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Cellular and Molecular Life Sciences

# Research Article

# Progesterone inhibits human endothelial cell proliferation through a p53-dependent pathway

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**Abstract.** Previous studies have shown that progesterone inhibits endothelial cell proliferation through a nuclear receptor-mediated mechanism. Here, we further demonstrate that progesterone at physiologic levels (5 – 500 nM) dose- and time-dependently inhibited DNA synthesis of cultured human umbilical vein endothelial cells (HUVEC). The mRNA and protein levels of p21, p27, and p53 in HUVEC were increased by progesterone. The formation of CDK2-p21 and CDK2-p27 were increased and the CDK2 activity was decreased in the progesterone-treated HUVEC. The progesterone-inhibited [3H]thymidine incorporation was completely blocked when the expressions of p21 and p27 were knocked-down together. Transfection of HUVEC with dominant negative p53 cDNA prevented the progesterone-induced increases in p21 and p27 promoter activity and protein level, decreases in thymidine incorporation, and capillary-like tube formation. Matrigel angiogenesis assay in mice demonstrated the anti-angiogenic effect of progesterone *in vivo*. These findings demonstrate for the first time that progesterone inhibited endothelial cell proliferation through a p53-dependent pathway.

**Keywords.** Progesterone, angiogenesis, p53, p27, p21.

## Introduction

The protective effect of sex hormones in cardiovascular diseases has been well documented. Several epidemiological and animal studies demonstrated that estrogen and progesterone together are more effective than estrogen by itself in protecting against some cardiovascular disease conditions [1 – 4]. However, there has been little evidence of independent progesterone effect on the cardiovascular system. Previously, we showed that progesterone directly inhibits proliferation of cultured vascular smooth muscle cells through a progesterone receptor-mediated pathway [5, 6]. The effect of progesterone on proliferation of vascular endothelial cells has been addressed by

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Vázquez et. al., who showed that progesterone could inhibit the proliferation of vascular endothelial cells and the rate of re-endothelialization, and thus impair vascular repair processes through progesterone receptor dependent pathway [7]. However, the underlying molecular mechanisms are still not clear, and the effect of progesterone on angiogenesis was not addressed in their study. Since endothelial cell proliferation is one of the major events essential for angiogenesis, the finding of the anti-proliferation effect of progesterone on endothelial cells led us to hypothesize that progesterone might also exert an anti-angiogenic activity.

Angiogenesis, the formation of new blood vessels from pre-existing vessels, is required in many physiological and pathological conditions [8, 9]. Normally, vascular proliferation occurs only during embryonic development, and is a very slow process in the adult with a few specific exceptions. The ovary and endometrium are two of the few tissues in the adult organism in which angiogenesis is a prominent feature in normal conditions. Studies of the regulation of angiogenesis in the primate endometrium suggest that physiological angiogenesis is under the control of sex steroids. It has been demonstrated that physiological concentrations of estradiol increase VEGF expression in endometrial epithelial and stromal cells [10–12]. There is a large body of literatures that describes the important role of estradiol in endometrial angiogenesis, but far less is known about the progesterone effect in this aspect.

In the present investigation, we studied some anti-angiogenic actions of progesterone and examined the underlying cellular and molecular mechanisms in both *in vitro* and *in vivo* experiments. These experimental findings reported below highlight certain molecular mechanisms of progesterone-induced inhibition of angiogenesis.

## Methods and Materials

**Cell and cell culture.** HUVEC were grown in M199 (GIBCO, Grand Island, NY) containing 10% FBS (Highveld Biological, Lyndhurst, RSA), endothelial cell growth supplement (ECGS, 0.03 mg $mL^{-1}$) (Biomedical Technologies, Stoughton, MA) and kanamycin (GIBCO) (50 U $mL^{-1}$) in a humidified 37 °C incubator. Cells from passages 3–10 were used.

### Preparation of plasmid constructs

**P21 promoter.** The pWWP-luc plasmid construct, containing promoter region of human p21 between positions −2,300 and +8 was a gift from Vogelstein (Johns Hopkins University, Baltimore, MD) [13]. P21 promoter was subcloned into pGL3-Basic luciferase reporter vector (Promega, Madison, WI) at the unique HindIII site.

**P27 promoter.** The fragment of human p27 promoter region spanning positions +132 to -1358 was amplified by genomic PCR, using genomic DNA from HepG2 cells as the template. The following primers were used: +132~-1358, 5'- TTTGATATCTTTCTCCC-GGGTCTGCACGAC-3' and 5'- TGTCTCTGCA-GTGCTTCTCCAAGTCCCGGG-3. P27 promoter was subcloned into pGL3-Basic luciferase reporter vector at Kpn I/XhoI sites.

**P53 dominant negative construct.** For amplification of the hot-spot mutant (V143A) of p53 [14, 15], recombinant PCR was performed. Synthesized cDNA (SuperScript III First-Strand Synthesis System for RT-PCR; Invitrogen, Carlsbad, CA) from HUVEC was used as a template and Phusion high-fidelity DNA polymerase (Finnzymes Diagnostics, Finland) with 3'→5' exonuclease activity was used in subsequent PCR. In the first round PCR, forward (5'-ATGGAG-GAGCCGCAGTCAG-3') and reverse (5'-TCAG-TCTGAGTCAGGCCCTTC-3') primers were used for amplified full-length wild type p53 (WT-p53). In the second round PCR, two primer sets 1 and 2 were used to synthesize fragment A and fragment B. Primer set 1: Forward (5'-CTTAAGCTTGCCGCCATG-GAGGAG -3'), and reverse (5'-CACAGATGCG-CAGGGCAGGT-3'). Primer set 2: Forward (5'-ACCTGCCCTGCGCAGCTGTG-3') and reverse (5'-GTGCTGGATATCTCAGTCTGAGTC-3'). For each fragment, the amplified PCR product was gel purified and used as a mixed template for synthesizing $p53^{V143A}$ in the PCR reaction. The synthesized PCR product of $p53^{V143A}$ was purified, digested with restriction enzyme HindIII and EcoRV, and cloned into pcDNA3.1+ (Invitrogen). The $p53^{V143A}$ sequence fidelity was confirmed by ABI PRISM 377 DNA Analysis System.

**RNA isolation and semiquantitative RT-PCR.** Total RNA was isolated from HUVEC with Trizol reagent (Invitrogen) according to the manufacturer's protocol. Two micrograms of total RNA were reverse transcribed to synthesize cDNA using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Two microliters of the first-strand reaction were used for PCR amplification with specific primers for p53, p21 and p27, together with the presence of the Aldolase A primers, and were carried out in the Gene Amp PCR system 2400 (Perkin-Elmer). The following

primers were used: p53 5'-CGGTTTCCGTCT-GGGCTTCT-3' and 5'-GCACCTCAAAGCTG-TTCCGTCCC-3'; P21 5'-GCCGCGACTGTGAT-GCGCTAATG-3' and 5'-CCGGCGTTTGGAGT-GGTAGA-3'; p27 5'-AGAGGCGAGCCAGCGCA-A-3' and 5'-CTGCTCCACAGAACCGGCA-3'; Aldolase A 5'-GGCACCGAGAACACCGAGGAGA-3' and 5'-TTGATGCCCACAACACCGCCC-3'. The PCR parameters comprised a cycle of 5 min at 95 °C, 30 s at 58 °C and 1 min at 72°C, followed by 30 s at 95 °C, 30 s at 58 °C and 1 min at 72 °C for 34 cycles. The number of cycles used in this study lies along a linearly increasing product amt/cycle curve (data not shown). PCR products were electrophoresized on a 2% agarose gel and visualized with ethidium bromide.

**Cell transfection.** For transient transfection of the indicated constructs into HUVEC, jetPEI-HUVEC transfection reagent (Polyplus Transfection, Bioparc, France) was used as per the manufacturer's protocol. Briefly, jetPEI-HUVEC/DNA mixture was added drop-wise onto the DMEM+Glutamax™ I medium (GIBCO) containing 2% FBS, mixed gently, and incubated in a humidified 37°C incubator for 4 h. The growth medium was then replaced and the cells were incubated for the next 24 h. The transfection efficiency was approximate 55% as determined by percentage of the cells transfected with EGFP.

**RNA interference.** Inhibition of p53 expression was performed by introducing small interfering RNAs (siRNA) into HUVEC according the protocol of *Trans*IT-TKO transfection reagent (Mirus Bio Corporation, Madison). Briefly, the cells were transfected with 25 nmol/L p53-specific siRNA duplex (cat #: sc-29435; Santa Cruz Biotechnology, CA) or non-targeting siRNA (cat #: 4390843; Applied Biosystems/ Ambion, TX) for 24 h, and then rendered quiescent by incubation of the cells in M199 containing 2% FBS for additional 24 h. M199 (phenol red free) supplemented with 10% charcoal-stripped FBS and DMSO (0.1%) with or without 500 nM progesterone (Sigma-Aldrich, St. Louis, MO) were added to the cells and cell extracts were harvested for protein detection at 24 h after progesterone treatment.

**Luciferase assay.** After treatment with DMSO (0.1%) or progesterone (500 nM) in DMSO (0.1%), the cells were washed twice with ice-cold PBS and lysed by cell culture lysis reagent (Promega). Luciferase activities were recorded in a $20/20^n$ luminometer (Turner Biosystems, Sunnyvale, CA) using the dual luciferase assay kit (Promega) according to the manufacturer's instructions.

**[$^3$H]thymidine incorporation.** The [$^3$H]thymidine (Amersham Biosciences, UK) incorporation was performed as previously described [16]. Briefly, HUVEC were applied to 24-well plates in growth medium (M199 plus 10% FBS and ECGS). After the cells had grown to 60–70% confluence, they were rendered quiescent by incubation for 24 h in M199 containing 2% FBS. M199 (phenol red free) supplemented with 10% charcoal-stripped FBS and DMSO (0.1%) with or without progesterone (5–500 nM) were added to the cells and the cultures were allowed to incubate for 24 h. During the last 3 h of the incubation, [$^3$H]thymidine was added at 1 μCi mL$^{-1}$ (1 μCi = 37 kBq). Incorporated [$^3$H]thymidine was extracted in 0.2 N NaOH and measured in a liquid scintillation counter.

**MTT assay.** Cell growth was estimated by a modified MTT [3-(4,5-Dimethyl-2- thiazolyl)-2,5-diphenyl-tetrazolium bromide] (USB/Amersham Life Science) assay after the cells were daily treated with DMSO (0.1%) or progesterone (5–500 nM) in DMSO (0.1%) for three days.

**Protein extraction and Western blot analysis.** To determine the protein levels in HUVEC, the total proteins were extracted and Western blot analyses were performed as described previously [17]. Electrophoresis was performed using 12% SDS-polyacrylamide gel (3 h, 70V, 50 μg protein per lane). Separated proteins were transferred to PVDF membranes (3 h, 400 mA), treated with 5% fat-free milk powder (Anchor, NZ) to block the nonspecific IgGs, and incubated for 1 h with specific antibody against p53,α-tubulin (Santa Cruz Biotechnology, CA), p21, p27, CDK2, CDK4 (BD Bioscience Pharmigen, CA), or G3PDH (Jackson ImmunoResearch Laboratories, PA). The blot was then incubated with anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch Laboratories) conjugated to horseradish peroxidase (HRP) for 1 h. Subsequently, the PVDF membrane was developed with chemiluminescence reagent plus (PerkinElmer Life Sciences, Boston, MA). To quantify the intensity of the protein expression levels, the exposed X-ray films (Fuji Photo Film, Tokyo, Japan) were scanned using an HP ScanJet scanner (HP ScanJet 5470C) and HP Precision ScanPro software, and the band densities were determined as arbitrary absorption units using the Image-Pro Plus 2 software program.

**Immunoprecipitation and kinase activity assay.** As previously described [16], the progesterone (5–500 nM)-treated cells were lysed in Gold lysis buffer (137 mM NaCl, 20 mM Tris, PH7.9, 10 mM NaF, 5 mM

EDTA, 1 mM EGTA, 10 % (v/v) glycerol, 1 % triton X-100, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, 100 μM β-glycerophosphate, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/mL leupeptin), and immunoprecipitated with anti-CDK2 or anti-CDK4 antibody (2 μg; BD Transduction Laboratories, CA). The phosphorylation levels of histone H1 (substrate for CDK2) and Gst-Rb fusion protein (substrate for CDK4) were measured by incubating the beads with 40 μL of kinase assay buffer containing 2 μg of Gst-Rb fusion protein (Santa Cruz Biotechnology) or 1 μg of histone H1 (Calbiochem, California), 0.5 μCi of [γ-$^{32}$P]ATP (Amersham), and 10 μM cold ATP at 37 °C for 20 min, and then stopped by boiling the samples in SDS sample buffer for 5 min. The samples were analyzed by 12 % SDS-PAGE, and the gel was then dried and subjected to autoradiography.

**Antisense oligonucleotide.** The antisense (AS) oligonucleotide was designed based on the coding sequence, which is complementary to the region of the initiation codon (5'-TCCCCAGCCGGTTCTGACAT-3' for AS p21 and 5'-GACACTCGCACGTTTGACAT-3' for AS p27). The scramble oligonucleotide of AS p21 (S p21) or AS p27 (S p27) was used for the control (S p21: 5'-GGACTCGCCTCGCCATCTTA-3'; S p27: 5'-GCAGTTACGCGTCTACACTA). The oligonucleotides purchased from Sigma-Genosys, Inc. were transfected with Oligofectamine™ reagent (Invitrogen) using the manufacturer's protocol into the cells at a final concentration up to 20 nM before the cells were challenged with 10 % FBS.

**Capillary-like tube formation assay.** Capillary-like tube formation assay was performed as described previously [18]. The 96-well plates were coated with 50 μL Matrigel (10 mg/mL) (BD Bioscience Pharmigen, CA, USA) by incubating at 37 °C for 1 h. HUVEC were suspended in M200 (Cascade Biologics, Portland, OR, USA) supplemented with 10 % FBS and endothelial cell growth supplement, and plated onto a layer of Matrigel at a density of 4x10$^4$ cells/well without or with progesterone (5 – 500 nM). The plates were then incubated for a further 3 h at 37°C, and capillary-like tube formation was observed using the microscope.

**Chromatin Immunoprecipitation (ChIP) Assay.** The ChIP assay was performed using a standard protocol with minor modifications. Briefly, HUVEC were cross-linked by adding 1 % (v/v) formaldehyde, and incubated at room temperature for 10 min. The cross-linking reaction was stopped by adding glycin to a final concentration of 0.125 M. HUVEC was washed twice with ice cold PBS, re-suspended, and homogenized in 0.5 ml swelling buffer (5 mM PIPES pH 8.0, 0.5 % Triton X-100, 0.5 mM PMSF) plus Complete Protease Inhibitor (Roche, Germany). Nuclei pellet was sonicated to shear an average DNA length in 0.5 mL sonication buffer (10 mM EDTA, 50 mM Tris-HCl pH 8.0, 0.5 mM PMSF) plus Complete Protease Inhibitor (Roche). Non-specific binding was reduced by adding 0.1 % SDS with 85 mM KCl together. Samples were divided into two equal aliquots and individually incubated for 1 h at 4 ºC with 1 μg of anti-p53 antibody (DO-1; Santa Cruz Biotechnology, CA) or 1 μg of anti-α tubulin antibody (Sigma-Aldrich, St. Louis, MO) as a control, added 30 μL of Protein A Agarose / Salmon Sperm DNA slurry (Upstate, Temecula, CA), and then incubated at 4 ºC overnight with constant rotation. The protein A agarose/antibody/chromatin complex was washed and cross-links were reversed using the Upstate ChIP protocol. DNA was purified using QIAquick PCR purification kit (Quiagen, Valencia, CA) and eluted in a 50 μL EB buffer (10 mM Tris-HCl pH 8.5). One μl of the DNA was assayed in a PCR cycle. Primers for consensus p53 binding site in p21 promoter were: forward: CTGGACTGGGCACTCTTGTC; reverse: GAGGTCTCCTGTCTCCTACCATC. P53 binding site within p27 promoter was analyzed in MOTIF Search (http://motif.genome.jp/), and the primers designed using Primer-BLAST of NCBI were: forward: CGTCTTTTCGGGGTGTTTT; reverse: GCCGAGTCTCCGCTGAT. All the PCR products were analyzed by 2 % agarose gels containing ethidium bromide.

**Matrigel angiogenesis assay.** The Matrigel angiogenesis assay, which has been used to evaluate angiogenesis *in vivo*, was performed as previously described [19]. A 500 μL portion of a liquid mixture of Matrigel (350 μL, BD Biosciences, CA), M199 medium (150 μL) and VEGF (50 ng/mL) with or without progesterone (500 nM) was injected subcutaneously into 7-week-old C57/BL6 male mice. Matrigel rapidly formed a single, solid gel plug. The plugs allowed VEGF to stimulate angiogenesis. On the 7th day, mice were sacrificed, and the Matrigel plugs were excised. Vascularization of Matrigel plugs was quantified by measuring hemoglobin (Hb) content using Drabkin reagent (Sigma-Aldrich) according to the instructions of the manufacturer. All *in vivo* procedures were approved by the Taipei Medical University Animal Care and Use Committee.

## Statistics

All data were expressed as the mean value ± s. e. mean. Comparisons were subjected to one way analysis of variance (ANOVA) followed by Fisher's

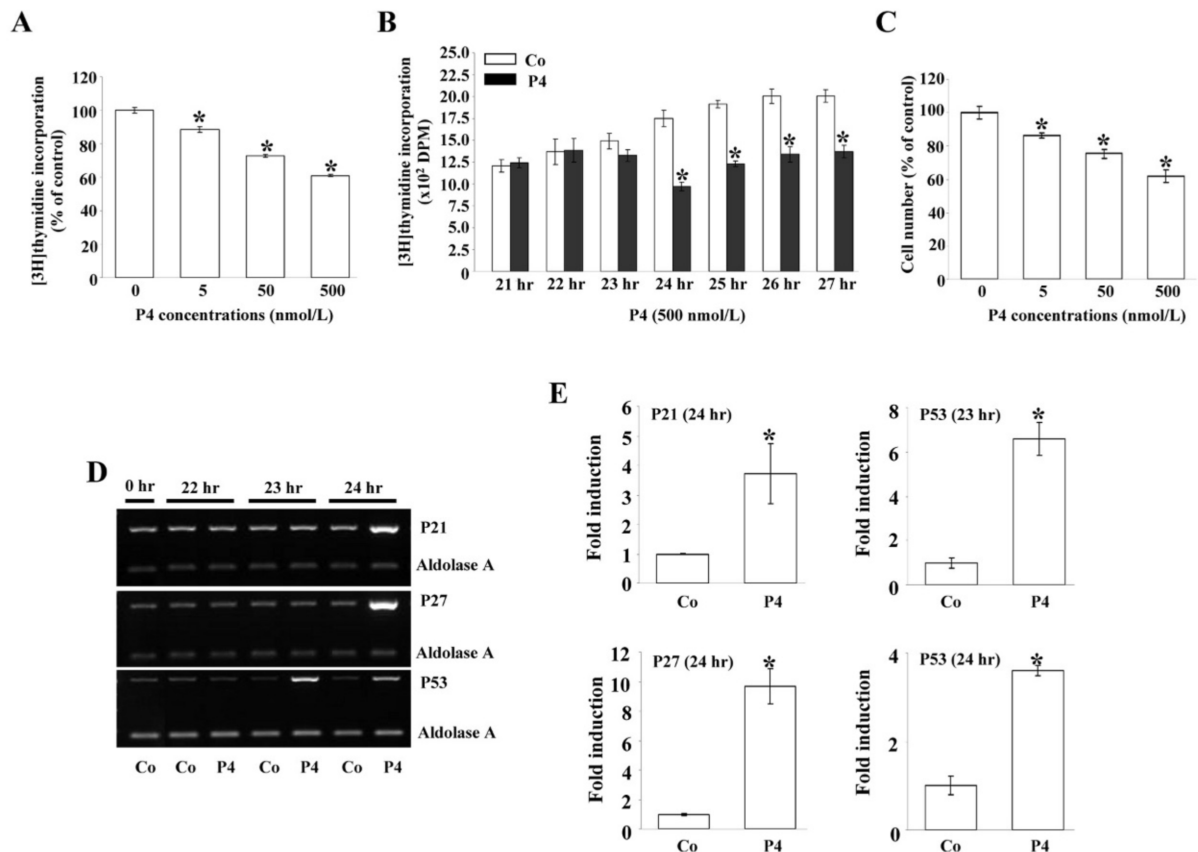


**Figure 1.** Progesterone inhibits DNA synthesis and increases the levels of p21, p27 and p53 mRNA in HUVEC. (*A*) Progesterone (5–500 nM) dose-dependently inhibited [$^3$H]thymidine incorporation. (*B*) Progesterone (500 nM) time-dependently inhibited [$^3$H]thymidine incorporation into HUVEC. (*C*) Progesterone (5–500 nM) dose-dependently decreased cell number in cultured HUVEC. Cell number was examined using MTT assay after daily treatment of cells with progesterone (5–500 nM) in DMSO (0.1 %) or DMSO (0.1 %) for three days. Values represent the means ± s. e. mean. (n = 4). * $P < 0.05$ different from control. (*D*) A representative measurement of the levels of p21 p27, and p53 mRNA. Progesterone (500 nM) increased the levels of p21 (at 24 h), p27 (at 24 h), and p53 mRNA (at 23 and 24 h) in HUVEC. The levels of p21, p27 and p53 mRNA were determined by semiquantitative RT-PCR analysis. Semiquantitative RT-PCR products of Aldolase A were used as an internal control to verify equivalent sample loading. (*E*) Quantitative results of p21, p27, and p53 mRNA, which were adjusted with Aldolase A mRNA level and expressed as fold-induction of control. Values represent the means ± s. e. mean. (n = 3). * $P < 0.05$ different from corresponding control. Co, control; P4, progesterone.

least significant difference test. Significance was accepted at $P < 0.05$.

## Results

**Progesterone inhibits thymidine incorporation into HUVEC.** To study the anti-angiogenic effect of progesterone, we examined the effect of progesterone on cell proliferation of HUVEC. As illustrated in Figures 1A and 1B, progesterone at a range of concentrations (5–500 nM) dose- and time-dependently decreased thymidine incorporation into cultured HUVEC. MTT assay demonstrated that progesterone also dose-dependently decreased cell number in cultured HUVEC (Fig. 1C).

**P21 and p27 are the key regulators for the progesterone-induced G0/G1 arrest.** It was previously demonstrated that progesterone inhibits the cell cycle of vascular endothelial cells at the G0/G1 phase [7]. To investigate the molecular mechanisms underlying of progesterone-induced G0/G1 arrest, HUVEC were switched to media with 2 % FBS for 24 h to render them quiescent at the G0/G1 phase. They were then returned to culture media supplemented with 10 % FBS and DMSO (0.1 %) without or with progesterone, and at different time points thereafter they were harvested for RNA and protein extraction. Semi-quantitative RT-PCR and Western blot analyses were conducted to examine the effects of progesterone on the expression of cell cycle regulatory proteins. Progesterone (500 nM) treatment increased the levels of mRNA (Figs. 1D and 1E) and protein (Figs. 2A–C) of p53, p21and p27 at 23, 24 and 24 h, respectively. The

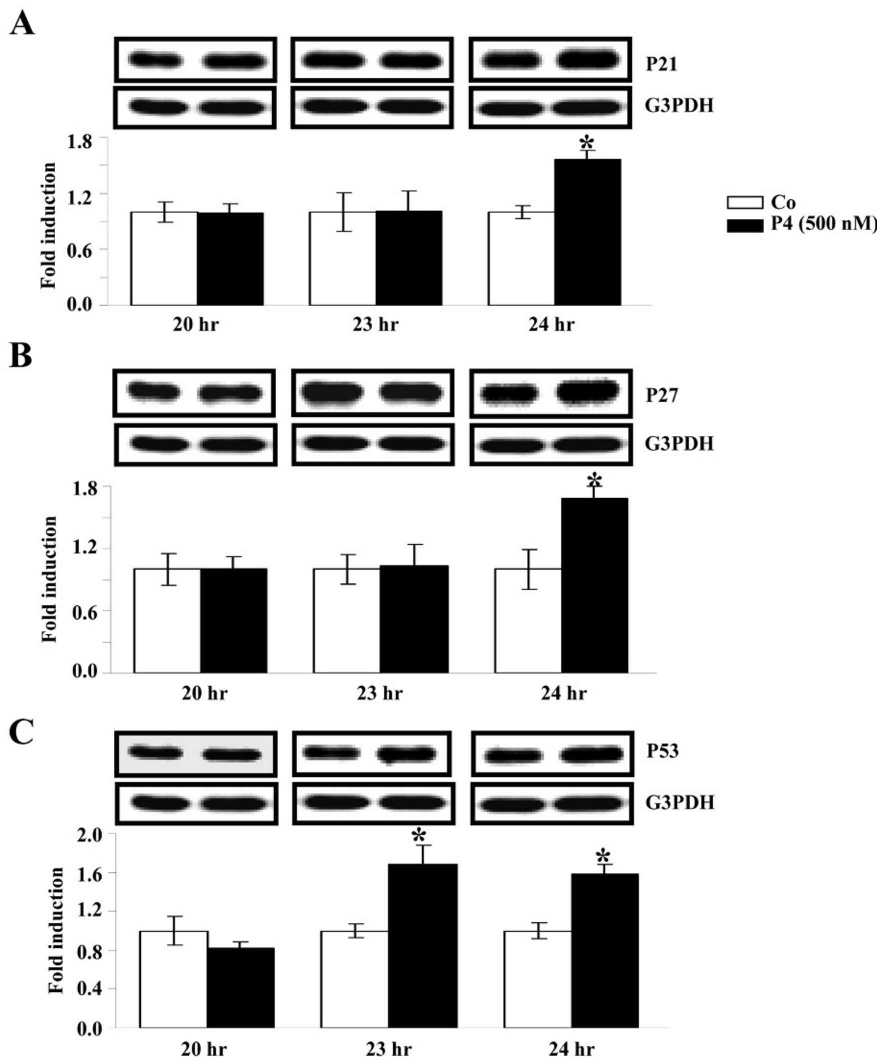


**Figure 2.** Effect of progesterone on the protein levels of p21, p27 and p53. Progesterone (500 nM) increased the protein levels of p21 (*A*), p27 (*B*), and p53 (*C*) in HUVEC. Top panels: representative results of p21, p27, p53 and G3PDH protein levels determined by Western blot analysis. Bottom panels: Quantitative results of p21, p27 and p53 protein levels, which were adjusted with G3PDH protein level and expressed as fold-induction beyond its own control. Values represent the means ± s. e. mean. (n = 4). * $P < 0.05$ different from corresponding control. Co, control; P4, progesterone.

CKI exerts its inhibitory effect on the kinase activity through binding to the cyclin-CDK complex. Accordingly, we further performed immunoprecipitation assay to examine the effect of progesterone on the formation of the CDK-CKI complex. In the progesterone-treated HUVEC, the production of the CDK2-p21 and CDK2-p27 complex, but not CDK4-p21 and CDK4-p27 complex, were increased (Fig. 3A). To demonstrate that the increased p21 and p27 proteins are associated with inhibition of CDK activation, which is necessary for cell cycle progression from $G_1$ to S phase, we examined the CDK kinase activity. In the progesterone-treated HUVEC, the kinase activity of CDK2, but not CDK4, was significantly decreased in a dose-dependent manner (Fig. 3B). To further demonstrate that the increased p21 and p27 expression observed in the progesterone-treated HUVEC correlated with G0/G1 arrest, the experiment illustrated in Figure 4 was conducted. Thus, in the sample labeled P4 (for 500 nM progesterone-treated alone), the [$^3$H]thymidine incorporation was decreased. Sample P4+AS p21 or P4+AS p27 was pretreated with a p21 or p27 antisense oligonucleotide (AS), which blocked the expression of p21 (Fig. 4A) or p27 (Fig. 4B) protein respectively. Treatment of HUVEC with AS p21 or AS p27 alone did not cause any significant change in [$^3$H]thymidine incorporation into HUVEC. Consequently, pretreatment of HUVEC with AS p21 or AS p27 partially blocked the progesterone-induced decrease in [$^3$H]thymidine incorporation. The progesterone-induced inhibition in [$^3$H]thymidine incorporation of HUVEC was completely blocked by a combined administration of both antisense oligonucleotides to p21 and p27 together (Fig. 4C). In contrast, sample P4+S p21 or P4+S p27 was pretreated with a p21 or p27 scramble antisense oligonucleotide, which did not cause any significant change in the expression of p21 and p27 protein, and did not

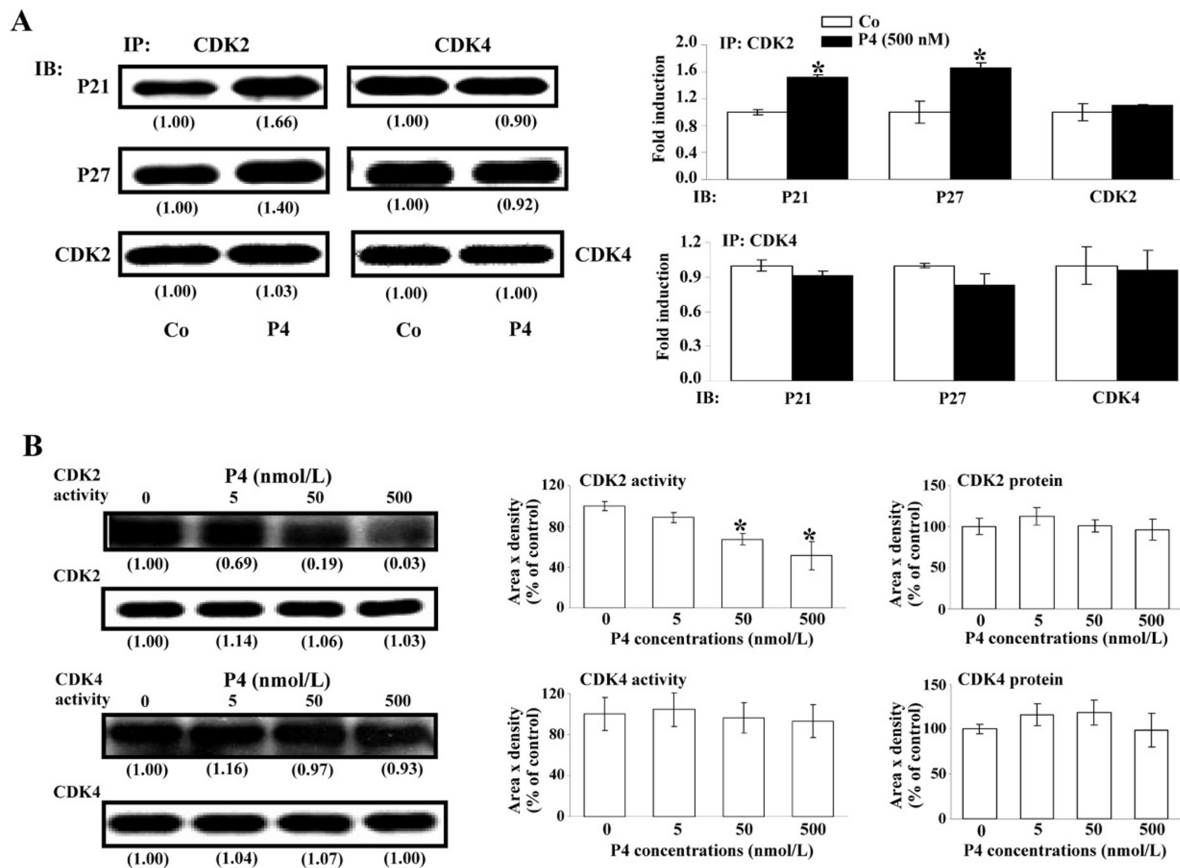


**Figure 3.** Effect of progesterone on the formation of CKI-CDK complex and CDK kinase activity. (*A*) Progesterone (500 nM) increased the formations of CDK2-p21 and CDK2-p27 complex, but not CDK4-p21 and CDK4-p27 complex, in HUVEC. Left panel: a representative result of the formations of CDK2-p21, CDK2-p27, CDK4-p21 and CDK4-p27 in HUVEC treated with or without progesterone (500 nM). The corresponding CDK2 and CDK4 protein levels are also shown. Right panel: quantitative results of three samples per group and values represent the means ± s. e. mean. (n = 3). * $P < 0.05$ different from control. (*B*) The CDK2, but not CDK4, kinase activity was decreased by progesterone treatment in a dose-dependent manner. The CDK2 and CDK4 kinase activities were determined as described in the Methods and Materials. Left panel: a representative result of the CDK2 and CDK4 activity and corresponding CDK2 and CDK4 protein levels in HUVEC treated without or with progesterone (5–500 nM). Middle panel: quantitative results of CDK2 and CDK4 activity. Values represent the means ± s. e. mean. (n = 3). * $P < 0.05$ different from control. Right panel: quantitative results of the corresponding CDK2 and CDK4 protein levels. Values represent the means ± s. e. mean. (n = 3). Co, control; P4, progesterone; IP, immunoprecipitation; IB, immunoblotting.

cause any significant change in [$^3$H]thymidine incorporation in the HUVEC.

**Involvement of p53 in progesterone-induced upregulation of p21 and p27 and decrease of thymidine incorporation.** To investigate whether the progesterone-induced increases of p21 and p27 protein and decreases of thymidine incorporation were regulated by p53, the dominant negative p53 cDNA was transfected into HUVEC. Pre-transfection of HUVEC with dominant negative p53 cDNA inhibited the progesterone-induced increases of p21 and p27 promoter activity (Fig. 5A) and protein level (Fig. 5B) and abolished progesterone-induced inhibition of thymidine incorporation (Fig. 5D, top panel). We confirmed these findings by using siRNA technique. Transfection of the cells with p53 siRNA blocked the progesterone-induced increases of p53, p21, and p27 protein (Fig. 5C) and decreases of thymidine incorporation (Fig. 5D, bottom panel). To further confirm the involvement of p53 on the progesterone-induced up-regulation of p21 and p27, we conducted the chromatin immunoprecipitation assay. As illustrated in Figure 5E, the p53 DNA binding activity on the p21 and p27 promoter was activated by progesterone (500 nM).

**Anti-angiogenic effect of progesterone.** To confirm the anti-angiogenic effect of progesterone, we performed the capillary-like tube formation assay. As illustrated in Figure 6A, progesterone (5–500 nM) dose-dependently inhibited the capillary-like tube

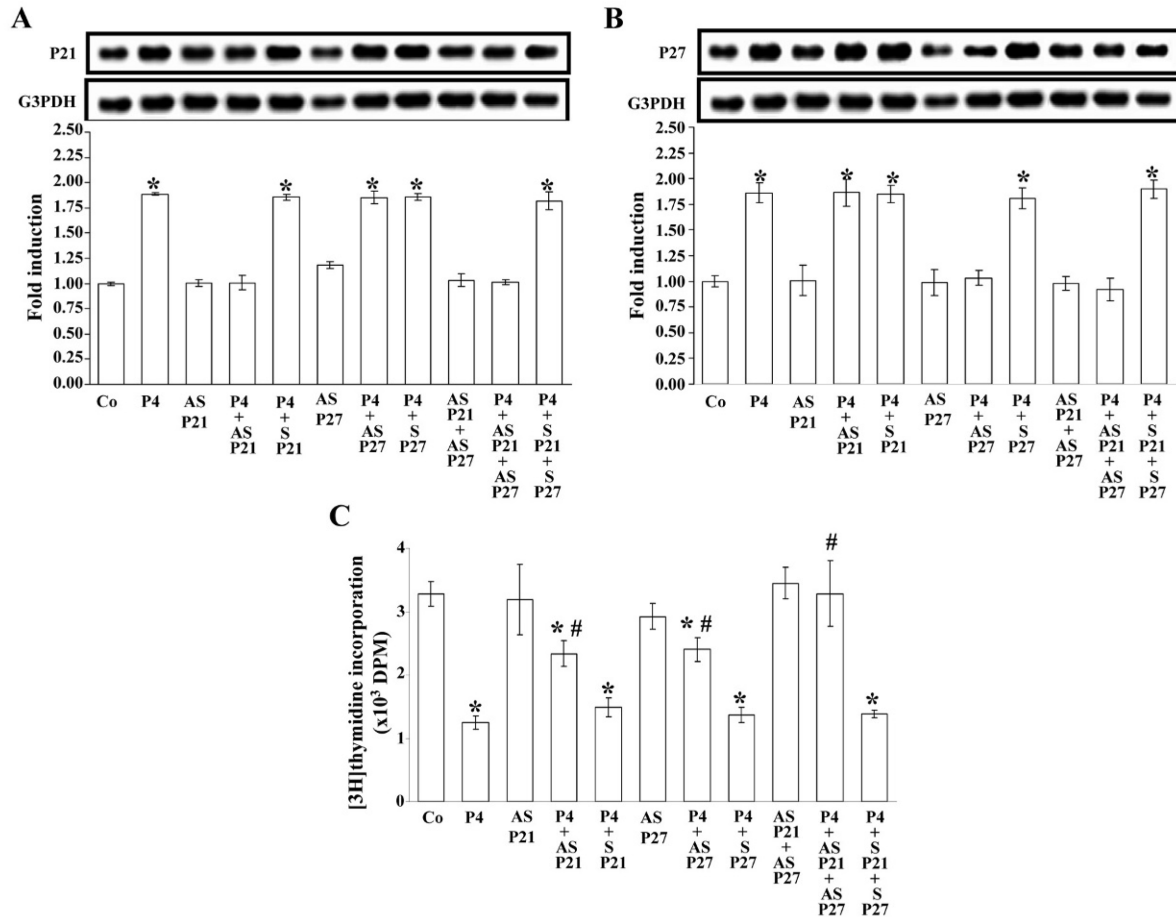


**Figure 4.** P21 and p27 are the key regulators for the progesterone-induced inhibition of DNA synthesis in HUVEC. Antisense (AS) p21 (*A*) and p27 (*B*) oligonucleotide blocked the progesterone (500 nM)-induced increases of p21 and p27 protein, respectively. In contrast, scramble (S) p21 and p27 oligonucleotide did not affect the progesterone (500 nM)-induced increases of p21 and p27 protein. Upper panel: a representative result of p21, p27 and G3PDH protein levels determined by Western blot analysis. The electrophoresis membrane was probed with G3PDH antibody to verify equivalent sample loading. Lower panel: Quantitative results of p21 and p27 protein levels, which were adjusted with G3PDH protein level and expressed as fold-induction of control (without oligonucleotide and P4 treatment). Values represent the means ± s. e. mean. (n = 3). * $P < 0.05$ different from control. (*C*) AS p21 or AS p27 oligonucleotide alone partially blocked the progesterone-mediated decrease of [$^3$H]thymidine incorporation. However, the progesterone-induced inhibition in [$^3$H]thymidine incorporation of HUVEC was completely blocked by a combined administration of both AS p21 and AS p27 together. [$^3$H]thymidine incorporation was conducted after HUVEC were released from quiescence by incubation in culture media supplemented with 10 % FBS and 0.1 % DMSO (Co) or 500 nM P4 in 0.1 % DMSO. Values represent the means ± s. e. mean. (n = 4). * $P < 0.05$ different from control. # $P < 0.05$ different from P4-treated. Co, control; P4, progesterone; AS, antisense oligonucleotide; S, scramble oligonucleotide.

formation. The progesterone-induced inhibition in the capillary-like tube formation was abolished by pre-transfection of the cells with dominant negative p53 cDNA (Fig. 6B). The anti-angiogenic effect of progesterone was further confirmed by using Matrigel angiogenesis assay *in vivo.* Figure 6C demonstrated that progesterone (500 nM) decreased the apparent plasma volume of the Matrigel plug.

## Discussion

Progesterone is a paradoxical hormone exerting either growth stimulatory or inhibitory effects, depending on the tissue and the treatment regimen [20]. For example, progesterone inhibits epithelial growth in the uterus [21]. On the other hand, in animals with established progesterone receptor-positive mammary tumors, progesterone usually stimulates proliferation [22]. We previously demonstrated that progesterone at physiologic levels (5–500 nM) dose-dependently inhibited the proliferation of cultured human and rat aortic smooth muscle cells [6], suggesting a direct inhibitory effect of progesterone on the development of atherosclerosis. In the present study, we show that progesterone at physiologic levels (5–500 nM) inhibited the proliferation of the cultured HUVEC, and the

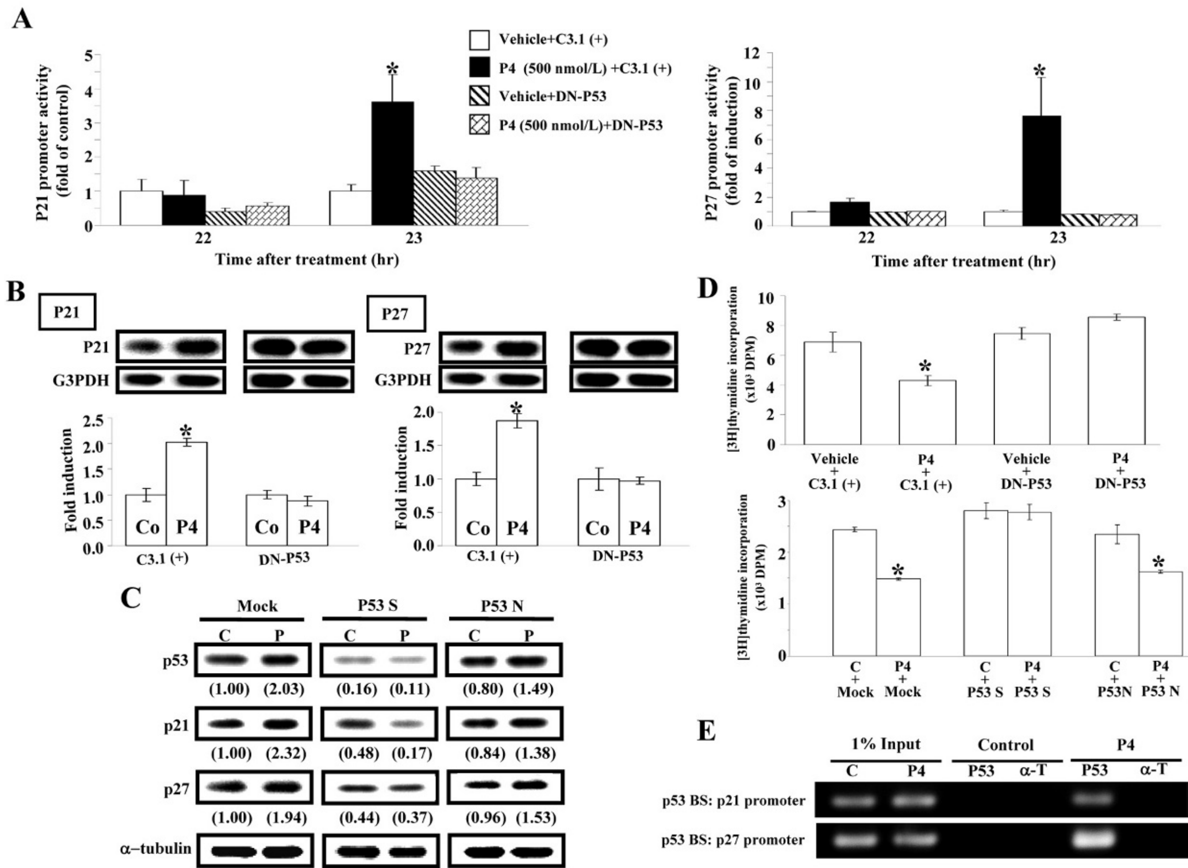


**Figure 5.** P53-dependent increases of promoter activities and protein levels of p21 and p27 by progesterone. (*A*) Transfection of the HUVEC with DN-p53 prevented the progesterone-induced increases of the p21 (left panel) and p27 (right panel) promoter activity. The HUVEC were transfected with either C3.1 (+) (control vector) or DN-p53. *Renilla* luciferase expression vector, used as the control, was co-transfected with promoter in the luciferase activity assay. Twenty-four h after transfection, the cells were rendered quiescent, and then treated with 10 % FBS and vehicle without or with progesterone (500 nM) for 22 or 23 h. Subsequently, the cells were processed for the luciferase activity assay. Values represent the means of fold of its own control ± s. e. mean. (n = 4). (*B*) Transfection of the HUVEC with DN-p53 inhibited the progesterone-induced increases of the p21 and p27 protein. Top panel: representative results of p21, p27 and G3PDH protein levels. Bottom panel: quantitative results of p21 and p27 protein levels, which were adjusted with G3PDH protein levels and expressed as fold-induction of its own control. Values represent the means ± s. e. mean. (n = 5). * $P < 0.05$ different from corresponding control. (*C*) The progesterone-induced increases of p53, p21 and p27 protein in HUVEC were suppressed by p53 siRNA, but not by non-specific siRNA. Results from a representative experiment are shown. Values shown in parentheses represent the quantified results after adjusted with their own α-tubulin levels. (*D*) Transfection of the HUVEC with DN-p53 (top panel) or p53 siRNA (bottom panel) inhibited the progesterone-induced decrease of thymidine. For dominant negative transfection, the HUVEC were transfected with either C3.1 (+) or DN-p53. For siRNA experiments, the cells were transfected with mock, p53 siRNA, or non-target siRNA. Twenty-four hours after the transfection, the cells were rendered quiescent, and then treated with 10 % FBS and DMSO (0.1 %) or progesterone (500 nM) in DMSO (0.1 %) for 24 h. Values represent the means ± s. e. mean. (n = 4) (*E*) The p53 DNA binding activity on the p21 and p27 promoter was activated by progesterone (500 nM). The p53 DNA binding activity was assessed by using chromatin immunoprecipitation assay (see Materials and Methods). P4, progesterone; DN-p53, dominant negative p53; P53 S, P53 siRNA; P53 N, nontarget siRNA; α-T, α-tubulin; p53 BS, p53 binding site. * $P < 0.05$ different from control.

formation of capillary-like tubular structure, and angiogenesis *in vivo.* To our knowledge, this is the first demonstration that progesterone alone can exert the anti-proliferation action of human vascular endothelial cells through a p53-dependent pathway.

Previously, Vázquez et. al. demonstrated that progesterone could inhibit the proliferation of vascular endothelial cells via a progesterone receptor-mediated pathway. In those cells, the cell cycle was arrested at the G1 phase, and it was inferred that this arrest was mediated through reduction in cyclin-dependent kinase activity [7]. Both forms of progesterone receptor, A and B, were detected in human dermal endothelial cells. They concluded that progesterone affected the endothelial cell cycle by regulating the CDK activity alone, but not by regulation of p21 and p27 expression levels. In agreement with their findings, we also observed an inhibitory effect of progesterone on the CDK2 kinase activity and on the proliferation of vascular endothelial cells. HUVEC also expresses the progesterone receptor (data not shown). However,

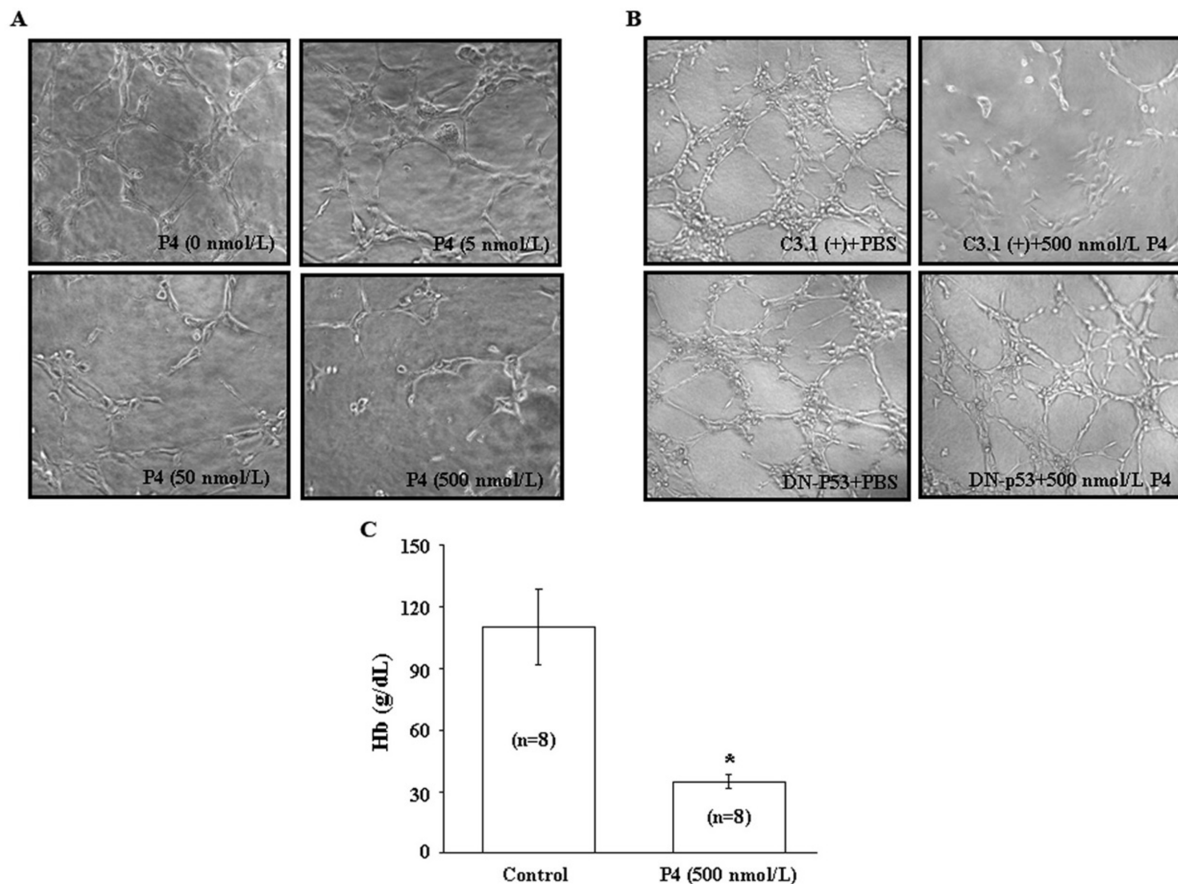


**Figure 6.** Anti-angiogenic activity of progesterone. (*A*) Progesterone (5–500 nM) dose-dependently inhibited capillary-like tube formation of HUVEC in Matrigel. (*B*) The progesterone-induced inhibition of capillary-like tube formation was prevented by pre-transfection of HUVEC with DN-p53. (*C*) Progesterone (500 nM) reduced the hemoglobin concentrations in the matrigel. Angiogenesis was assessed by using the matrigel angiogenesis assay (see Materials and Methods). The mean Hb concentration of plugs excised from animals treated with vehicle without progesterone is 100 %. Values represent the means ± s. e. mean. * $P < 0.05$ different from control. P4, progesterone; DN-p53, dominant negative p53; Hb, hemoglobin.

our data showed that progesterone increased the promoter activities (Fig. 5A), the mRNA levels (Figs. 1D and 1E), and the protein levels (Fig. 2) of p21 and p27 in HUVEC, suggesting that p21 and p27 might be involved in the progesterone-induced inhibition of DNA synthesis. Treatment of HUVEC with p21 and p27 antisense oligonucleotides together completely blocked the progesterone-induced inhibition of [3H]thymidine incorporation (Fig. 4C). Moreover, pre-treatment of HUVEC with DN-p53 inhibited the progesterone-induced increases of p21 and p27 promoter activity (Fig. 5A) and protein level (Fig. 5B) and decreases of thymidine (Fig. 5D, Top panel). These data further support the involvement of p21 and p27 in the progesterone-induced growth inhibition in HUVEC. It seems that Vázquez et. al. missed the time points of the increased p21 and p27 expression in their study.

The role of p21 and p27 in the regulation of cell cycle progress has been well documented [23–25]. Due to a high level of homology in their primary structure, it is believed that p21 and p27 inhibit their targets through similar mechanisms [24, 26]. Both p21 and p27 arrest the cell cycle through binding and inactivating the CDK system [23, 26]. P21 and p27 can bind isolated cyclin and CDK subunits independently, but they have a higher affinity for the cyclin-CDK complex [26, 28, 29]. It has been indicated that low concentrations of p21 and p27 protein inhibit CDK2, but not CDK4/6 [23, 26]. P21 is a transcriptional target of the tumor suppressor gene p53 [13, 27]. Expression of p21 is inducible by wild-type but not mutant p53 [13]. Several studies have shown that expression of p53 in the cells can induce cell growth arrest through transcriptional activation of p21 [31–33]. In this study, we showed that progesterone increased the levels of p53 mRNA and protein in HUVEC. When HUVEC were transfected with DN-p53 cDNA, the progesterone-induced increases in p21 and p27 promoter activity and protein level, and also the accompanying decrease

in DNA synthesis were all prevented (Fig. 5). P53 siRNA, which blocked the induction of p53, prevented the progesterone-induced increases of p53, p21 and p27 (Fig. 5C), and decreases of thymidine incorporation (Fig. 5D, bottom). Moreover, the p53 DNA binding activity on the p21 and p27 promoter was activated by progesterone (Fig. 5E). These findings further support the notion that progesterone arrests endothelial cell cycle through induction of p53, which in turn up-regulates p21 and p27 expression, and then inhibits CDK2 activity.

In accord with the established concept that p21 and p27 are two known CDK inhibitors, we found in progesterone-treated cells that the formation of the CDK2-p21 and CDK2-p27, but not CDK4-p21 and CDK4-p27, were increased and the CDK2, but not CDK4, activity was decreased (Fig. 3). The progesterone effect on the CDK activity appears to be tissue specific and more analogous to the effects on the uterine epithelium than on breast cancer cells. Pretreatment of uterine epithelial cells with progesterone abolished estrogen-induced cyclin E-CDK2 activation [34]. In breast cancer cells, on the other hand, the progestin-induced growth inhibition was preceded by inhibition of cyclin D1-CDK4, cyclin D3-CDK4, and cyclin E-CDK2 kinase activities [23, 24, 35]. Previously, it has been demonstrated that regulation of transcription of the p21 promoter is involved in the progesterone-induced cell cycle arrest at the transition of the cells from the G1 phase to the S phase [36]. The important role that p21 and p27 play on the progesterone-induced inhibition of DNA synthesis in HUVEC was further confirmed by the demonstration that a combined treatment of HUVEC with p21 and p27 antisense oligonucleotides completely reversed the progesterone-induced inhibition of [$^3$H]thymidine incorporation (Fig. 4C).

In conclusion, the results from the present study indicate that progesterone inhibited HUVEC proliferation through a p53-dependent mechanism, by which the levels of p21 and p27 protein were increased, and consequently inhibited the CDK2 kinase activity, and finally impair the transition of the cells from the G1 phase to the S phase. The results from our animal study show that in the intact, living rat, progesterone at physiologic levels exerts an anti-angiogenic action. The findings from the present study suggest the potential applications of progesterone in the treatment of angiogenesis-related disease conditions.

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