Estrogen Receptor-α and Endothelial Nitric Oxide Synthase Nuclear Complex Regulates Transcription of Human Telomerase

Annalisa Grasselli, Simona Nanni, Claudia Colussi, Aurora Aiello, Valentina Benvenuti, Gianluca Ragone, Fabiola Moretti, Ada Sacchi, Silvia Bacchetti, Carlo Gaetano, Maurizio C. Capogrossi, Alfredo Pontecorvi, Antonella Farsetti

Abstract—We report that in endothelial cells, the angiogenic effect of 17β-estradiol (E2) is inhibited by the estrogen receptor (ER) antagonist ICI or the NO synthase (NOS) inhibitor 7-nitroindazole via downregulation of hTERT, the telomerase catalytic subunit, suggesting that E2 and NO are involved in controlling hTERT transcription. Quantitative Real-Time PCR and chromatin immunoprecipitations in E2-treated human umbilical vein endothelial cells, showed recruitment of ERs on the hTERT promoter and concomitant enrichment in histone 3 methylation at Lysine 79, a modification associated with transcription-competent chromatin. Confocal microscopy and re-chromatin immunoprecipitations revealed that on E2 induction, endothelial (e)NOS rapidly localized into the nucleus and associated with ERα on the hTERT promoter. Transfections of a constitutively active eNOS mutant (S1177D) strongly induced the hTERT promoter, indicating a direct role of the protein in hTERT transcriptional regulation. Mutation of the estrogen response element in the promoter abolished response to both ERs and active eNOS, demonstrating that the estrogen response element integrity is required for hTERT regulation by these factors. To investigate this novel regulation in a reduced NO environment, pulmonary endothelial cells were isolated from eNOS$^{-/-}$ mice and grown with/without E2. In wild-type cells, E2 significantly increased telomerase activity. In eNOS$^{-/-}$ cells, basal telomerase activity was rescued by exogenous eNOS or an NO donor, whereas responsiveness to E2 demanded the active protein. In conclusion, we document the novel findings of a combinatorial eNOS/ERα complex at the hTERT estrogen response element site and that active eNOS and ligand-activated ERs cooperate in regulating hTERT expression in the endothelium. **(*Circ Res*. 2008;103:34-42.)**

Key Words: eNOS/ERα complex ■ telomerase transcription ■ angiogenesis

Estrogens play a pivotal role in many physiological processes (reproduction, cardiovascular health, nervous system function, and skeletal homeostasis)[1–4] and are also implicated in the development or progression of numerous pathologies, including cancer, osteoporosis, neurodegenerative, and cardiovascular diseases.[5,6] The biological effects of 17β-estradiol (E2) is mediated by the estrogen receptors (ERs) ERα and ERβ, which function as ligand-induced transcriptional factors and belong to the nuclear receptor superfamily.[7–9]

ERs regulate gene expression in a ligand-responsive manner through nuclear and non-nuclear mechanisms,[10–12] by direct interaction of their DNA binding domain with estrogen-response elements (EREs) in the promoter of target genes, or through protein–protein interaction with other classes of molecules.[13] In this case, ERs modulate the activity of transcription factors such as activator protein-1 or stimulatory protein 1–1 by stabilizing their DNA binding capacity and/or coactivator recruitment to the transcription complexes.[14,15] In addition, ERs mediate important nongenomic effects of E2[16] and related steroid hormones in a variety of tissues, regulating multiple transducing signals,[17,18] including the functional activation of the endothelial NO synthase (eNOS).[9,10,18–22]

In endothelial cells (ECs), ERs localize to caveolae and activate eNOS upregulating NO production through protein kinase–mediated eNOS phosphorylation.[23–25] Active eNOS changes its subcellular localization from the endoplasmic reticulum to the cell membrane. Recently, however, eNOS has also been detected in the nucleus.[26–28] Although eNOS nuclear function(s) remains obscure, it seems important in mediating NO-dependent nuclear signaling. NO is, in fact, an

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From the Department of Experimental Oncology (A.G., S.N., A.A., V.B., F.M., A.S., S.B., A.F.), Regina Elena Cancer Institute, Rome; Endocrinology (A.G., V.B., A.P.), Catholic University, Rome; Laboratory of Vascular Biology and Gene Therapy (S.N., C.C.), Centro Cardiologico "I. Monzino," Milan; Laboratorio di Oncogenesi Molecolare (G.R.) and Laboratorio di Patologia Vascolare (C.G., M.C.C.), Istituto Dermopatico dell' Immacolata, Rome; and Institute of Neurobiology and Molecular Medicine (A.A., F.M., A.F.), National Research Council, Rome, Italy.

Correspondence to Antonella Farsetti, INeMM, National Research Council and Department of Experimental Oncology, Regina Elena Cancer Institute, Rome, Italy via delle Messi d'Oro 156, 00158 Rome, Italy. E-mail farsetti@ifo.it or afarsetti@gmail.com

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important signaling molecule acting in many tissues to regulate diverse processes, including apoptosis and gene expression.[29–31] In this context, previous reports indicate that NO synthesis is important for the expression and function of hTERT, the catalytic subunit of human telomerase, during angiogenesis.[32,33] In an independent line of research, we provided evidence that E2 and ERs are also involved in hTERT transcriptional regulation[34,35] and consequently in telomerase activation, through specific interaction of ERs with their cognate ERE site in the hTERT promoter.[35] Here, we document the novel findings that NO production is important for E2-dependent induction of hTERT and that eNOS is bound to the hTERT ERE, together with ligand-activated ERs. Thus, both active eNOS and ligand-activated ERs may cooperate in regulating hTERT transcription during estrogen-dependent angiogenesis.

Materials and Methods

Hormones and Inhibitors

The following hormones and inhibitors were used: 17β-Estradiol (E2) (Sigma-Aldrich), 7-nitroindazole (7N) (Biomol), ICI 182,780 (ICI) (Sigma-Aldrich), KT5823 (Calbiochem), DETA-NO (Sigma), N^G-nitro-L-arginine methyl ester (L-NAME), and L-6-Hydroxymethyl-chiro-inositol-2-[(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate] (AKTi) (Alexis).

Antibodies

The following antibodies were used: anti–ER-α (HC-20 [Santa Cruz Biotechnology]; Ab-10 and Ab-3 ([NeoMarkers]); anti–ER-β (L-20 [Santa Cruz Biotechnology], 14C8 [Abcam], D7N [ZYMED Laboratories Inc]); anti-H3K79^m polyclonal antibody and anti–dimethyl-histone H3 (Lys79) (Upstate); anti-eNOS (eNOS/NOS Type III [BD Biosciences]).

Cell Culture and Treatments

Human umbilical vein ECs (HUVECs) were cultured according to the protocols of the supplier (Lonza). Bovine vein ECs (BAECs) were cultured in DMEM with 10% FBS. Cells were treated with E2 (10^{-7} mol/L), ICI (10^{-7} mol/L), 7N (0.5 or 1 mmol/L), and diethylenetriamine/nitric oxide (DETA-NO) (500 μmol/L) as indicated.

Immunofluorescence Analysis

Cells were immunostained with the telomerase-specific antibody K-370,[36] and nuclei were stained with Hoechst 33258. Images were captured with a Zeiss fluorescence microscope.

RNA Extraction and RT-PCR Analysis

Total RNA was isolated with TRIzol (Invitrogen). cDNA synthesis was performed with High-Capacity cDNA Archive kit (Applied Biosystems). Expression of hTERT mRNA was analyzed by semi-quantitative RT-PCR amplification using primers and condition as described.[34] Amplified PCR products were electrophoresed and the intensity of each band evaluated by densitometry using NIH ImageJ 1.24 software (NIH, Bethesda, Md).

Telomere Repeat Amplification Protocol Assay

Telomerase activity was measured by the telomere repeat amplification protocol (TRAP) method.[37] Assays were repeated at least 3 times with three different preparations of cell lysates.[34] Quantitative analysis was performed with the NIH Image 1.61 software. Telomerase activity was quantified by integrating the signals of the telomerase ladder, and relative activity was calculated as ratio to the internal standard.

Matrigel

Formation of capillary-like structure was performed as described,[33] quantified on 10 randomly chosen fields and expressed as the mean±SE.

Transfection and Luciferase Reporter Assay

Transient transfections were performed with Lipofectamine Plus (Invitrogen). BAECs were transfected with 0.5 μg of hTERT promoter fragment-luc with wild-type (P-1009)[35,38] or mutated ERE (P-1009Mut)[35] and an eNOS vector encoding S1177D (gift of S. Dimmeler, University of Frankfurt, Germany).[29] Cytomegalovirus (CMV)–β-galactosidase was used as internal control of transfection efficiency. Luciferase and β-galactosidase activities were assayed using reagents and protocols from Promega. Each experiment was repeated at least 3 times.

Generation and Characterization of Murine Pulmonary Endothelial Cells

All experimental procedures were approved by the Internal Animal Research Ethical Committee (protocol HH39) according to the Italian Ministry of Health and complied with the NIH Guide for the Care and Use of Laboratory Animals.

Endothelial cells (ECs) were isolated from lungs of 8 weeks old wild-type and eNOS$^{-/-}$ mice (Charles River Laboratories). Isolated lungs were washed in ice-cold PBS/Hepes 20 mmol/L and cut in pieces of ≈2 mm. Collagenase A (1.5 mmol/L) digestion was for 30 to 45 minutes and was stopped by addition of EGM-2 medium with 10% serum (Lonza). Undigested tissue was removed by filtration with a 40 μm cell strainer and cells were recovered by centrifugation at 800 rpm for 10 minutes. After two washes in PBS, cells were plated in gelatin-coated dishes in EGM-2 10% serum and grown for 1 week. ECs were recovered and plated on collagen-coated dishes in EGM2-bullet kit (Lonza) with 10% FCS and their purity evaluated by LDL-Dil uptake and immunofluorescence with anti-Von Willebrand factor. ECs were characterized by uptake of acetylated LDL (Ac-LDL) labeled with the fluorescent probe 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanide perchlorate (Dil). Cells were incubated with 10 μg/mL of Dil-Ac-LDL for 4 hours at 37°C, fixed in 4% formaldehyde and red fluorescence was visualized using standard rhodamine excitation:emission filters. For factor VIII expression cells were fixed in 4% formaldehyde and stained with anti factor VIII (Santa Cruz Biotechnology) 1:500. Nuclei were visualized by 4′,6-diamidino-2-phenylindole (DAPI) incorporation.

Infections and Treatments

Replication-deficient recombinant adenoviruses were prepared and stored as described.[39,40] Briefly, E1 was replaced by the CMV-immediate early gene promoter/enhancer driving the cDNA for human eNOS (S1177D)[29] (C.G., unpublished data, 2008) or green fluorescent protein (GFP). Wild-type and eNOS$^{-/-}$ pulmonary ECs (PECs) were plated on collagen-coated dishes at a density of 30000 cells/cm2, and a day after were infected with adenovirus encoding active eNOS (S1177D) or GFP at a multiplicity of infection of 100 pfu/cell. Two days after infection cells were treated with E2 (10^{-7} mol/L) or the NO donor DETA-NO (500 μmol/L) and collected 4 hours later for TRAP assay.

Chromatin Immunoprecipitation and Re–Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation (ChIP)[34] and Re-ChIP[41] assays were performed as described. Real-time PCR was performed using the hTERT promoter up 5′-GCGTTTGTTAGCATTTCAGTGTTT-3′; hTERT promoter down 5′-CGGGTTGCTCAAGTTTGGA-3′; and c-Jun promoter as in.[42] Standard curves were generated by serially diluting the input (5-log dilutions in triplicate). Quantitive RT-PCR was performed in ABI Prism 7500 PCR instrument (Applied Biosystems) using SYBR Master mix (Applied Biosystems) with evaluation of dissociation curves.

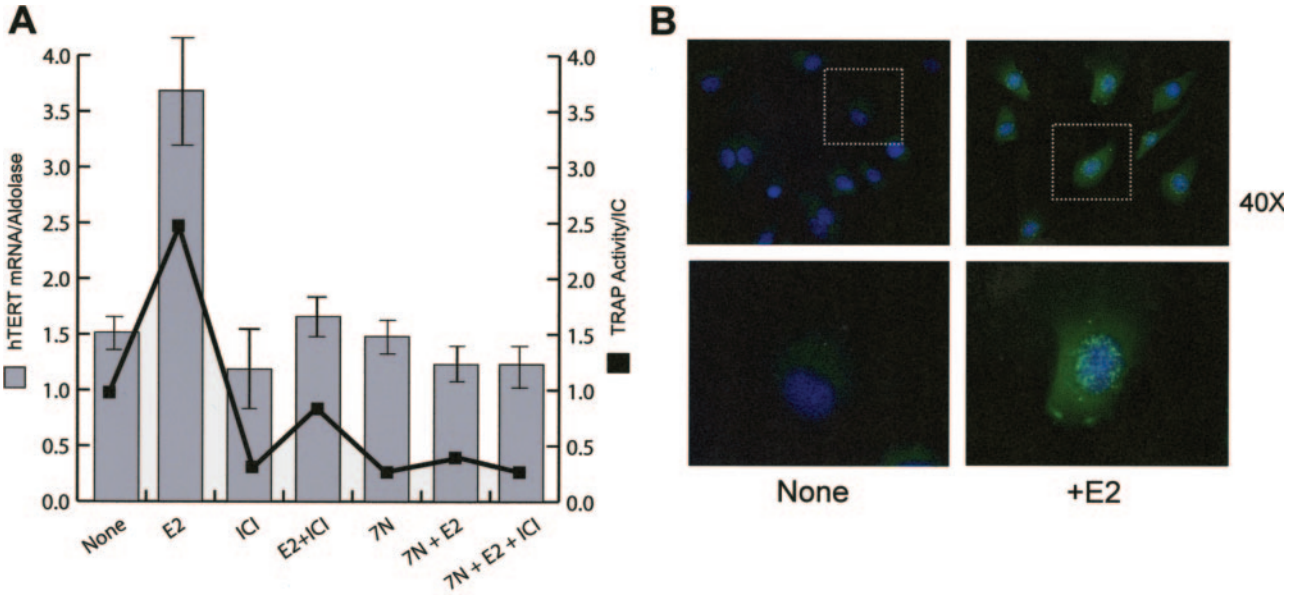

Figure 1. Effect of 17β-estradiol on hTERT mRNA level, hTERT expression, and telomerase activity. A, Semiquantitative RT-PCR analysis of hTERT mRNA levels in HUVECs treated for 4 hours with E2 (10^{-7} mol/L), ICI (10^{-7} mol/L) and 7N (0.5 mmol/L), or vehicle alone. 7N or ICI was added 1 hour before E2. mRNA levels were normalized to values of the housekeeping gene aldolase. Cell extracts from HUVECs cultured in the same conditions were assayed for telomerase activity by TRAP in the presence of an internal control (IC) (36 bp). Average values from 3 independent experiments are expressed as the ratio of hTERT to aldolase (hTERT/aldolase) in the left axis or as relative telomerase activity calculated as the ratio to the internal control (TRAP activity/internal control) in the right axis. B, HUVECs were cultured in medium with hormone-deprived serum, incubated for 6 hours with or without E2 (10^{-7} mol/L), and stained with anti-hTERT antibody K-370 (green) or Hoechst 33258 (blue). Magnification: $\times$40 (upper images); the lower images show higher magnification of indicated areas (dotted lines).

Confocal Microscopy

HUVECs were fixed in 4% formaldehyde, permeabilized with 0.2% Triton X-100 for 10 minutes, and incubated in 10% BSA/PBS for 1 hour to block nonspecific protein-binding sites. Cells were incubated overnight at 4°C with primary antibodies against eNOS (1:100 NOS Type III, BD) or ERα (1:100 Ab10, Neomarkers), followed by mouse cy5- or rabbit fluorescein isothiocyanate–labeled secondary antibodies (Jackson). Nuclei were stained with TOPRO3 dye. Samples were analyzed using a Zeiss LSM510 Meta Confocal Microscope. Lasers power, beam splitters, filter settings, pinhole diameters, and scan mode were the same for all examined samples. Fields in the figures are representative of all examined fields.

Statistical Analysis

Continuous variables were analyzed by the Student *t* test and 1-way ANOVA. Post hoc tests according to the Student–Newman–Keul method were used when the ANOVA probability value indicated a statistically significant difference among groups. Data are expressed as the means$\pm$SE. A value of $P<0.05$ was deemed statistically significant.

Results

eNOS Activity Is Required for E2-Dependent hTERT Expression

E2 exerts potent genomic and nongenomic effects on eNOS expression and function,[23,25,43] leading to increased NO production.[25,44] To investigate the contribution of NO in E2-dependent regulation of EC function, we examined the E2 effects on hTERT, a known estrogen target. Basal and E2-dependent levels of hTERT mRNA were evaluated in HUVECs by RT-PCR and densitometry (Figure 1A). Notably, a 2.5-fold increase in hTERT expression was observed on E2 treatment. The role of E2 in hTERT induction was further investigated using the ER antagonist ICI, which completely prevented E2-dependent hTERT expression, suggesting that induction could be mediated by ER-dependent transactivation of the hTERT promoter (Figure 1A). To assess whether eNOS activity was important for hTERT expression in the presence of ligand-activated ERs, HUVECs were cultured with the NOS inhibitor 7N (Figure 1A). E2 stimulation of hTERT mRNA was significantly decreased by 7N, indicating a functional link between E2, NO production, and hTERT expression in human ECs. In all of these conditions, telomerase activity correlated with the hTERT mRNA profile (Figure 1A). These observations were paralleled by abundant nuclear accumulation of the hTERT protein on E2 induction (Figure 1B).

E2 Induction of Angiogenesis and hTERT Promoter Activity Depends on NO Production

Numerous observations suggest that estrogens modulate endothelial proliferation, migration, differentiation, and angiogenesis in ECs.[2,3,45] To investigate the role of NO in E2-induced angiogenesis, in vitro differentiation assays were performed (Figure 2A) with HUVECs cultured in hormone-free medium on growth factor-reduced Matrigel and treated with E2 (10^{-7} mol/L), plus/minus ICI (10^{-7} mol/L) and 7N (0.5 mmol/L), or either ligand alone. As expected,[46] E2 strongly promoted formation of capillary-like structures. Remarkably, addition of ICI abrogated this effect, indicating that ERs are required for E2-dependent angiogenesis. Moreover, the NOS inhibitor 7N significantly reduced the number of capillary-like structures formed in the presence of E2, evidencing a functional link between estradiol and NO.

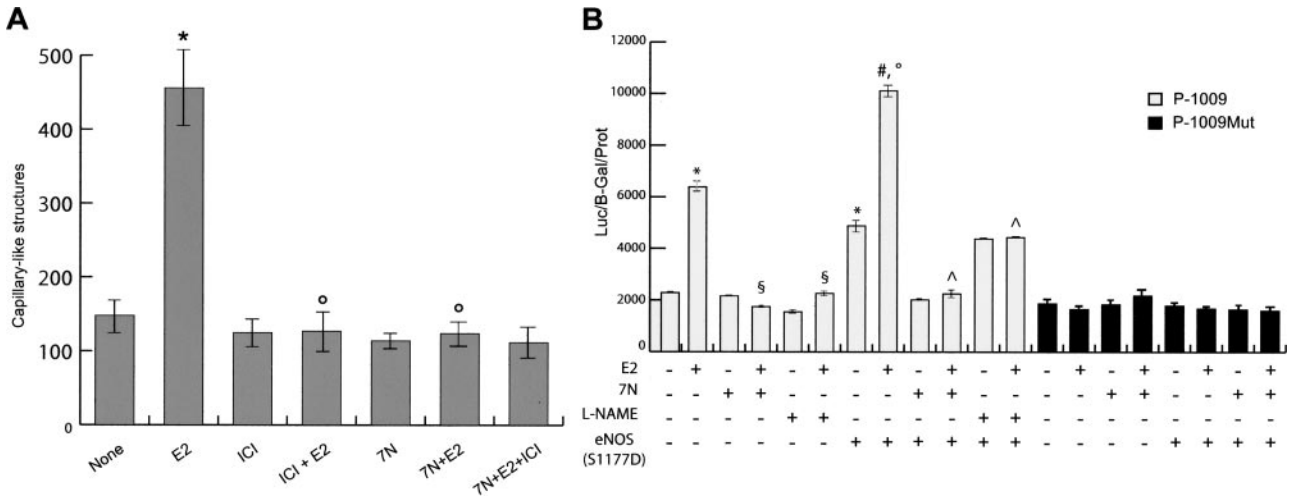

Figure 2. NO role in estrogen regulation of capillary-like tubes formation and hTERT promoter activity in ECs. A, HUVECs were incubated at 37°C on growth factor–reduced Matrigel with or without E2 (10^{-7} mol/L), ICI (10^{-7} mol/L), and 7N (0.5 mmol/L). The number of tube networks from triplicate wells (10 fields/well) was quantified at ×20 magnification after 3 to 24 hours of differentiation. *$P<0.05$ vs basal condition, °$P<0.05$ vs E2. B, BAECs were transfected with the hTERT promoter-reporter, wild-type (P-1009) or with a mutated ERE (P-1009 mutant) in the presence of CMV-βgal, alone or together with the eNOS mutant at serine 1177 (eNOS S1177D). Cells were treated with vehicle, E2 (10^{-7} mol/L), 7N (1 mmol/L in for P-1009 and 0.5 mmol/L for P-1009 mutant), and L-NAME (5 mmol/L) alone or in combination, and relative activities were determined in cell lysates after 48 hours. Results represent the averages (±SEM) of 6 independent experiments, each in duplicate. Symbols indicate: *$P<0.05$ vs P-1009 basal activity; §$P<0.05$ and °$P<0.05$ vs P-1009 plus E2; #$P<0.05$ vs P-1009 plus eNOS mutant (S1177D); $\hat{P}<0.05$ vs P-1009 plus eNOS S1177D in the presence of E2.

To investigate the role of eNOS on hTERT promoter activity in response to E2, we transiently transfected BAECs with the luciferase reporter fused to an hTERT promoter fragment wild-type (P-1009) or with a mutated ERE (P-1009Mut), alone or with the eNOS mutant (S1177D), which mimics a phosphorylated enzyme and determines a significant increase in enzyme activity. Transfections were performed in the presence of E2, ICI, and/or the NOS inhibitors 7N and L-NAME (Figure 2B). With the wild-type promoter, basal activity was detectable and E2 increased it by ≈3-fold. This increase was prevented by both NOS inhibitors, supporting an important role of NO function in the estrogen-dependent activity of the hTERT promoter. This observation is emphasized by evidence that the constitutively active eNOS mutant induced hTERT promoter activity, which was further augmented by E2, whereas addition of 7N or L-NAME again abolished the E2 effect. As expected, ICI prevented the E2-dependent promoter activity (data not

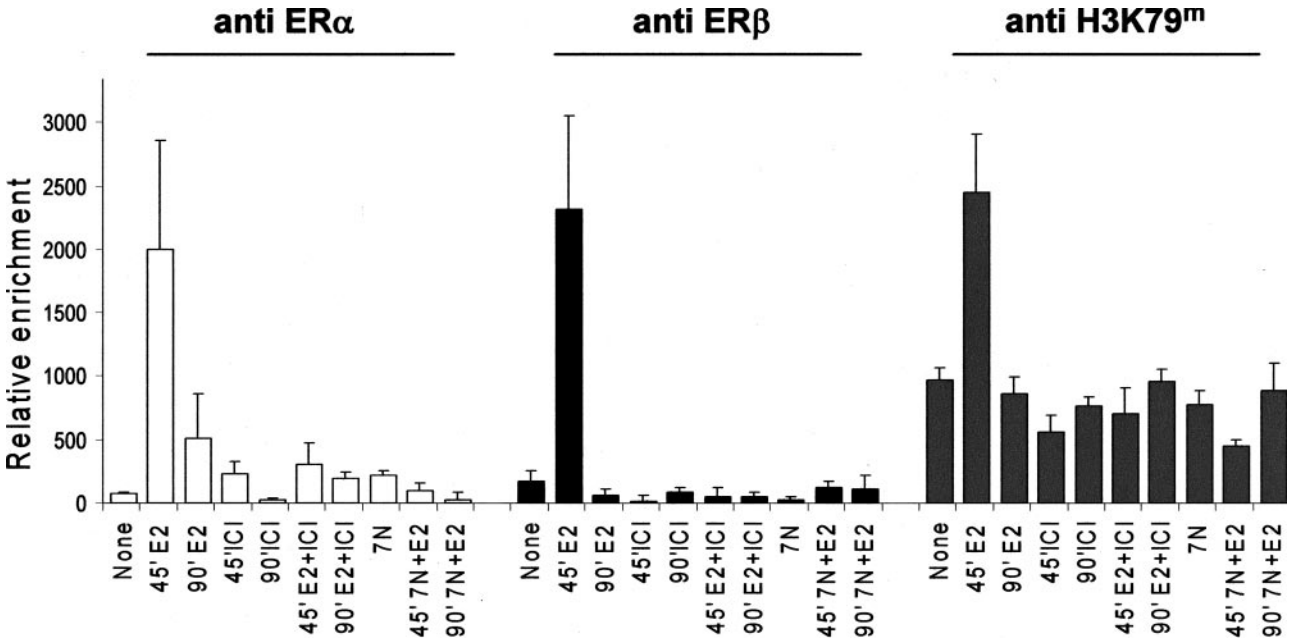

Figure 3. In vivo estrogen receptor recruitment onto hTERT promoter region. After formaldehyde crosslinking and chromatin precipitation with ERα, ERβ, and $H3K79^m$ antibodies or in the absence of antibody, the DNA was subjected to quantitive RT-PCR amplification using primers for the hTERT promoter. Analyses were in duplicate, and values were normalized to the corresponding DNA input after subtracting the no antibody values. Results are expressed as relative enrichment.

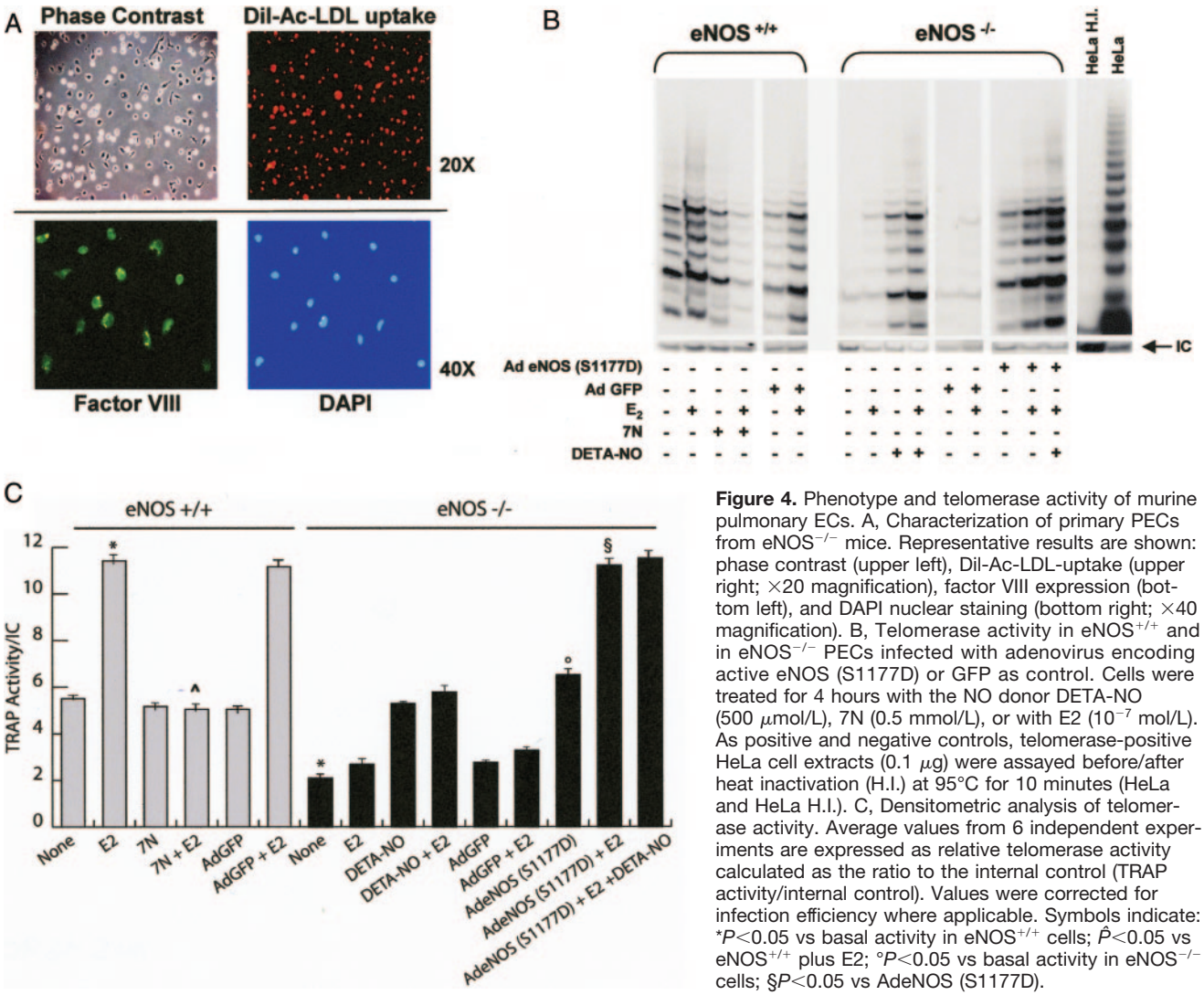

Figure 4. Phenotype and telomerase activity of murine pulmonary ECs. A, Characterization of primary PECs from eNOS$^{-/-}$ mice. Representative results are shown: phase contrast (upper left), Dil-Ac-LDL-uptake (upper right; ×20 magnification), factor VIII expression (bottom left), and DAPI nuclear staining (bottom right; ×40 magnification). B, Telomerase activity in eNOS$^{+/+}$ and in eNOS$^{-/-}$ PECs infected with adenovirus encoding active eNOS (S1177D) or GFP as control. Cells were treated for 4 hours with the NO donor DETA-NO (500 μmol/L), 7N (0.5 mmol/L), or with E2 (10^{-7} mol/L). As positive and negative controls, telomerase-positive HeLa cell extracts (0.1 μg) were assayed before/after heat inactivation (H.I.) at 95°C for 10 minutes (HeLa and HeLa H.I.). C, Densitometric analysis of telomerase activity. Average values from 6 independent experiments are expressed as relative telomerase activity calculated as the ratio to the internal control (TRAP activity/internal control). Values were corrected for infection efficiency where applicable. Symbols indicate: *$P<0.05$ vs basal activity in eNOS$^{+/+}$ cells; $\hat{P}<0.05$ vs eNOS$^{+/+}$ plus E2; °$P<0.05$ vs basal activity in eNOS$^{-/-}$ cells; §$P<0.05$ vs AdeNOS (S1177D).

shown). Intriguingly, transfection of the hTERT promoter with mutated ERE abrogated responsiveness to E2 or to eNOS, indicating that the integrity of the ER-binding site is important for the hTERT response to both agents. Furthermore, E2 induction of hTERT mRNA was abrogated by inhibition of the NO downstream effector protein kinase G (see Figure I in the online data supplement, available at http://circres.ahajournals.org), indicating that integrity of the NO signaling cascade is important in this process.

ChIPs Reveal ERα and ERβ In Vivo Recruitment onto the hTERT Promoter

To mechanistically investigate the in vivo interaction between ERs and the hTERT promoter and the NO contribution in this context, HUVECs grown in hormone-deprived medium for 3 days, followed by treatment with E2, 7N, or ICI for 45 and 90 minutes, were analyzed by ChIP (Figure 3). Strong ERα and ERβ signals were observed only at the 45-minute time point on E2 treatment, indicating recruitment of both ERs onto the hTERT promoter. Binding dissociated by 90 minutes with a dynamic profile previously described[34,41,47] and was abolished by ICI. Consistently, no ERs signal was observed on E2 addition in the presence of 7N, confirming the importance of NO in the E2-dependent regulation of hTERT expression. Amino acids residues at specific positions within histones tail or protein core may undergo post-translational modifications. Specifically, lysine (K) methylation of histone 3 (H3) at positions 4, 36, and 79 marks transcriptionally active chromatin regions, whereas H3 K methylation at positions 9 or 27 or on histone 4 (H4) at position 20 defines regions of repressed chromatin.[48,49] After 45 minutes of estrogen stimulation, using an antibody specific for methylated H3K79, we observed a positive signal (Figure 3) commonly correlating with unfolded chromatin and active transcription.[48,49] Altogether, these experiments indicated that ligand-activated ERs and active eNOS are required for hTERT promoter function. No ERs recruitment was observed on the control c-Jun promoter (data not shown).

Loss of Estrogen Regulation of Telomerase Activity in PECs Derived From eNOS$^{-/-}$ Mice

To further investigate the role of eNOS on hTERT function in E2-stimulated ECs, PECs were isolated from eNOS$^{-/-}$ and

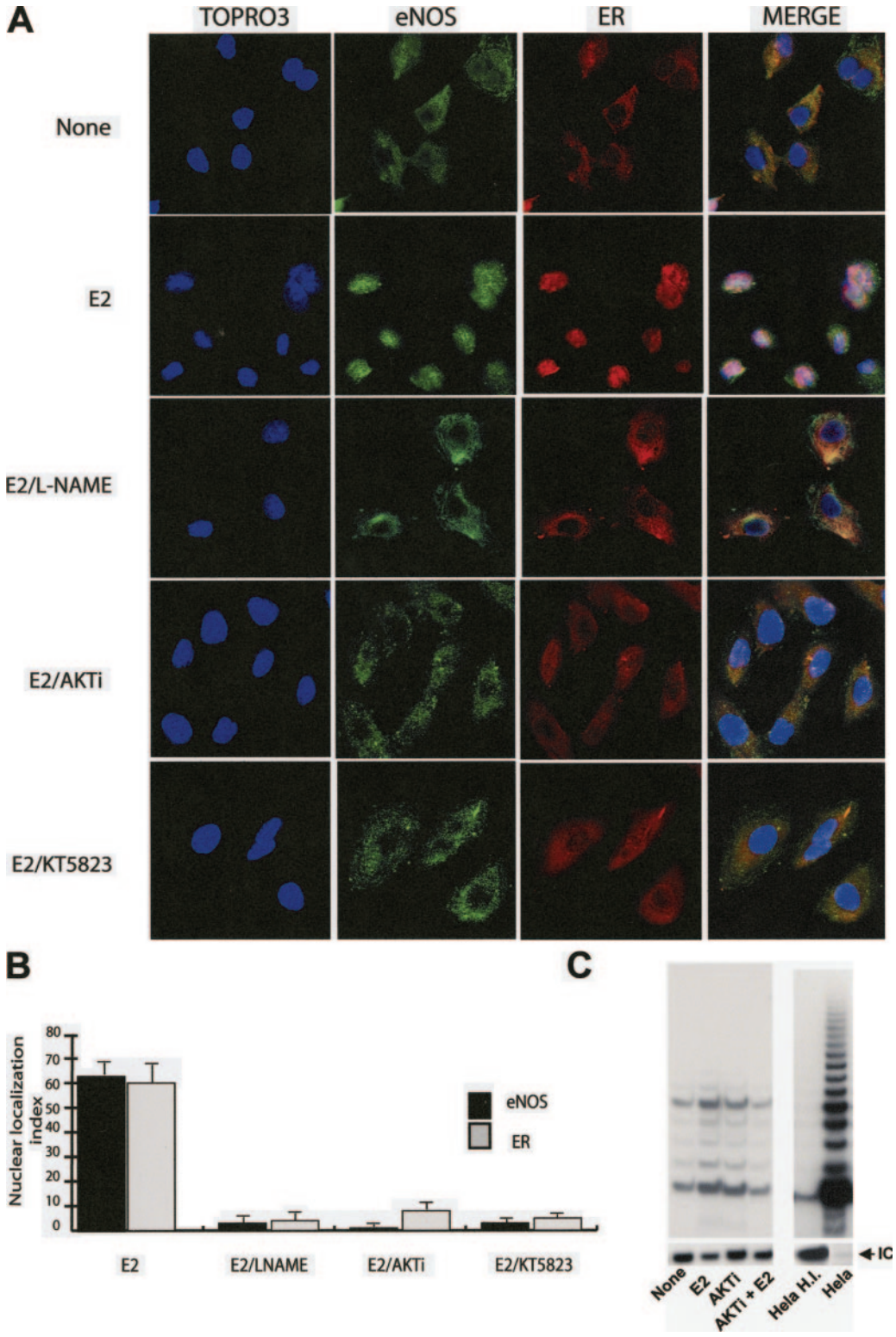

Figure 5. Nuclear colocalization of ERα and eNOS. A, HUVECs were incubated in the presence or absence of E2 (10^{-7} mol/L), L-NAME (5 mmol/L), AKTi (10 μmol/L), and KT5823 (1 mmol/L) for 45 minutes. Cells were fixed, stained with anti-eNOS (green) and anti-ERα (red) antibodies, and examined by confocal microscopy. Individual and merged fluorescence signals of red and green cells are shown. Images are from a typical experiment of 2 performed. B, Nuclear localization indexes of the eNOS and ERα proteins were obtained from confocal images by counting the number of nuclear positive cells in treated samples compared with control. Two hundred cells per sample were analyzed. Images shown are representative of all examined fields. The results were replicated in 2 independent experiments. C, Extracts from HUVECs cultured in presence of E2 (10^{-7} mol/L) and AKTi (20 μmol/L) were assayed for telomerase by TRAP in the presence of an internal control (IC) (36 bp).

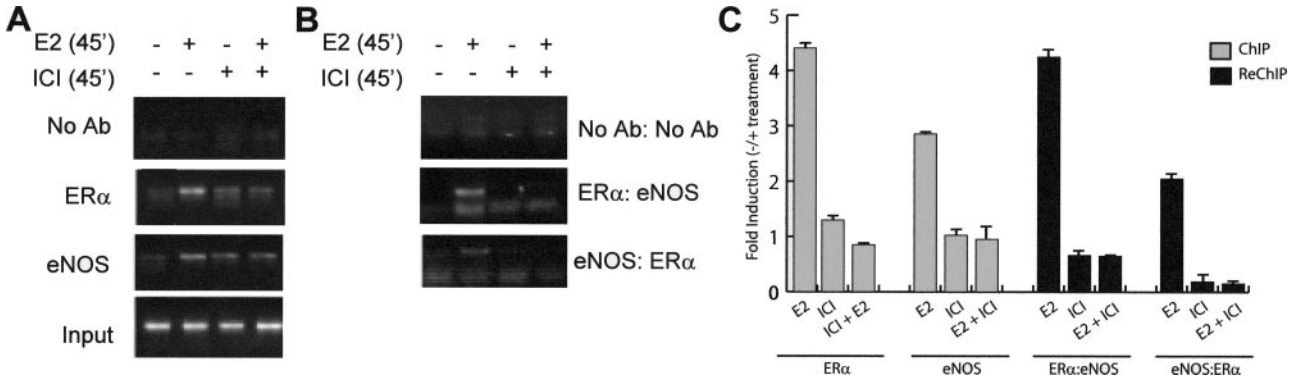

Figure 6. ChIP and Re-ChIP analysis of eNOS and ERα on E2 treatment. A and B, Soluble chromatin was prepared from HUVECs and divided into 2 aliquots, which were immunoprecipitated with antibodies to ERα and eNOS, respectively. Immunocomplexes were collected and eluted, and soluble chromatin fractions were reimmunoprecipitated (Re-ChIP) with the reciprocal antibodies, respectively. Controls for the ChIP and Re-ChIP fractions include input chromatin (Input) and no antibody (No Ab). Input, ChIP, and Re-ChIP DNAs were analyzed by PCR using hTERT promoter primers. C, ERα and eNOS recruitment on the hTERT promoter. Densitometric values were normalized to the corresponding input DNA after subtraction of No Ab and are expressed as fold inductions (±treatments). The bars represent the mean plus SEM of 3 independent experiments each performed in duplicate.

wild-type ($eNOS^{+/+}$) syngeneic mice, grown in the presence or absence of E2 and infected with adenovectors encoding a constitutively active form of eNOS (S1177D) or GFP as control.

Figure 4A shows the endothelial phenotype of these cells in terms of LDL uptake and expression of factor VIII. Telomerase activity was evaluated by TRAP (Figure 4B and 4C). In $eNOS^{+/+}$ animals, basal activity was significantly increased by estrogen, but inhibition of NO production by 7N abrogated the estrogen effect. In contrast to this, in $eNOS^{-/-}$ cells, basal telomerase activity was significantly compromised and enhanced by E2 to a significantly lower extent than in wild-type cells. Of note, in $eNOS^{-/-}$ cells, E2 alone was incapable of restoring even the basal activity exhibited by $eNOS^{+/+}$ cells, indicating that eNOS is required for optimal EC response to E2.

Delivery of the eNOS mutant (S1177D), alone or with E2, respectively, restored the basal or E2-dependent telomerase activity of $eNOS^{-/-}$ cells to the levels of wild-type cells. Addition of DETA-NO had similar effect on basal activity but was unable to restore E2 responsiveness, confirming that NO is a crucial signaling molecule for telomerase regulation and revealing a novel role for the eNOS protein itself on hTERT activity in the presence or absence of estrogen.

Estradiol Induces eNOS Nuclear Translocation, Colocalization With ERα, and Association In Vivo on the hTERT Promoter

NO and ERs are both important mediators of signal transduction in a variety of tissues, and numerous studies indicate a functional link between ERs and eNOS.[17,23,25] To assess the cellular expression pattern of these factors, we analyzed eNOS and ERα localization by confocal microscopy (Figure 5A and 5B). On estrogen stimulation, we observed nuclear colocalization of both proteins, suggesting their potential coregulation and functional crosstalk. Notably, inhibition of AKT, NO synthesis, or protein kinase G (PKG) function prevented nuclear translocation of eNOS and ERα and reduced telomerase activity (Figure 5C), indicating that the integrity of the AKT-eNOS-PKG signaling pathway is required for ERα function in response to E2 in ECs.

To investigate whether the ERα/eNOS interaction occurs at DNA level, ChIPs (Figure 6A and 6C) and Re-ChIPs (Figure 6B and 6C) were performed on the hTERT promoter region encompassing the ERE using HUVECs cultured with or without E2 and/or ICI. Surprisingly, we observed significant recruitment of both proteins on the hTERT promoter under E2 stimulation. Re-ChIPs showed the formation of a novel combinatorial complex between ERα and eNOS. Our results support a model in which ERα and eNOS jointly operate to stimulate hTERT expression and their complex is important in mediating hTERT response to E2.

Discussion

This study documents that the eNOS protein and its product NO are important for telomerase expression and activity in ECs stimulated by estrogens. We have shown that both ERα and ERβ bind dynamically to a specific hTERT promoter site determining a local wave of chromatin remodeling that correlates with active gene transcription. Notably, in this context, ER binding occurs in association with active eNOS. The inhibition of NO synthesis, in fact, prevents these effects and, at the biological level, the reduction of hTERT activity diminishes the proangiogenic functions of estrogen. Altogether, these findings suggest that estrogen regulation of telomerase, which requires eNOS-ER complex formation and NO synthesis, may be relevant for the effect of estrogen on ECs. Estrogens are important atheroprotective molecules that regulate vascular function exerting actions at genomic and nongenomic levels. Ligand-activated ERs affect eNOS expression and activity by transactivating the eNOS gene and stimulating NO synthesis in the caveolae.[24,25] The nongenomic effect of estrogens on eNOS activity is mediated by phosphatidylinositol 3-kinase (PI3K) activation, which, in turn, regulates the AKT-eNOS signaling.[18] Our previous experiments have indicated that the PI3K-AKT pathway is also important for TERT activity in the presence of vascular endothelial growth factor (VEGF).[33] The present study pro-

vides evidence that eNOS and ERα colocalize in the nucleus in the presence of E2, a process requiring an active NO signaling cascade being largely prevented by AKT, eNOS, and PKG inhibition. This expands our knowledge by indicating that in the estrogen/NO-dependent regulation of hTERT, both AKT and PKG kinases are involved, thus establishing a novel molecular hierarchy in the ER-dependent regulation of telomerase. Classically, ligand-activated ERs relocate to the nucleus and directly modify expression of target genes.[10,50] Recent evidence suggests that ERE are widely dispersed in the human genome and potentially regulate expression of a large number of genes.[50–52] Our results indicate that, concomitantly with ER binding, the histone H3 in the hTERT promoter is covalently modified conferring to this region a potentially open transcription–competent configuration. This effect, which is modulated in time, is abolished by the estrogen antagonist ICI or the NOS inhibitor 7N. This evidence suggests that both active eNOS and ER are required for estrogen-dependent hTERT chromatin recognition and remodeling. The presence of eNOS on the hTERT promoter in association with ERα further expands our knowledge on NO contribution to gene expression and provides novel insights about eNOS function in the nuclear environment.

Remarkably, recent evidence has shown that NO is able to inactivate HDAC2, a member of the histone deacetylase family often involved in negative regulation of gene expression as part of nuclear hormone receptor corepressor complexes.[53,54] This effect seems to involve cysteine-*S*-nitrosylation C.C., C. Mozzetta, A. Gurtner, B. Illi, J. Rosati, S. Straino, G.R., M. Pescatori, G. Zaccagnini, A. Antonini, G. Minetti, F. Martelli, G. Piaggio, P. Gallinari, C. Steinkulher, E. Clementi, L. Altucci, A. Mai, M.C.C., P.L. Puri, C.G., manuscript in preparation). In our experimental system, the estrogen-mediated ER-eNOS association may be involved, therefore, in the recruitment of ER cofactors including chromatin remodelling enzymes controlling DNA unwinding and transcription of target genes.[47] Hence, the importance of active eNOS complexed with ERα may reside on its potential capacity of providing localized production of NO with regulatory effects on ER function, perhaps through changes of the redox balance at local chromatin level.[55]

Human telomerase has been previously identified as an estrogen responsive gene[35,56]; however, the present work provides the first evidence that the estrogen-dependent mechanism of TERT regulation is active in ECs and depends on the presence of eNOS and NO synthesis. Previous work indicated that NO is important for VEGF signaling and for the VEGF effect on TERT function in vitro and in vivo,[33] which, in turn, modulates the angiogenic response to VEGF. The production of NO decreases in aged animals and senescent cells,[32,57] and this reduction affects telomerase activity. Therefore, the age-dependent reduction of estrogens, associated with reduced NO levels, may have a direct effect on TERT activity contributing to the diminished angiogenic response of ECs in aged patients.

In conclusion, here, we document for the first time that the presence of an active eNOS and NO production play a major role in the regulation of TERT by estrogens. This effect, in fact, requires NO synthesis and is achieved by an ER-eNOS complex recruited onto the hTERT ERE genomic site. Given that telomerase, on its own, can induce angiogenesis and protect from apoptosis,[33] it is reasonable to assume that its induction is essential for estrogen regulation of endothelial function. These findings widen our views on the effect of estrogens and nitric oxide in the vascular system and may lead to the design of novel therapeutic interventions based on the regulation of hTERT to delay the onset of age-dependent endothelial dysfunction.

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Disclosures

None.

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