

Supplementary material

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

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Supplementary methods

Quantitative combined bisulfite restriction analysis (COBRA)

COBRA was performed to confirm the DNA-methylation states in various samples. Following PCR amplification and the bisulfite reaction, the PCR product was digested with the restriction enzyme *Taq*^α I (New England BioLabs, Beverly, MA). After digestion, quantitative COBRA coupled with the Shimadzu MCE®-202 MultiNa platform (Shimadzu, Japan) was conducted to quantitatively determine the DNA-methylation ratio. The sequences of the primers used in this assay are T4 in Supplemental Table 2.

Electrophoretic Mobility Shift Assay (EMSA) experiments

Crude nuclear extracts were prepared from MRC-5 cells and MRC-iPSCs. The cells were scraped from the culture dishes, suspended in 5 ml of PBS, and centrifuged at 1,000 rpm for 3 min. The cell pellet was resuspended in 400 µl of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 1× protease inhibitor cocktail; Roche, Basel, Switzerland). After a 20-min incubation on ice, the cells were vortexed for 1 min and centrifuged at 15,000 rpm for 10 sec. All steps to recover the nuclear proteins were performed at 4°C. The protein concentrations of nuclear extracts were determined by the BCA method (Thermo). EMSA experiments were performed with the DIG Gel Shift Kit, 2nd generation (Roche) according to the manufacturer's instructions. Briefly, DNA fragments of the human *TERT* promoter region were obtained by PCR amplification of genomic DNA with specific primers. The sequences of primers used in this study are summarized in Supplemental Table 2. The fragments were labelled with digoxigenin-11-ddUTP (DIG) by terminal transferase. Then, 2 µg of crude nuclear extract derived from MRC-5 cells and MRC-iPSCs was incubated with DIG-labelled fragments in a buffer (20 µl) containing 1 µg of poly(d[A-T]) and 1 µg poly L-lysine. Following a 20-min incubation at room temperature, the samples were loaded on 4% non-denaturing polyacrylamide gels. For the competition assay, a 40-fold excess of unlabelled fragment probe was premixed with the crude nuclear extract for 20 min before adding DIG-labelled fragments. Signals were captured by chemiluminescent detection with an alkaline phosphatase-labelled anti-DIG antibody, using CSPD (Roche) as a substrate.

Performing Chromosome conformation capture (3C) and Splinkerete-3C (S3C) assays

The 3C assays were conducted as described by Tomikawa *et al.* [1] with some modification. Chromatin crosslinked with 1% formaldehyde was digested overnight at 37°C with 500 U of *PvuII* (#R0151T, NEB), and was self-ligated. The 3C products were extracted in phenol/chloroform, ethanol precipitated, and dissolved in Tris-EDTA buffer (pH 8.0; NAKARAI Tesque, Japan). The *TERT*-DMR chromatin loop was analyzed by PCR, using specific primers. The S3C assay was developed by slightly modifying the 3C assay. Chromatin crosslinked with 1% formaldehyde was digested with *PvuII* and self-ligated. Then the DNA was digested by *BglII* (#R0144, NEB), *BstYI* (#R0523, NEB) and *BamHI* (#R0136, NEB), which are compatible-end restriction enzymes. Digested fragments were ligated with an adaptor sequence and amplified by PCR using a target-specific primer and adaptor sequence primer. Then the amplicons were cloned in to the *LacZ* region of the *EcoRV*-digested pBSII-SK(-) vector and sequenced. The primer positions are indicated in Supplemental Fig. 2a. The sequences of the primers used in this study are summarized in Supplemental Table 2.

Supplementary reference

1. Tomikawa J, Uenoyama Y, Ozawa M, Fukunuma T, Takase K, Goto T, et al. Epigenetic regulation of *Kiss1* gene expression mediating estrogen-positive feedback action in the mouse brain. *Proc Natl Acad Sci USA*. National Acad Sciences; 2012;109: E1294–301. doi:10.1073/pnas.1114245109

Supplemental Table 1. List of used cell lines used in this study.

Cell ID	Description	Karyotype	Ref.	Accession No.		
				Expression	Ref.	Methylation
MRC-5	Fetal lung fibroblast cells	46 XY	[10]	GSM520665	[13]	GSM2650818
AM936EP	Amnion-derived cells	46 XX	[9]	GSM520690	[13]	GSM1906516
PAE551	Placental artery endothelial cells	46 XY	[9]	GSM520704	[13]	GSM2650820
Edom22	Menstrual blood-derived cells	46 XX	[8]	GSM608016	[13]	GSM1906515
UtE1104	Endometrium-derived cells	46 XX	[8]	GSM520692	[13]	GSM1906514
MRC-iPS-25	MRC-5-derived iPSC	46 XY	[12]	GSM608022	[13]	GSM2650819
AM-iPS-8	AM936EP-derived iPSC	46 XX	[9]	GSM1032389	[13]	GSM1906535
PAE-iPS-1	PAE551-derived iPSC	46 XY	[13]	GSM1032440	[13]	GSM2650821
Edom-iPS-2	Edom22-derived iPSC	46 XX	[13]	GSM608018	[13]	GSM2650827
UtE-iPS-6	UtE1104-derived iPSC	46 XX	[13]	GSM1032434	[13]	GSM2650831
201B7	Fibroblast-derived iPSC	46 XX	[1]	GSM608028	[16]	GSM2650822
HUES-2	Embryonic stem cells	46 XX	[14]	-	-	-
HUES-3	Embryonic stem cells	46 XY	[14]	GSM608029	[13]	GSM2650816
HUES-6	Embryonic stem cells	46 XX	[14]	-	-	-
HUES-8	Embryonic stem cells	46 XY	[14]	GSM608030	[13]	GSM2650817
NCR-G3	Embryonal carcinoma cells	XY	[11]	-	-	-
NCR-G4	Embryonal carcinoma cells	XY	[11]	-	-	-

Platform of the expression profiling array data is Agilent Whole Human Genome Microarray chip G4112F, and that of the DNA methylation profiling array data is Illumina HumanMethylation450K BeadChip.

HumanMethylation450K data were submitted to the GEO under accession numbers GSE73938, GSE99716 in this study.

Supplemental Table 2. Sequences of primer sets and adaptors used in this study.

Primers for RT-PCR

	Forward (5' -> 3')	Reverse (5' -> 3')
<i>TERT</i>	GAGCAAGTTGCAAAGCATTG	TTTCTCTGCGGAACGTTCTG
<i>NANOG</i>	CCAGACCCAGAACATCCAGT	CAGAGCAAGACTCCGTTTCA
<i>SOX2</i>	GTTTGC AAAAGGGGGAAAGT	GAGGAAAATCAGGCGAAGAA
<i>SALL4</i>	GAAAACGGTTCCTGGAGAG	ACGAGAAGTTCTTCCACACC
<i>EPHA1</i>	GTCGTCACAAAGCGAAAGC	AGCACCTCCCACATCACAAT
<i>GAPDH</i>	GCTCAGACACCATGGGGAAGGT	GTGGTGCAGGAGGCATTGCTGA

Primers for Bisulfite-PCR

	Forward (5' -> 3')	Reverse (5' -> 3')
T1	TagttTaTtTtggTtTtaggTt	ActtctacctaAacccaAatAttct
T2	agaaaTatTtgggtTtgggttaggaagT	tcttctaAaaatcccctaAtAatc
T3	gatTaTtaaggggattTtagaaga	AatccaAAcacctAActcaaAcct
T4	gaggTtggagTtaggtTtTtgaT	aAacccaAAAActAcctccaAatc
T5	gaTtTggaggTagTTTtgggtTt	aatccactAAAaAccCGAcctAAc
T6	TagaTTTTcggtTcgTTCggagTagT	AAAtAAccGAAAccaAAActccca

*Upper cased in primer sequences represent converted nucleotide for bisulfite PCR.

Primers for ChIP-PCR

	Forward (5' -> 3')	Reverse (5' -> 3')
Distal	CTCCTGTGGAGGAAGGAATG	CAGCCTGAGCAACAAGAGTG
<i>TERT</i> -DMR 1	TTTGCCCTAGTGGCAGAGAC	GCTGGAGGTTAGCCTCGTC
<i>TERT</i> -DMR 2	CTGGGAAGTCCTCAGCTGTC	GACTTCCAGCCCAGCAGTAG
<i>TERT</i> -DMR 3	AGATTTGGGGTGGTTTGCTC	ACTTGGGCTCCTTGACACAG
Proximal	TCGCGGCGGAGTTTCAGGCAG	AGCACCTCGCGGTAGTGG

Primers for PCR to generate EMSA fragment

	Forward (5' -> 3')	Reverse (5' -> 3')
E1	TTGTGTTTTCTATGTTGGCTTCTC	CTTCTAGAAATCCCCTTAGTGATCC
E2	CTAGTGGCAGAGACAATTCACAAAC	GGAGCTAGCATTTGAACAGG
E3	GCTCCATAAAATAAAGCAATTCCTC	GTTGCTCAAGTTTGGATCTAAGG
E4	TACATTTAAGGTTGCGTTTGTAGC	GCAGGACAGCTGAGGACTTC
E5	CTTAGATCCAAACTTGAGCAACC	CTCGGACTGCAGAGCTAGGAG
E6	CTACTGCTGGGCTGGAAGTC	GATGAGGCCAACATCTGGTC
E7	GGGATGTGACCAGATGTTGG	CATTTCTCTTGCAGGTTCTCAG
E8	GTGGTTTGCTCATGGTGGGG	GGACGTGAAGGGGAGGACG

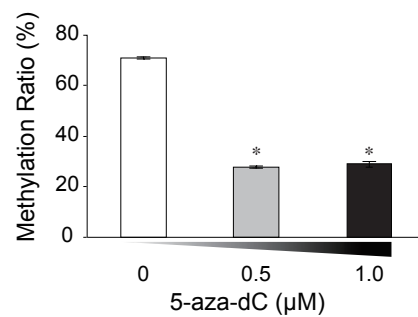
Primers for 3C assay

	Sequence (5' -> 3')	Region
a	AAGCACAGCCATCACTTAGC	Upstream of <i>TERT</i> gene
b	TGTAGTGAGGCCTGGGTCAG	Upstream of <i>TERT</i> gene
c	CTGCGTCTGCTTCTGCAGTC	Upstream of <i>TERT</i> gene
d	GGACGTGAAGGGGAGGACG	<i>TERT</i> -DMR
e	GCTCCATAAAATAAAGCAATTCCTC	<i>TERT</i> -DMR
f	AAGCACATGGCTCACACGTG	Downstream of <i>TERT</i> gene
g	ATGTGCTGCAGCGTGCCAG	Downstream of <i>TERT</i> gene
h	CTGTGTGCTGCTGCTCTTGC	Downstream of <i>TERT</i> gene
i	GGCCTCAGAGAGCTGAGTAG	Downstream of <i>TERT</i> gene
j	CTGTGGAAGCAGAGGTCAG	Downstream of <i>TERT</i> gene

Primers and adaptor for S3C assay

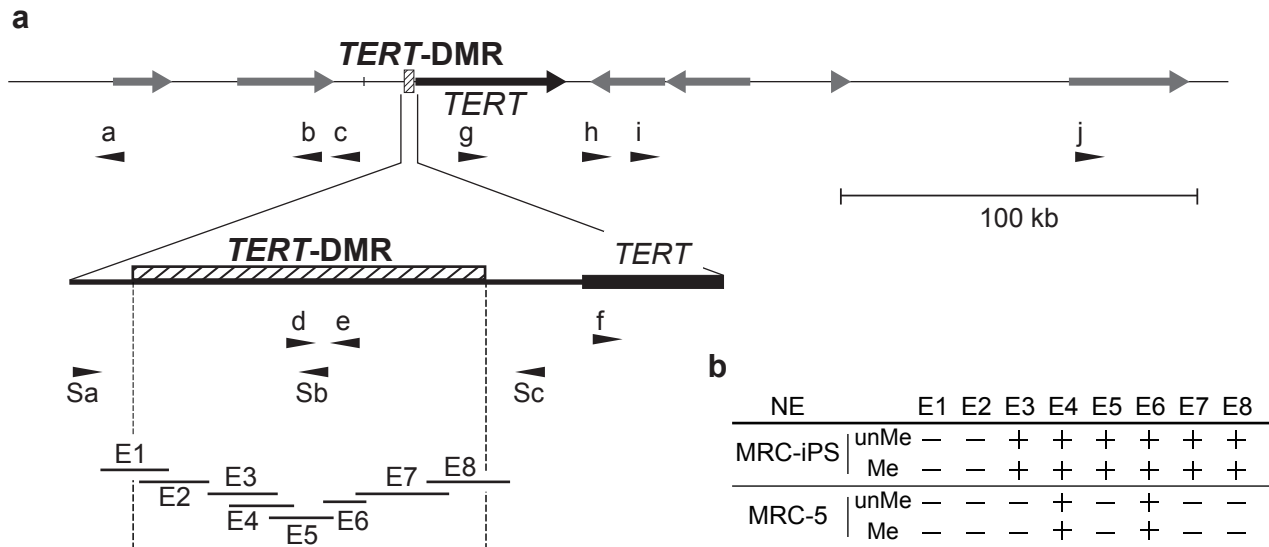
	Forward (5' -> 3')	What for
SPLNK-BOT	CGAAGAGTAACCGTTGCTAGGAGAGACCGTGGCTG AATGAGACTGGTGTGACACTAGTGG	Splinkerrete adaptor
SPLNK-TOP	GATCCCACTAGTGTGACACCAGTCTCTAATTTTT TTTTCAAAAAA	Splinkerrete adaptor
SPLNK 1 st	CGAAGAGTAACCGTTGCTAGGAGAGACC	Splinkerrete primer
SPLNK 2 nd	ctgcaggaattcgatGTGGCTGAATGAGACTGGTGTGAC	Splinkerrete primer
Sa-1 st	CTAGGCCGATTTCGACCTCTCT	Gene specific primer
Sa-2 nd	atcgataagcttgatCCTCGCTGGCGTCCCTGCAC	Gene specific primer
Sb-1 st	CTTAGATCCAAACTTGAGCAACC	Gene specific primer
Sb-2 nd	atcgataagcttgatCTTGAGCAACCCGGAGTCTG	Gene specific primer
Sc-1 st	TCGCGCCCTGATTCTACATC	Gene specific primer
Sc-2 nd	atcgataagcttgatACTGGGACCTGAAGCCTGCAG	Gene specific primer

*Lower cased in primer sequences represent arm sequences of *EcoRV* digested pBSII-SK(-).



Supplemental Fig. 1. Methylation levels of *TERT*-DMR following treatment with 5-aza-dC in MRC-iPSCs.

DNA-methylation levels of the *TERT*-DMR, as determined by combined bisulfite restriction analysis (COBRA). MRC-iPSCs were treated with 5-aza-dC for 4 days. Each bar in the figure represents the means $\pm$ SEM.



Supplemental Fig. 2. EMSA in MRC-iPSCs and MRC-5 somatic cells.

(a) Schematic representation of human chr5:1,044,900-1,389,100 indicating the *TERT* gene. Hatched boxes indicate the *TERT*-DMR. The black arrow indicates the *TERT* gene, and the gray arrows indicate other genes neighboring *TERT*. The black bars (E1-E8) indicate the positions of DNA fragments for electrophoretic mobility shift assays (EMSAs).

Arrow heads indicate the positions of primers used for the chromosome conformation capture (3C) and Splinkerete-3C (S3C) assays. (b) Summary of EMSA results for fragments E1-E8 in MRC-iPSCs or MRC-5 somatic cells. Me: methylated EMSA fragment; unMe: unmethylated EMSA fragment; NE: crude nuclear extracts from MRC-iPSCs or MRC-5 somatic cells. Several shifted bands were observed; however, no factor that bound to the *TERT*-DMR in a methylation status-dependent manner was detected. None of the genomic regions interacting with *TERT*-DMR were detected in 3C and S3C assays.