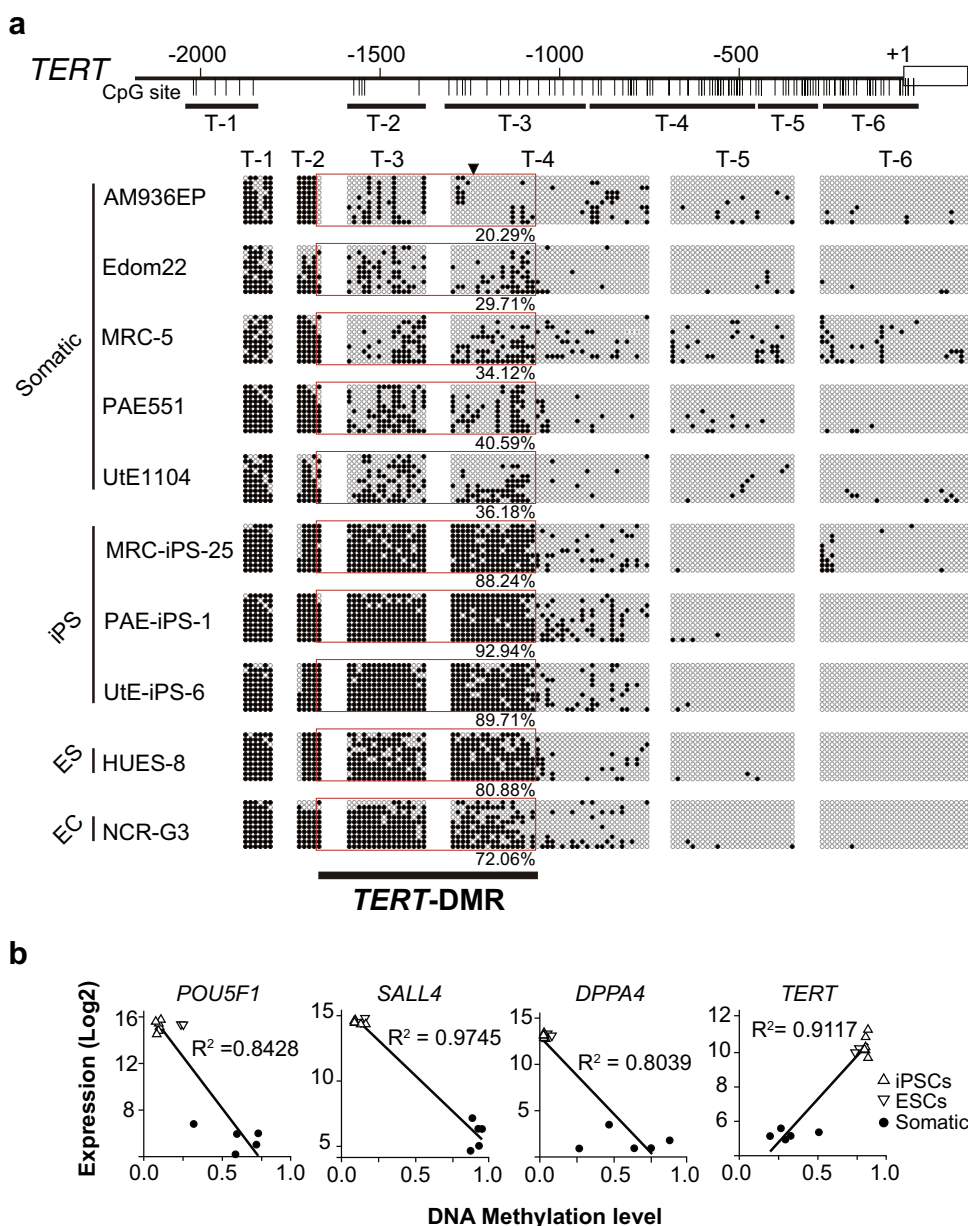
To investigate whether *TERT* expression was regulated by the DNA methylation status of the *TERT*-DMR, we conducted demethylation assay using MRC-iPSCs. In the demethylation assay, MRC-iPSCs were treated with a demethylating reagent (5-aza-dC) for 4 days, and *TERT* expression was measured. Treatment with 5-aza-dC caused significant demethylation at the *TERT*-DMR (Supplemental Fig. 1) and significant suppression of the *TERT* gene in iPSCs (Fig. 3). Expression of typical stem cell marker genes, *POU5F1*, *NANOG*, *SOX2*, *SALL4*, and *EPHA1* was still maintained in 5-aza-dC-treated iPSCs (Fig. 3). These results suggested that hypermethylation of the *TERT*-DMR is important for maintaining *TERT* expression in iPSCs.

Methylation at the *TERT*-DMR enhanced promoter activity

To further analyze of the effect of DNA methylation at the *TERT*-DMR on *TERT* transcription, region-specific methylated-promoter assays were performed using MRC-iPSCs and the parental somatic cells. We designed four reporter constructs: empty vector (pGL4.10), *TERT*-626, *TERT*-1560, and *TERT*-1560_Me. The DNA sequences of *TERT*-1560 and *TERT*-1560_Me were identical, with the only difference being in their DNA methylation states. The *TERT*-626 construct, containing the *TERT*-proximal region but not the *TERT*-DMR, showed a significant increase in luciferase activity relative to that of the empty pGL4.10 vector in iPSCs. The *TERT*-1560 construct showed significantly less reporter activity relative to *TERT*-626, being comparable to pGL4.10. Interestingly, the *TERT*-1560_Me construct showed significantly higher reporter activity than the unmethylated construct, *TERT*-1560 (Fig. 4). In the parental somatic cells (MRC-5), none of the constructs showed significantly increased activity relative to that of the pGL4.10. These results suggested that DNA hypermethylation of the *TERT*-DMR was indispensable for *TERT* expression and that the *TERT*-DMR included an expression regulatory element.

Combining the results of the demethylation and methylation assays revealed the possibility that the *TERT*-DMR contains a region that suppresses *TERT* expression. Thus,

Fig. 2 Bisulfite sequencing analysis and correlation between DNA methylation and gene expression. **a** Bisulfite sequencing analysis of the 5'-flanking regions of the *TERT* gene. (Top) diagram of the *TERT* gene. The white box indicates the 1st exon of the *TERT* gene. Black vertical lines indicate the position of individual CpG sites. The position of the TSS was designated as +1. Black horizontal lines (T1–T6) indicate the regions studied by bisulfite sequencing. (Bottom) the open and closed circles indicate the unmethylated and methylated states of each CpG site, respectively. Black arrowhead represents the position of the CpG site in Infinium HumanMethylation450K assay (cg07380026). The horizontal line at the bottom of the figure indicates the defined *TERT*-DMR extending from positions –1415 to –638 nt. **b** Scatter plot of DNA methylation and gene expression levels for *POU5F1*, *SALL4*, *DPPA4*, and *TERT*. DNA methylation levels were determined by performing the Illumina Infinium HumanMethylation450K assay. The methylation probes used for *POU5F1*, *SALL4*, *DPPA4*, and *TERT* (shown in the plot) are cg15948871, cg10941835, cg14836960, and cg07380026, respectively. White triangles, iPSCs ($n = 6$). White inverted triangles, ESCs ($n = 2$). Black circles, somatic cells ($n = 5$). Black lines, linear regression lines



we performed electrophoretic mobility shift assay (EMSA) to determine whether hypermethylation of the *TERT*-DMR prevents certain transcriptional suppressors from binding the *TERT*-DMR. However, no factors that depend on the methylation status were detected (Supplemental Fig. 2a, b).

Methylated *TERT*-DMR maintained open chromatin

Histone modification is another epigenetic mechanism for regulating gene expression. Trimethylated histone H3 Lys 4 (H3K4me3) tends to distribute in “active” gene loci [19], and distribute in hypomethylated DNA region [20]. In contrast, trimethylated histone H3 Lys 27 (H3K27me3) tends to distribute in “inactive” gene loci [19]. In addition, inactive

gene loci are generally enriched for the heterochromatic protein HP-1 α and form heterochromatin. The genomic DNA in heterochromatin is usually hypermethylated [21]. Therefore, we performed ChIP-qPCR for H3K4me3, H3K27me3, and HP-1 α to determine the histone modification status at the *TERT*-DMR. ChIP-qPCR revealed that H3K4me3 was significantly enriched in iPSCs, whereas the H3K27me3 and HP-1 α were significantly enriched in somatic cells (Fig. 5a–d). Furthermore, analyzing the dataset of ATAC-seq analysis [22] of human ESCs (GSM2433022) [23] and human somatic cells (GSM1939273) revealed that the chromatin structure around the *TERT*-DMR was “open” in *TERT*-expressing pluripotent cells, but was closed in somatic cells. These data indicated that histones at the *TERT*-DMR

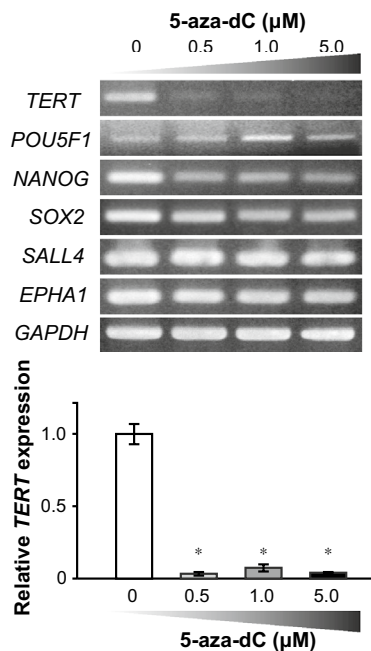


Fig. 3 Demethylation of the *TERT*-DMR led to suppression of *TERT* expression. Effect of a demethylation reagent (5-aza-dC) on the expression of *TERT* and pluripotent marker genes in MRC-iPSCs. (Top) *TERT*, *POU5F1*, *NANOG*, *SOX2*, *SALL4*, *EPHA1*, and *GAPDH* gene expression. *GAPDH* was detected as an internal control. (Bottom) relative *TERT* gene expression levels measured by qPCR, with normalization to *GAPDH* expression using the delta-delta CT method. The data shown represent the mean \pm SE. * $p < 0.01$

were active state, even though the DNA was highly methylated in human iPSCs.

Role of hypermethylation of the *TERT*-DMR in regulating *TERT* expression

In human iPSCs that express the *TERT* gene, the *TERT*-DMR was hypermethylated under the active state of histone modification. These results suggested that hypermethylation of the *TERT*-DMR promoted *TERT* expression. To clarify the role of hypermethylation at the *TERT*-DMR in regulating *TERT* gene expression, we searched for factors that interact with the *TERT*-DMR. First, we performed chromosome conformation capture (3C) and Splinkerete-3C (S3C) assays (see Supplementary methods) to determine whether hypermethylation of the *TERT*-DMR affects the genomic interaction between the *TERT*-DMR and other genomic loci. No genomic interaction was detected (data not shown).

Next, we focused on the nuclear lamina. The nuclear lamina is a polymeric protein meshwork associated with the nucleoplasmic surface of the inner nuclear membrane [24, 25] that has been associated with gene silencing by interacting with genomic DNA [26, 27]. We performed ChIP-qPCR for Lamin B1, which is a representative component of the

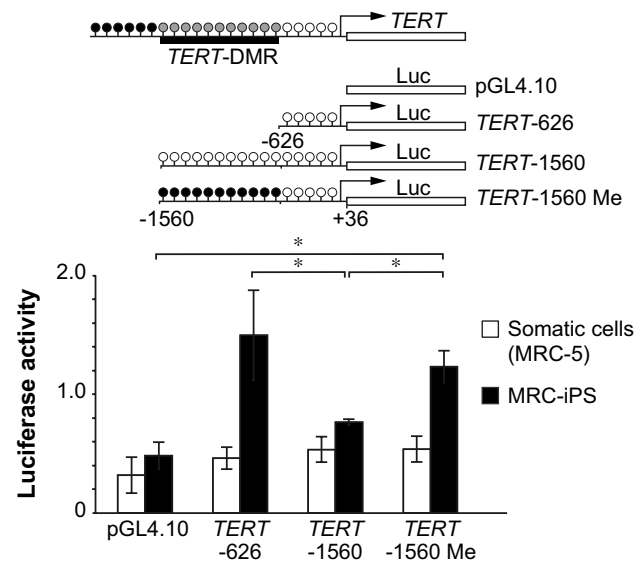
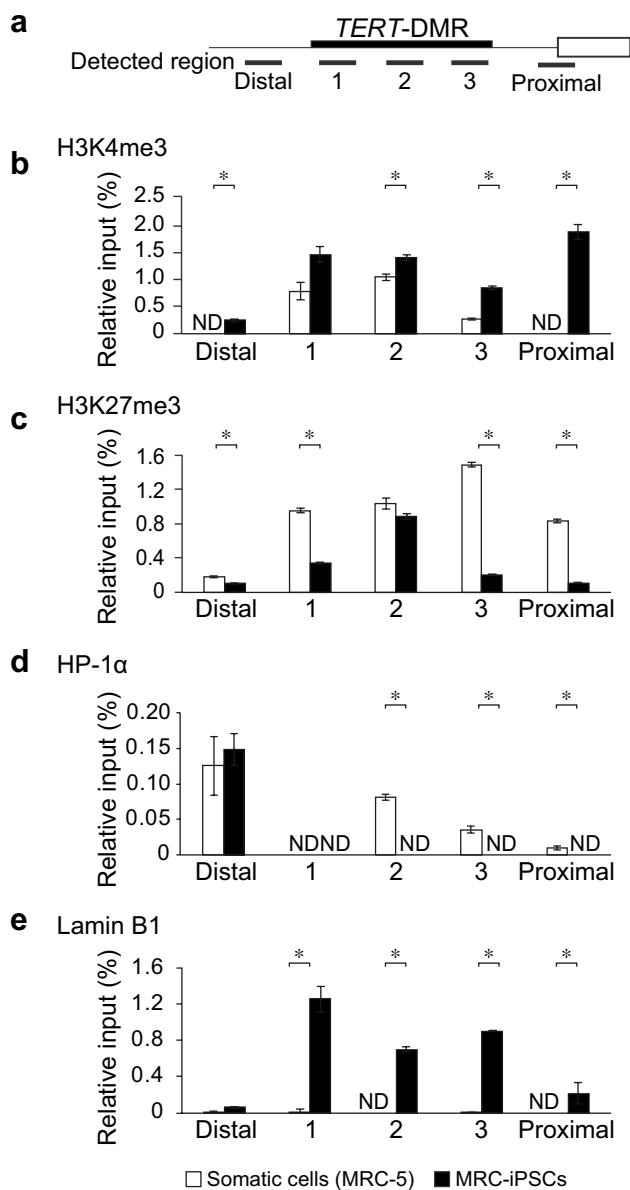


Fig. 4 Methylation of the *TERT*-DMR enhanced the promoter activity. Region-specific methylated-reporter assay in MRC-iPSCs and parental somatic cells (MRC-5). (Top) schematic representation of the *TERT* gene and reporter constructs. Open and closed circles indicate unmethylated and methylated CpG sites in the constructs, respectively. (Bottom) luciferase activity of each reporter construct. White bars indicate luciferase activities in parent somatic cells (MRC-5). Black bars indicate luciferase activities in MRC-iPSCs. Each bar shown in the figure represents the mean \pm SEM. * $p < 0.05$

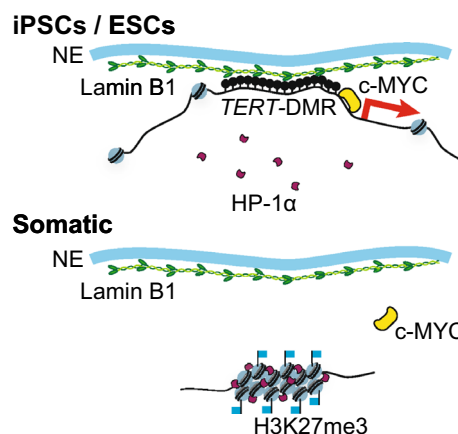
nuclear lamina, to determine whether hypermethylation of the *TERT*-DMR inhibited binding to the nuclear lamina. Unexpectedly, Lamin B1 was enriched at the *TERT*-DMR in iPSCs, but not in somatic cells (Fig. 5e), meaning that Lamin B1 was selectively interacted with methylated *TERT*-DMR.

Our findings suggested the model whereby human *TERT* expression is regulated: in the *TERT*-expressing cells such as iPSCs and ESCs, the *TERT*-DMR is highly methylated and binds to the nuclear lamina via Lamin B1, which lead to an open chromatin structure, enabling transcription factors, such as c-MYC [28, 29], to bind the proximal promoter and drive *TERT* expression. In contrast, in cells not expressing *TERT* such as most somatic cells, the unmethylated *TERT*-DMR is free in the nucleus. H3K27me3 and HP-1 α become enriched at the unmethylated *TERT*-DMR, forming heterochromatin (Fig. 6).

Several groups have discussed the epigenetic regulation of the human *TERT* gene [30–33]; however, no clear relationship between DNA methylation and *TERT* expression has been demonstrated. One reason why the epigenetic regulation of *TERT* has been poorly understood may be that previous studies were focused on the proximal core promoter region (approximately 500-bp upstream from the TSS). In this study, we focused on the distal promoter region of the *TERT* gene and identified the *TERT*-DMR, which provides additional information for the human *TERT* gene.



Of note, the demethylating reagent 5-aza-dC (also known as decitabine) has been used clinically for treating acute myeloid leukemia (AML) in patients whose malignant cells express *TERT* gene [34]. In addition, treatment with decitabine has been reported to suppress expression and inhibit malignant cell proliferation [35, 36]. However,



exactly how decitabine suppresses *TERT* expression remains unclear. In this study, demethylating *TERT*-DMR with 5-aza-dC suppressed *TERT* expression. Therefore, the therapeutic effect of decitabine in AML potentially involves the inhibition of *TERT* expression through *TERT*-DMR demethylation. Moreover, monitoring DNA methylation at the *TERT*-DMR may be useful for diagnosis, prognosis, and as a biomarker for estimating the risk for developing AML.

In conclusion, we identified *TERT*-DMR at the distal *TERT* promoter region. Hypermethylation of the *TERT*-DMR promoted activation of *TERT* gene expression by binding to the nuclear membrane. Our findings shed light on a new functional aspect of DNA methylation with respect to gene expression. This novel epigenetic regulation mechanism is required for the reprogramming the transformation of somatic cells into iPSCs.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was approved by the research ethics committee of University of Miyazaki and Institutional Review Board of the National Institute for Child Health and Development. All procedures performed in studied involving the handling of human cells were in accordance with the ethical standards of the 1964 Helsinki declaration and its later amendments or comparable ethical standard.

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