Homocysteine Accelerates Senescence of Endothelial Cells via DNA Hypomethylation of Human Telomerase Reverse Transcriptase

Donghong Zhang, Xiaoli Sun, Jianlan Liu, Xina Xie, Wei Cui, Yi Zhu

**Objective**—Homocysteine can accelerate the senescence of endothelial progenitor cells or endothelial cells (ECs) via telomerase inactivation and length shortening. However, the underlying mechanism is unclear. Here, we investigated whether homocysteine promotes endothelial senescence by reducing the expression and activity of human telomerase reverse transcriptase (hTERT) by DNA methylation to reduce ECs telomerase activity.

**Approach and Results**—When compared with primary human umbilical vein endothelial cells grown under standard conditions, ECs with chronic homocysteine treatment showed accelerated upregulation of p16, p21, and p53, markers of cellular senescence, during 6 to 10 passages. Interestingly, homocysteine-stimulated but not angiotensin II–stimulated ECs senescence could be reversed by hypermethylation induced by folic acid or s-adenosylmethionine supplementation. Meanwhile, homocysteine promoted the shortening of telomere length specifically related to restoration of hTERT transcriptional expression and CCCTC-binding factor binding sites with hTERT promoter hypomethylation, as detected by quantitative real-time polymerase chain reaction, Western blot, methylation-specific polymerase chain reaction, and bisulfite sequencing assay. Electrophoretic mobility shift assay and chromatin immunoprecipitation results showed that homocysteine-reduced telomere activity and homocysteine-induced EC senescence might contribute to hTERT promoter demethylation by increasing CCCTC-binding factor repression and interfering in the SP1 binding to the demethylated hTERT promoter, which might relate with reduced of DNA methyltransferase 1. Furthermore, the CCCTC-binding factor–dependent mechanism of homocysteine-reduced hTERT expression via DNA demethylation was confirmed in aortic endothelia of mice with hyperhomocysteine levels.

**Conclusions**—CCCTC-binding factor and SP1 cross talk may contribute to homocysteine-reduced hTERT DNA methylation and expression in endothelial senescence. (Arterioscler Thromb Vasc Biol. 2015;35:71-78. DOI: 10.1161/ATVBAHA.114.303899.)

**Key Words:** aging ▪ endothelial cells ▪ homocysteine ▪ telomerase reverse transcriptase, human

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Extensive cause and histology evidence demonstrated that age-related senescence and dysfunction of vascular endothelial cells (ECs) play a critical role in human atherosclerotic lesions.1 Age-associated change in ECs includes decreasing compliance and increasing inflammation, which promote atherosclerosis.2 Shear stress–induced nitric oxide (NO) production and endothelial NO synthase (eNOS) activity were reduced in senescent human ECs.3 Homocysteine, an independent risk factor of atherosclerosis, accelerated the onset of senescence in endothelial progenitor cells and ECs via telomerase inactivation and led to cellular dysfunction.3,5 Epidemiological studies have shown that elevated homocysteine levels are associated with shortened human leukocyte telomere length.1,6 As well, some reports have shown that inhibitors of oxidative stress could prevent homocysteine-activated ECs aging.1,5,7,8 However, the precise mechanisms by which homocysteine reduces telomerase activation and length remain to be determined.

Telomerase activity, which maintains telomere length and stabilizes the termini of linear chromosomes, is tightly regulated by the expression of the human telomerase reverse transcriptase (hTERT) gene.9 Moreover, with the characterization of the genomic sequence of hTERT, demethylation with 5-aza-2-deoxycytodine (5-aza) or trichostatin A strongly reduced or even suppressed hTERT gene expression and telomerase activity and shortened telomere length in cancer cells.10,11 Thus, epigenetic mediation of hTERT activity may be critical to carcinogenesis. Recently, our in vivo and in vitro experiments indicated that homocysteine could affect EC dysfunction and vascular smooth muscle cell migration or proliferation via an epigenetic mechanism involving...
Atherosclerosis-related genes. Therefore, DNA demethylation of hTERT may be a candidate target of epigenetic regulation in homocysteine-induced EC senescence. However, homocysteine might reduce transcriptional hTERT expression by DNA hypomethylation, which reversed with homocysteine demethylation of soluble epoxide hydrolase or platelet-derived growth factors and promoted their expression in our previous study. This contradictory hypothesis prompted us to study how DNA methylation of the hTERT promoter can lead to its activation by homocysteine in ECs.

In the core region of the 5'-hTERT promoter (−200 to +100 bp), several binding sites for activators (c-Myc, Sp1, AP2, E2F, and E-box) or repressors (p53, Mad1, MZF-2, ZRF, WT1, and CCCTC-binding factor [CTCF]) might be methylated and coregulate the expression of hTERT in human ECs. Zinn et al demonstrated high expression of hTERT, despite DNA hypermethylation in several cancer cells, which contrasts with low expression with DNA hypomethylation in non-transformed cells, so some repressors might play a major role in hTERT expression and activity especially for DNA hypermethylation in some cancer cells. Similarly, hypermethylation of the CTCF repressor binding site on the hTERT promoter is necessary for reducing its expression in some cancer cells, and treatment with trichostatin A or 5-aza could allow for hTERT transcriptional expression with demethylation.

In the present study, we first showed changes in hTERT core-promoter methylation patterns and the potential role of SP1 and CTCF cross talk in hTERT gene regulation in homocysteine-induced EC senescence in vivo and in vitro. Homocysteine transcriptionally decreased hTERT expression by increasing CTCF but interfered in SP1 binding to the demethylated hTERT promoter in accelerated EC senescence. This effect could be markedly attenuated by pretreatment with folic acid (FA) or S-adenosylmethionine (SAM). Thus, epigenetic regulation of hTERT activity and EC senescence may play a critical role in homocysteine-related cardiovascular diseases.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results
Effect of SAM and FA on Homocysteine-Induced EC Senescence
To test the effects of clinically relevant doses of homocysteine on EC senescence in vitro, we exposed serial passages (P2–P10) of cultured human umbilical vein endothelial cells (HUVECs) to standard extracellular matrix in the presence or absence of 25 μmol/L homocysteine. The expression of p16, p21, and p53, markers of EC senescence, was spontaneously and persistently increased from P4 to P10 with homocysteine treatment (Figure 1A–1D, n=4 for quantitative real-time polymerase chain reaction [qRT-PCR] and Western blot). As expected, homocysteine could accelerate EC senescence when compared with standard conditions. Interestingly, homocysteine-increased p16, p21, and p53 expressions (Figure 1E, n=5) as well as SA-b-Gal–positive cells, apoptosis cells, and cell cycle (Figure 1F and 1G, n=5; Figure 1A and IB in the online-only Data Supplement, n=4 for each experiment), could be almost completely prevented with treatment with 50 μmol/L FA, which lowers plasma homocysteine level by increasing methionine metabolism, or partially prevented by the addition of 50 μmol/L SAM, causing DNA hypermethylation. However, oxidative stress can induce or accelerate the development of cellular senescence. FA, an L-arginine mimic, could also reverse homocysteine-decreased bioavailability of NO by upregulating eNOS expression and downregulating inducible NOS (iNOS) expression in ECs under senescence (Figure II A and II B in the online-only Data Supplement, n=4 for each experiment). Therefore, FA- or SAM-induced DNA hypermethylation involved homocysteine-accelerated human endothelial senescence, independent of NO availability.

Homocysteine Demethylating the CTCF-Binding Site on the hTERT Promoter Is Associated With Telomere Attrition in ECs Under Senescence
Several reports have demonstrated that early onset senescence may be attributed to accelerated telomere attrition, probably from the generation of single-strand breaks in telomeric DNA. We used a previously described telomere-length assay system and found that homocysteine shortened telomere length in 2-cell passages when compared with cells grown under standard conditions (Figure III A in the online-only Data Supplement, n=4). Among 4 major essential elements of human telomerase, including the RNA subunit (hTERT), catalytic subunit (hTERT), TRF2, and TP1, only hTERT expression was reduced with homocysteine stimulation at 50 μmol/L for 72 hours in ECs (Figure III B in the online-only Data Supplement, n=5). Indeed, homocysteine also accelerated the reduction in hTERT mRNA and protein expression from P6 to P10 (Figure 2A, for Western blot assay, n=4; qRT-PCR, n=5). In addition, homocysteine decreased the expression of hTERT in a dose-dependent manner in P4-5 HUVECs (Figure 2B, for Western blot assay, n=4; qRT-PCR, n=5).

Because hTERT expression is often modulated by epigenetic processes in cancer cells, to understand the epigenetic regulation mechanism in the repression of hTERT in ECs with homocysteine treatment better, we performed bisulfite sequencing and methylation-specific PCR assay and examined changes in methylation of the core region of CpG sites in the hTERT promoter (from −200 to +100 bp, surrounding TSS and containing 37 CpG sites; Figure 2C, n=5). Bisulfite sequencing sequencing revealed only partial methylation

Nonstandard Abbreviations and Acronyms

<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>5-aza</td>
<td>5-aza-deoxycytidine</td>
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<tr>
<td>CTCF</td>
<td>CCCTC-binding factor</td>
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<tr>
<td>DNMT1</td>
<td>DNA methyltransferase 1</td>
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<td>EC</td>
<td>endothelial cells</td>
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<td>FA</td>
<td>folic acid</td>
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<tr>
<td>hTERT</td>
<td>human telomerase reverse transcriptase</td>
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<td>HUVEC</td>
<td>human umbilical vein endothelial cell</td>
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<tr>
<td>qRT-PCR</td>
<td>quantitative real-time polymerase chain reaction</td>
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<td>SAM</td>
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Zhang et al. Hcy Accelerates EC Senescence by Reducing hTERT

(16.9%, 4 clones, 25 of 148 CpG sites) in the core region of the hTERT promoter in P4 to P5 HUVECs without homocysteine treatment. The CpGs in these regions mainly contained the binding sites for SP1, E2F, and CTCF, and the methylation was decreased by prolonged homocysteine treatment (50 μmol/L) for 72 hours. Specially, the methylation of 3 CpG sites (32–34) for CTCF binding was reduced from 66.7% to 16.7%. Also, 4 CpG sites (16–19) for SP1 and E2F were transformed from hyper- to hypomethylation (62.5%–12.5%). Interestingly, this effect was completely reversed by FA treatment (≤83.3% for CTCF, 68.8% for SP1 binding sites). To examine the effect of CTCF-binding site CpG methylation on the aging of HUVECs, we performed methylation-specific PCR assay with primers designed to focus on CTCF-binding sites. Methylation ratios were greatly reduced from P4 (79.9±3.9% to 61.3±2.3%) to P10 (73.2±4.8% to 39.1±2.4%) with homocysteine treatment when compared with control treatment (Figure 2D, n=4). Similarly, FA or SAM presupplementation could abolish homocysteine demethylation of CTCF-binding sites for hTERT, as well as hTERT mRNA and protein expression (Figure 2D–2G, for Western blot assay, n=4; for qRT-PCR and methylation-specific PCR, n=5).

Specifically, angiotensin II–caused SA-b-Gal positivity in ECs could be partially inhibited by l-arginine or FA but not by SAM-stimulating NO bioavailability (Figure IIIC–IIIF in the online-only Data Supplement, n=5 for quantitative real-time PCR, methylation-specific PCR, and senescence-associated β-galactosidase staining assay). Homocysteine and 5-aza demethylation of the hTERT promoter and reduced hTERT expression also led to increased SA-b-Gal–positive ECs, not related to the NO pathway.

Homocysteine Increases CTCF and Interferes in SP1 Binding to the hTERT Promoter in ECs In Vivo

Sp1 could cooperate with the oncogene c-Myc to activate transcription of hTERT gene. CTCF is a repressor of hTERT activity by demethylating its recognition sequence. To understand the mechanism of cross talk of SP1 and CTCF directly regulating hTERT activity in aging HUVECs better, we first identified the putative SP1 and CTCF-binding sites in the core region of the hTERT promoter and generated their probes. EMSA (electrophoretic mobility shift assay) revealed a super-shift band after the addition of anti–CTCF-specific antibody, and homocysteine or 5-aza treatment increased the binding of the hTERT promoter but with FA abolished (Figure 3A, n=4). Moreover, demethylation by homocysteine or 5-aza treatment also increased the binding of the hTERT promoter but with FA abolished (Figure 3A, n=4). However, demethylation by homocysteine or 5-aza treatment also increased the binding of the hTERT promoter but with FA abolished (Figure 3A, n=4). Furthermore, DNA demethylation by homocysteine and 5-aza could interfere in SP1 binding to the hTERT promoter (0.44-fold; P=0.031 and 0.43-fold;
Reduced hTERT Expression and Activity

Our previous study indicated that homocysteine treatment could specifically decrease the protein expression and activity of DNA methyltransferase 1 (DNMT1; maintaining methyltransferase activity) in ECs and was related to platelet-derived growth factor transcriptional upregulation. We transfected siRNA-DNMT1 in P4 to P5 of HUVECs and found that DNMT1 knockdown (Figure IVC in the online-only Data Supplement, n=4) could reduce the expression of hTERT, completely independent of homocysteine concentration, but induce inactivity of hTERT and EC senescence, independent of homocysteine only in part. hTERT activity was further decreased and SA-b-Gal–positive cell numbers were increased with homocysteine treatment at 200 to 500 μmol/L when DNMT1 was knocked down (Figure 4A–4C, n=5 for qRT-PCR, relative telomere activity and senescence-associated β-galactosidase staining). Homocysteine-repressed DNMT1 expression might contribute to DNA global demethylation, including the hTERT gene, and enhance CTCF binding to the hTERT promoter, thus resulting in reduced hTERT expression and activity.

Homocysteine-Upregulation of TERT Was Confirmed in the Aortic Intima of Mice With Hyperhomocysteinemia

Previously, we established a mild and moderate hyperhomocysteinemia model (total plasma homocysteine level 27.6±4.5 and 61.5±31.4 μmol/L) with activity in the aortic endothelium in C57BL/6J mice by feeding with a diet supplemented with 2% methionine for 4 or 8 weeks (n=8 for each group). Real-time RT-PCR revealed 2.23±1.30-fold (P=0.043) and 4.29±1.65-fold (P=0.014) increased p16 level

Figure 2. Homocysteine (Hcy) restores human telomerase reverse transcriptase (hTERT) expression by demethylating the CCCTC-binding factor (CTCF) binding site on the hTERT promoter in endothelial cells (ECs). Western blot and quantitative real-time polymerase chain reaction (qRT-PCR) analysis of hTERT protein and mRNA levels, respectively, in P2 to P10 (A) or P4 (B) HUVECs treated with Hcy. TATA binding protein (TBP) was an internal control for Western blot, and β-actin was an internal control for qRT-PCR. C, Structure of CpG sites (short vertical lines) and CpG island (blue) on the hTERT promoter (−2000 to 1735 bp). Location of transcriptional factors in the core region (−200 to +100 bp) of the hTERT promoter (black box). Bisulfite sequencing (BGS) analysis of P4 to P6 HUVECs treated with Hcy, 50 μmol/L, for 72 hours with or without presupplementation with folic acid (FA; 100 μmol/L) for 1 hour. Each row indicates a clone from BGS to obtain a representative sampling of 37 CpG methylation patterns in the core promoter. Each circle corresponds to a single CpG site. Methylated sites are shown as filled circles, unmethylated sites as empty circles, and deletion or mutation sites as filled triangles. D, Methylation-specific PCR (MSP) of CTCF binding sites in hTERT promoter methylation patterns in P2–P10 HUVECs treated with Hcy. Histogram of mean±SD ratio of DNA methylation to total methylation and unmethylation of hTERT promoters. hTERT promoter methylation patterns (E), mRNA (F), and protein (G) expression in P4 to P6 HUVECs by Hcy stimulated with or without FA and SAM pretreatment. For Western blot assay in A, n=4; For BGS, MSP, and qRT-PCR analysis, n=5. Data are mean±SD and analyzed by Student t test. *P<0.05 vs controls; †P>0.05 vs PBS control in their corresponding passages, ‡P<0.05 vs Hcy treatment. DM indicates DNA methylation marker; M, methylated; and U, unmethylated.

P=0.035, respectively) when compared with the control (Figure 3C and 3D, n=4), and this effect is reversed by CTCF knockdown. In addition, we did not find the interaction of CTCF and SP1 by Co-IP assay (Figure V in the online-only Data Supplement, n=4).

Furthermore, hTERT expression and activity were decreased and EC senescence was increased with siRNA knockdown of SP1 expression, and reversed effect was observed with CTCF knockdown at basal level. However, homocysteine-decreased hTERT expression and activity and increased EC senescence could be reversed with CTCF but not SP1 knockdown (Figure 3E–3G, n=4), and this effect is reversed by CTCF knockdown. In addition, we did not find the interaction of hTERT and EC senescence was increased with siRNA knockdown. In addition, we did not find the interaction of CTCF and SP1 by Co-IP assay (Figure V in the online-only Data Supplement, n=4). Thus, homocysteine-repressed DNMT1 expression might contribute to DNA global demethylation, including the hTERT gene, and enhance CTCF binding to the hTERT promoter, thus resulting in reduced hTERT expression and activity.

Homocysteine-Decreased DNA Methyltransferase 1 Expression Involves Reduced hTERT Expression and Activity

Our previous study indicated that homocysteine treatment could specifically decrease the protein expression and activity of DNA methyltransferase 1 (DNMT1; maintaining methyltransferase activity) in ECs and was related to platelet-derived growth factor transcriptional upregulation. We transfected siRNA-DNMT1 in P4 to P5 of HUVECs and found that DNMT1 knockdown (Figure IVC in the online-only Data Supplement, n=4) could reduce the expression of hTERT, completely independent of homocysteine concentration, but induce inactivity of hTERT and EC senescence, independent of homocysteine only in part. hTERT activity was further decreased and SA-b-Gal–positive cell numbers were increased with homocysteine treatment at 200 to 500 μmol/L when DNMT1 was knocked down (Figure 4A–4C, n=5 for qRT-PCR, relative telomere activity and senescence-associated β-galactosidase staining). Homocysteine-repressed DNMT1 expression might contribute to DNA global demethylation, including the hTERT gene, and enhance CTCF binding to the hTERT promoter, thus resulting in reduced hTERT expression and activity.
Although homocysteine-reduced telomerase activity accelerated endothelial senescence has been reported for decades, the detailed mechanism has not yet been elucidated. Here, we describe and confirm a novel hypothesis that cross talk of the transcription factors CTCF and SP1 may contribute to homocysteine-related endothelial senescence. The detailed mechanism has not yet been elucidated. Here, we describe and confirm a novel hypothesis that cross talk of the transcription factors CTCF and SP1 may contribute to homocysteine-related endothelial senescence.

**Discussion**

Although homocysteine-reduced telomerase activity accelerating endothelial senescence has been reported for decades, the detailed mechanism has not yet been elucidated. Here, we describe and confirm a novel hypothesis that cross talk of the transcription factors CTCF and SP1 may contribute to homocysteine-related endothelial senescence in vitro and in vivo. Thus, maintaining telomerase activity by hypermethylating hTERT could be protective in homocysteine-related cardiovascular diseases.

Homocysteine is considered an independent risk factor of atherosclerosis; the factor has been reported to induce telomere overactivation which extends the lifespan of ECs,1 which suggests a possible mechanism by which homocysteine promotes atherosclerosis.2 Experimentally, evidence in normal human ECs also confirmed telomerase activity, which decreases with aging of ECs and expression in endothelial senescence.3 4 Therefore, the degree of telomere shortening may be related to the risk of developing atherosclerotic plaque and is a predictor of mortality in patients with coronary artery disease. However, defining with certainty the role of telomeres in and the effect of telomere length on the atherosclerotic process is still difficult.6 Experimental evidence in normal human ECs also confirmed telomerase activity, which decreases with aging of ECs and expression in endothelial senescence.

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Reduced DNA methyltransferase 1 (DNMT1) activity contributes to homocysteine (Hcy) downregulation of human telomerase reverse transcriptase (hTERT) expression and activity. Quantitative real-time polymerase chain reaction (A), relative telomere activity (RTA; B), and senescence-associated β-galactosidase (SA-β-gal) staining (C) analyzed by DNMT1 knockdown on transfection with 40-nmol/L siRNA for 12 hours, and with or without Hcy treatment for 72 hours in P4 of human umbilical vein endothelial cells (HUVECs). β-Actin was an internal control. Data (n=5) are mean±SD and analyzed by Student t test. *P<0.05 and †P<0.05 vs si-Ctrl transfection with or without Hcy treatment, respectively.

Figure 4. Reduced DNA methyltransferase 1 (DNMT1) activity contributes to homocysteine (Hcy) downregulation of human telomerase reverse transcriptase (hTERT) expression and activity. Quantitative real-time polymerase chain reaction (A), relative telomere activity (RTA; B), and senescence-associated β-galactosidase (SA-β-gal) staining (C) analyzed by DNMT1 knockdown on transfection with 40-nmol/L siRNA for 12 hours, and with or without Hcy treatment for 72 hours in P4 of human umbilical vein endothelial cells (HUVECs). β-Actin was an internal control. Data (n=5) are mean±SD and analyzed by Student t test. *P<0.05 and †P<0.05 vs si-Ctrl transfection with or without Hcy treatment, respectively.

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NO reduction by oxidative stress may be the critical step in homocysteine-induced telomerase inactivity.

In the current study, for markers of EC senescence, levels of TIMP4 (tissue inhibitor of metalloproteinases 4) and NOX4 (NADPH oxidase 4), 2 markers of oxidative stress, were increased in a passage-dependent manner and were significantly enhanced by homocysteine treatment (data not shown). Preincubation with a repressor of oxidative stress (L-arginine), by increasing NO synthesis, could markedly abolish the homocysteine-induced level of p16 or p21 and the ratio of senescence-associated β-galactosidase–positive cells but not the expression of hTERT. Interestingly, homocysteine but not angiotensin II–induced EC senescence and reduced hTERT level could be attenuated by DNA hypermethylation with treatment with FA or SAM. Moreover, inhibition of DNA methylation by 5-aza alone could mimic the homocysteine effect on hTERT expression and demethylation. Therefore, homocysteine might downregulate hTERT level independently to induce telomerase inactivity, which differs from oxidative stress and NO reduction. Thus, we found another novel mechanism of homocysteine inactivating telomerase activity by directly downregulating hTERT expression with DNA demethylation during endothelial aging. Previously, we and others suggested that homocysteine reduced the expression and activity of DNMT1 and further demethylated promoters of atherosclerosis-related genes and enhanced their expression.13,33 Thus, our current report clarifies that hTERT is a novel target gene in homocysteine-induced endothelial dysfunction by an epigenetic mechanism, just as hTERT is a candidate target gene via DNA hypomethylation in various carcinogenesis paths.4

Similar to previous studies,14 we found that the core region of the hTERT promoter showed only low-density methylation (16.9%) in normal ECs and mainly focused on the SP1 (62.5%) and CTCF-binding sites (66.7%) in the core region. Importantly, both sites showed a decreasing methylation pattern by inhibiting histone deacetylase by trichostatin A (data not shown) or DNA demethylation by homocysteine or 5-aza, which facilitated CTCF but interfered SP1 binding to the hTERT promoter but did not alter their expression. Surprisingly, different from Choi et al11 and Meeran et al,17 our ChIP analysis confirmed that homocysteine- or 5-aza-induced demethylation promoted CTCF but prevented SP1 binding to the hTERT promoter. SP1 binding to homocysteine-induced unmethylated CpGs on the promoter of hTERT might be an activator for hTERT expression in normal ECs because siRNA knockdown of SP1 repressed hTERT expression and activity in ECs without but not with homocysteine treatment. However, siRNA knockdown of CTCF could upregulate hTERT expression whether SP1 was deleted, which suggests that homocysteine enhanced CTCF (repressor) but interfered in SP1 (activator) binding to the hTERT promoter and coordinately contributed to hTERT downregulation. Although the inhibitory effect of CTCF recruitment on SP1 binding to the homocysteine-demethylated hTERT promoter is not clear, CTCF might affect the interaction of SP1 and p300 or c-Myc in intrinsic histone acetyltransferase activity, which explains the cross talk of CTCF and SP1 contributing to homocysteine-repressed hTERT activity in ECs.

In our previous study,13 we found that treatment with a moderate concentration of homocysteine for long-term stimulation could inhibit the activity and expression of DNMT1 in HUVECs. DNMT1 knockdown could reduce the expression and activity of hTERT completely independent of homocysteine but induce EC senescence independent of homocysteine only in part. Together with other data,14,33 our data support that hTERT transcriptional expression can be controlled by DNMT1 in ECs and other specific cancer cells; decreased DNMT1-induced demethylation focuses on fully methylated CpGs of the CTCF-binding site in cancer cells and on partially methylated CpGs in ECs.

According to Horikawa et al16 and Ritz et al,32 humans and mice might share a similar pattern of TERT expression. Thus, we examined homocysteine-reduced hTERT activity via DNA demethylation in an hyperhomocysteinemia mouse model. Indeed, cellular senescence, shortened telomere length, and mTERT protein reduction in the intima were found only with moderate hyperhomocysteinemia (8 weeks). Although we detected elevated mTERT mRNA levels and hypomethylation
of CTCF-binding sites on mTERT in the mouse aortic intima with short-term (4 weeks) mild hyperhomocysteinemia treatment, which indicates that DNA demethylation is an early event in hyperhomocysteinemia, it was not sufficient to cause EC senescence. Hyperhomocysteinemia-activated oxidative stress might play a crucial role combined with hTERT reduction in EC senescence.37

The limitations of our study is that we performed no atherosclerotic lesion experiments in vivo to identify whether homocysteine-induced hTERT repression and shortened telomere length is a cause or consequence in atherosclerosis. However, homocysteine affecting the cross talk of CTCF and SP1 in demethylating the hTERT promoter and reducing hTERT expression is a rapid and critical step for telomere dynamics in circulating cells, cultured ECs, and aortic intima. These areas are affected directly by homocysteine at the early stage of atherosclerotic lesion development, and combined with oxidative stress, cocontribute to telomerase inactivation, thus further leading to EC senescence in homocysteine-related cardiovascular diseases.

In conclusion, our in vivo and in vitro findings show a novel mechanism of CTCF and SP1 cross talk coordinately contributing to homocysteine-induced DNA demethylation on the hTERT promoter and restoring its expression, for accelerated EC telomerase inactivation and senescence. In addition, our study also supports that hTERT mRNA expression or methylation ratio is an early risk event in homocysteine-induced cardiovascular disease.

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Disclosures
None.

References


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Supplemental Material and Methods

Reagents

DL-Homocysteine (Hcy), FA, L-arginine (LAR), L-methionine, L-cysteine (L-cys), S-adenosylmethionine (SAM) and 5-aza-2’ deoxycytidine (5-Aza) were from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies for hTERT, β-actin, polyperoxidase-anti-mouse/rabbit IgG and protein A/G PLUS-agrous were from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibodies for TATA binding protein (TBP), SP1, P16, P21, P53 and CTCF were from Abcam (Cambridge, UK). Cy3-labeled goat anti-rabbit IgG (H+L) was from Beyotime (Beijing) and DAPI was from Vector Labs (Burlingame, CA). IRDye 800CW goat anti-rabbit IgG (H+L) and IRDye 680 goat anti-mouse IgG (H+L) were from LI-COR Biosciences (Lincoln, NE, USA).

Cell culture

Primary human umbilical vein ECs (HUVECs) were isolated in our lab\(^1\). The investigation conformed to the principles outlined in the Declaration of Helsinki for use of human umbilical cord. The protocol was approved by University of Shantou Human Sample Use Committee. All experiments involved primary cells at passages 0 to 12. Cells were cultured in ECM with 5% fetal bovine serum (Sciencell, USA) at 37°C in a humidified atmosphere containing 5% CO\(_2\). Human embryonic kidney (HEK) 293 (HEK293) cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (Highclone, USA).

Quantitative PCR (qPCR)

Quantitative RT-PCR (qRT-PCR) was used to examine gene expression as described\(^1\). Cellular or tissue total RNA was extracted by the Trizol reagent method (Invitrogen, USA). An amount of 0.5 \(\mu\)g of RNA was used for cDNA synthesis, and qPCR amplification involved use of Applied Biosystems Prism 7300 (ABI, USA). Relative mRNA expression was normalized to that of β-actin.

The qPCR-based method was used for measuring mean telomere length as described\(^2\). Cellular or tissue genomic DNA was extracted by use of the TIANamp Genomic DNA Kit (TIANGEN, China) and used for telomere (T) and single copy gene (S), 36B4u and β-globin amplification. The relative telomerase length was the ratio of telomere (T) repeats to single-copy gene (S) copies (T/S ratio) in human ECs and normalized to that in HEK293 cells. qPCR involved use of a LightCycler 480 (Roche, Switzerland).

Western blot analysis

Hcy-treated HUVECs protein was extracted and quantified by use of the BCA protein assay kit (NovasyGen, China). Equal amounts of protein (80 mg) were separated by electrophoresis on a 12% SDS-polyacrylamide gel and blotted onto a PVDF membrane, which was blocked with 10% non-fat dry milk, then incubated with
primary antibodies against hTERT (1:200), SP1 (1:1000), CTCF (1:1000), DNA methyltransferase 1 (DNMT1; 1:1000) or β-actin (1:2000) overnight at 4°C, then with IRDye 800CW goat anti-rabbit IgG or IRDye 680 goat anti-mouse IgG for 1 hr at room temperature. LI-COR Odyssey (Li-Cor, Lincoln, NE) was used to detect fluorescent immunocomplexes, and the densities of the bands were quantified and normalized to that of β-actin.

Small interfering RNA (siRNA) transfection

SP1, CTCF and DNMT1 knockdown with siRNAs were performed as we and others described. Briefly, siRNA for SP1 or CTCF and scramble siRNA were synthesized and purchased from GenePharma (Shanghai). Then 40-nM siRNA or vehicle was transfected into primary HUVECs by use of Lipofectamine RNAiMAX Reagent (Invitrogen Corporation, CA) for 12 hr. qRT-PCR and western blot were used to confirm the knockdown effect of siRNA in transfected cells.

Standard and quantitative chromatin immunoprecipitation (S-ChIP and Q-ChIP)

HUVECs were treated with 50 µM Hcy or 8 µM 5-aza for 72 hr. S- and Q-ChIP assays were performed as described to examine the in vivo binding of CTCF or SP1 on the hTERT promoter. Normal IgG was used as an immunoprecipitation (IP) negative control, and the supernatant of IP was an input control. For Q-ChIP, immunopurified DNA was amplified with use of the PrimeScript Real-Time PCR reagent kit (Takara Biotechnology [DALIAN] Co.) with LightCycler 480 and normalized to the input control. For S-ChIP analysis, semiquantitative PRC was used for immunopurified DNA amplification, and products were analyzed on 2% agarose gel after ethidium bromide. All ChIP experiments were carried out in triplicate.

Electrophoretic mobility shift assay (EMSA)

Oligonucleotides containing putative binding sequences for CTCF (5′-CCGGATGCCGCTCCGCTCCCCGC-3′) and SP1 (5′-TCTTCTCCGCCGCTCCCCT CT-3′) of the hTERT promoter were labeled at the 3′-end with biotin (Takara Biotechnology [DALIAN] Co.) and annealed to their respective antisense strands to form labeled double-stranded DNA for 20 min at room temperature, then incubated with nuclear extracts harvested from P6 HUVECs, with 50 µM Hcy, or 8 µM 5-aza treatment according to the manufacturer’s protocol (Beyotime, China). 6% Electrophoresis was performed and detected on a phosphoimager plate. For the supershift assay, CTCF or SP1 antibody was preincubated in the reaction mixture at a final dilution of 1:30 at 4°C for 20 min. In the competition assay, excess amounts of unlabelled competitors were added before the labelled probes.

Methylation-specific PCR (MSP) and bisulfite sequencing (BGS)

Tissue or cellular genomic DNA was extracted with use of the TIANamp Genomic DNA Kit and modified by use of the BisulFlashTM DNA Modification Kit (Epigentek Group, USA). The modified DNA was used as a template for BGS assay. The procedure and primers were as described. Specifically, for MSP amplification, we designed methylated and unmethylated primers focusing on CTCF binding sites by using a bioinformatics program (http://www.urogene.org/methprimer/index1.html). For BGS sequencing, bisulfite-treated DNA was amplified by use of the Premix Ex
Taq Hot Start Version (Takara Biotechnology [DALIAN] Co., China) and analyzed on agarose gels. All positive PCR products were ligated into the pGEM-T vector and confirmed by sequencing. With use of NIH ImageJ as previously described, the DNA methylation ratios were calculated as methylation/(methylation and unmethylation) x100% from the densitometry of the visualized band by ethidium bromide staining. We used human placental genomic DNA (gDNA; Sigma-Aldrich) methylated in vitro with SssI methylase (NEB) and converted, as a fully methylated (100%) MSP positive control, and used the same unmethylated placental gDNA and converted with SB, as a negative MSP control.

Relative telomere activity (RTA) assay

We examined 2,000 HUVECs after treatment with the TeloTAGGG telomerase PCR ELISAPLUS Kit (Roche Diagnostic GmbH, Mannheim, Germany) based on telomeric repeat amplification protocol (TRAP) assay to determine telomerase activity. Telomerase adds telomeric repeats (TTAGGG) to the 3’-end of the biotin-labeled synthetic primer, which is elongated by PCR. The PCR products were denatured and hybridized separately to digoxigenin-labeled detection probes, specific for the telomeric repeats and for the internal standard. Then, the products were immobilized and detected with an antibody against digoxigenin and warmed with TMB substrate solution for 10-20 min and stopped. Relative telomere activity was analyzed by use of a microplate reader (Infinite M1000 PRO, Tecan Group Ltd., Switzerland) within 2 to 10 min at 450 nm (with a reference of 690 nm), and calculated as follows: [(OD sample – OD RNAS-treated sample)/OD internal standard]/ [(OD control temple – OD lysis buffer)/OD internal standard of control]x 100.

Senescence-associated β-galactosidase (SA-β-gal) assay

SA-β-gal activity was measured by use of the β-Galactosidase Staining Kit (Beyotime, China). HUVECs were washed and fixed with fixative solution for 10 to 15 min and further incubated overnight at 37 °C with the staining solution mix. Green-colored stained images were analyzed by confocal laser scanning microscopy (OLYMPUS, Japan).

TUNEL Assay

Cell apoptosis was assessed by detection of DNA fragmentation using the One Step TUNEL Apoptosis Assay Kit (Beyotime, China). P2, P6 and P10 of HUVECs were subjected to 50 µM Hcy treatment or not. As protocol, adherent ECs were fixed in 4% paraformaldehyde and incubated with the reaction mixture containing terminal deoxynucleotidyl transferase and fluorescein-12-dUTP for 1 hour at 37°C. Cell images were analyzed with a fluorescence microscope (Leica DMIRB). The number of apoptosis cells was measured by counting the number of stained nuclei from 5 randomly chosen fields under a fluorescence microscope.

Flow cytometry

P2, P6 and P10 of HUVECs were treatment with or without 50 µM Hcy. Flow cytometry assay as our previous report, 10⁶ of ECs were digested and fixed with 70% ethanol for 30 min at room temperature. Then, cells were re-suspended in PBS containing propidium iodide and RNase (10 µg/mL each), incubated for 30 min at 37°C and analyzed with the use of a FACStar Plus flow cytometer (Becton Dickinson, Franklin Lakes, NJ). S phase cells were calculated to indicate the cell proliferation.
Animal experiment

We previously described the hyperhomocysteinemia (HHcy) mice model \(^\text{3, 6}\). Briefly, 6- to 8-week-old male C57BL/6J mice were fed standard mouse chow diet with or without 2% (wt/wt) L-methionine for 4 or 8 weeks (n=8 in each group). Mouse serum was harvested for measuring the concentrations of total Hcy, and aortas were removed after mice were anesthetized (intraperitoneally, 100 mg/kg ketamine and 10 mg/kg xylazine) until the loss of the forepaw righting reflex. Immunohistochemistry involved the primary antibody for hTERT (1:50) with the VECTASTAIN ABC System (Vector Labs) on mouse aorta sections. Phosphate-buffered saline was used as a negative control \(^\text{7}\). Total RNA and genomic DNA were extracted from the intima layer scraped from thoracic and abdominal aortas of mice for qRT-PCR or MSP assay.

All treatments and procedures for in vivo study followed the Guide for the Care and Use of Laboratory Animals by the US National Institutes of Health (NIH Publication, 8th Edition, 2011) and were approval by Laboratory Animals of the University of Shantou and the local governmental authorities.

**Statistical analysis**

All in vitro experiments were performed in 4-6 independent times in triplicate as labeled in each figure legend. We performed the Shapiro-Wilk Test for the normality of all continuous variables, and if the p-value were more than 0.05, which indicated all data was normal distribution and could be expressed as mean ± SD and analyzed with Student's t test. A two-tailed P<0.05 was considered statistically significant. Statistical analysis involved use of SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

References: