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Enhanced Stromal Cell CBS-H_2S Production Promotes Estrogen-Stimulated Human Endometrial Angiogenesis

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Abbreviations: BCA, β-cyano-L-alanine; CBS, cystathionine-β synthase; CSE, cystathionine-γ lyase; DMEM, Dulbecco's Modified Eagle Medium; DPN, diarylpropionitrile; EC, endothelial cells; ECM, endothelial cell medium; EMEC, endometrial microvascular endothelial cell; ER, estrogen receptor; ESC, endometrial stromal cell; E_2, estradiol-17β; FBS, fetal bovine serum; H_2S, hydrogen sulfide; Ig, immunoglobulin; mRNA, messenger RNA; NaHS, sodium hydrosulfide; NP, nonpregnant; P_4, progesterone; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; POM, postmenopausal; PRM, premenopausal; pPRM, proliferative premenopausal; Preg, pregnant; siRNA, small interfering RNA; sPRM, premenopausal secretory.

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Abstract

Angiogenesis is a physiological process for endometrial regeneration in the menstrual cycle and remodeling during pregnancy. Endogenous hydrogen sulfide (H_2S), produced by cystathionine-β synthase (CBS) and cystathionine-γ lyase (CSE), is a potent proangiogenic factor; yet, whether the H_2S system is expressed in the endometrium and whether H_2S plays a role in endometrial angiogenesis are unknown. This study was to test whether estrogens stimulate endometrial H_2S biosynthesis to promote endometrial microvascular endothelial cell (EMEC) angiogenesis. CBS messenger RNA/protein and H_2S production significantly differed among endometria from postmenopausal (POM), premenopausal secretory (sPRM), and proliferative (pPRM) nonpregnant (NP) and pregnant (Preg) women ($P < .05$) in a rank order of POM approximately equal to sPRM is less than pPRM is less than Preg, positively correlating with angiogenesis indices and endogenous estrogens and with no difference in CSE expression. CBS and CSE proteins were localized to stroma, glands, and vessels in endometrium, and greater stromal CBS protein was observed in the pPRM and Preg states. Estradiol-17β (E_2) (but not progesterone) stimulated CBS (but not CSE) expression and H_2S production in pPRM endometrial stromal cells (ESCs) in vitro, which were attenuated by ICI 182 780. The H_2S donor sodium hydrosulfide promoted in vitro EMEC angiogenesis. Co-culture with sPRM, pPRM, and Preg ESCs all stimulated EMEC migration with a rank order of sPRM less than pPRM approximately equal to Preg. CBS (but not CSE) inhibition attenuated ESC-stimulated

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EMEC migration. E_2 did not affect EMEC migration but potentiated ESC-stimulated EMEC migration. Altogether, estrogens stimulate specific receptor-dependent stromal CBS-H_2S production to promote endometrial EMEC angiogenesis in women.

Key Words: estrogens, H_2S, angiogenesis, endometrium, women

Angiogenesis is a process of new vessel formation from existing ones; it occurs with enhanced local production of angiogenic factors that stimulate endothelial cell proliferation and migration as well as formation of tube-like structures that are stabilized by recruitment of pericytes and smooth muscle cells (1). Angiogenesis is a normal physiological process required for endometrial regeneration in the proliferative phase during the menstrual cycle (2); it is also a key mechanism for the massive vascular expansion at the maternal-fetal interface in the third trimester in pregnancy (3, 4). Aberrant endometrial angiogenesis is associated with many reproductive disorders such as abnormal uterine bleeding, endometriosis, implantation failure and early pregnancy loss, placenta accrete spectrum, preeclampsia, fetal growth restriction, and endometrial cancer. (5, 6).

Enhanced endometrial angiogenesis in the proliferative phase and pregnancy coincides with elevated endogenous estrogens in these physiological states (7). Previous studies have shown that estrogens play a key role in endometrial angiogenesis (8, 9). Human endometrial microvascular endothelial cells (EMECs) specifically express estrogen receptor (ER) β (ERβ) (10, 11). Noteworthy standing is that neither estradiol-17β (E_2) nor the ERβ-specific agonist diarylpropionitrile (DPN) stimulates human EMEC angiogenesis in vitro; instead, DPN decreases EMEC tube formation (11), leading to the speculation that estrogens regulate endometrial angiogenesis indirectly through interactions with other cell types (11). Because EMECs are in direct contact with endometrial stromal cells (ESCs) that express both ERα and ERβ (12, 13), we posited that elevated estrogens stimulate ESC production of angiogenic factor(s), which in turn play a paracrine role in stimulating EMEC angiogenesis in the proliferative and pregnant endometria.

The gaseous molecule hydrogen sulfide (H_2S) is a potent angiogenesis promoter. H_2S donors such as sodium hydrosulfide (NaHS) promote proliferation, migration, and tube-like structure formation in endothelial cells in vitro and in a rat hind limb ischemia model of angiogenesis in vivo (14-16). Endogenous H_2S is primarily synthesized from L-cysteine by cystathionine-β synthase (CBS) and cystathionine-γ lyase (CSE) (17, 18). The expression of these enzymes is tissue/cell specific because both are needed to generate H_2S in some tissues whereas only one is sufficient in others (19, 20). Uterine artery CBS expression and H_2S production are significantly stimulated by treatment with exogenous estrogens in ewes in vivo (21) and are positively associated with endogenous estrogens during the proliferative phase of the estrus cycle and pregnancy in ewes (22) and women (23), contributing to pregnancy-associated uterine vasodilation (23). Dysregulation of placental CSE/H_2S results in preeclampsia-like conditions that are linked to placental vascular malformation (24) and shallow trophoblast invasion and impaired spiral artery remodeling (25). CBS and CSE are highly expressed in human myometrium, but only CBS seems to be augmented during pregnancy and sharply drops during labor initiation (26), playing a key role in maintaining uterine quiescence during pregnancy (27). CBS-deficient female mice are infertile because of impaired decidualization and placentation (28, 29), implicating a role of H_2S in preparing the endometrium for implantation and placentation. Trophoblast-derived H_2S stimulates placental artery endothelial cell angiogenesis in vitro (16) and maintains early pregnancy by regulating maternal-fetal interface immune balance (30). Human endometrium produces H_2S (31); yet, whether endometrial H_2S biosynthesis is regulated during the menstrual cycle and pregnancy and whether H_2S regulates endometrial angiogenesis in women are unknown.

We hypothesized herein that endometrial H_2S biosynthesis is augmented to play a role in endometrial angiogenesis in the proliferative phase and pregnancy in women, 2 physiological states with significantly elevated estrogens (32). The objectives of this study were to determine during the menstrual cycle and pregnancy whether: 1) CBS and/or CSE are differentially expressed in different endometrial cells; 2) CBS/CSE expression and H_2S production are associated with altered endometrial angiogenesis; 3) estrogen and progesterone (P_4) regulate endometrial stromal H_2S biosynthesis; and 4) H_2S plays a role in endometrial angiogenesis.

Materials and Methods

Human participants and tissue collection

Human tissues were collected with written consent from women who underwent clinically indicated hysterectomy at the University of California Irvine Medical Center. Ethical approval (HS No. 2013-9763) was granted by the University of California Irvine Institutional Review

Board. All study participants were not on steroid hormone treatments. Endometrium samples were collected from 9 postmenopausal (POM) women, 17 nonpregnant (NP) premenopausal (PRM) women, and 13 pregnant (Preg) women, and their demographic data are summarized in Supplemental Table S1 (33). The POM participants were age 48 to 65 years, undergoing elective hysterectomy because of fibroids (2 cases), pelvic organ prolapse (5 cases), and abnormal uterine bleeding (2 cases). The 17 PRM participants were age 35 to 50 years, undergoing elective hysterectomy for fibroids (5 cases), abnormal uterine bleeding (11 cases), and pelvic organ prolapse (1 case); their menstrual status was determined by the last menstrual period recorded and confirmed with endometrial histology. Among them, 13 were in proliferative (pPRM) and 4 were secretory (sPRM) phases, respectively; none were on hormone replacement therapy at the time of tissue collection. Pregnant women were recruited with suspected placental accreta spectrum based on previous ultrasound findings in the event a hysterectomy was indicated, without any other complications; pregnant endometria were collected at the time of cesarean delivery hysterectomy at gestational age 33 to 38 weeks. Pregnant endometria were collected from the area without trophoblast villi invasion. Endometrium tissues were dissected from adjacent myometrium, placed in chilled DMEM (Dulbecco's Modified Eagle Medium), and transported to the laboratory. Portions of the endometrium were fixed in 4% paraformaldehyde and the rest was frozen in liquid N_2, and stored at –80°C until analyzed.

Antibodies and chemicals

The sources and dilution factors of the antibodies are summarized in Supplemental Table S2 (33). Prolong Gold antifade reagent with 4,6-diamidino-2-phenylindole (DAPI) was obtained from Invitrogen. β-cyano-L-alanine (BCA: inhibitor of CSE) was obtained from Cayman Chemical. O-(carboxymethyl) hydroxylamine hemihydrochloride (CHH: inhibitor of CBS), E_2, progesterone (P_4), and all other chemicals were obtained from Sigma, unless specified.

Cell isolation and culture

Human ESCs were isolated by collagenase digestion and cultured as described previously (34). ESCs were cultured in DMEM with 10% fetal bovine serum (FBS) with 1% antibiotics and used within 4 passages. Human EMECs were isolated and cultured as detailed previously (16, 35). Briefly, endometrium tissue was dissected free of connective and smooth muscle tissues under a stereoscope and finely minced (~1 mm^3). After being rinsed with phosphate-buffered saline (PBS), the tissues were digested with 2 mg/mL collagenase II at 37°C for 45 minutes. The mixed cell suspension was pulled down by sedimentation and then resuspended in endothelial cell medium (ECM, ScienCell) containing 5% FBS. The cells were cultured with ECM containing 5% FBS and 1% antibiotics in 100-mm culture dishes with series dilutions (1:5; 1:10; 1:50), and medium was changed every other day. After 5 to 7 days in culture, endothelial cell (EC) colonies were labeled on the bottom of the dishes under a microscope; each colony was picked up by a sterile cloning disc (3.2 mm in diameter) soaked with 0.25% trypsin-EDTA, then placed into a well of 24-well plate with 1 mL ECM culture medium. Following 5 to 7 days in culture, cells in each well were transferred into a 100-mm culture dish and defined as passage 1 (P1). Cells were passaged and stored in liquid N_2 at P2 and used for this study within 10 passages.

Endometrial angiogenesis

Tissues were chosen from 4 to 6 women enrolled in each group for angiogenesis analysis according to sample availability and convenience. Endometrium sections were deparaffinized in xylene and rehydrated. Antigen retrieval was achieved by proteinase K digestion for 30 minutes at 37°C. Endogenous peroxidase was quenched in 3% H_2O_2 in 60% methanol and nonspecific binding was blocked with 2.5% normal horse serum for 30 minutes. The sections were incubated with mouse anti-CD31 overnight at 4°C, followed by incubation with horse antimouse immunoglobulin (Ig) for 30 minutes. The CD31 immunoreactive signal was detected by the VECTASTAIN Universal Elite ABC Kit following the manufacturer's instructions. After counterstaining with hematoxylin, sections were mounted and examined under a Leica microscope for image acquisition using a charge-coupled device camera with SimplePCI software. Vessels were selected by using the "region of interest" (ROI) selection tool offline using SimplePCI; total vessel counts, average area, perimeter, and diameter of each vessel were measured for calculating angiogenesis indices, which were expressed as fold of POM.

Hydrogen sulfide determination—methylene blue assay

Endometrium tissues (~100 mg/sample) were homogenized in ice-cold 50 mM potassium phosphate buffer, pH 8.0. Equal amount of proteins (100 μg/reaction) were used to determine H_2S production with or without CHH or BCA by the methylene blue assay as described previously (21). H_2S concentration was calculated based on a calibration curve generated from NaHS solutions.

RNA extraction, reverse transcription, and real-time quantitative polymerase chain reaction

Total RNAs were extracted from endometrial samples (~100 mg) using Trizol reagent (Invitrogen) and quantified by optical density$_{260/280}$. Complementary DNA was synthesized by reverse transcription with random primers and AMV Reverse Transcriptase (Promega) and then used for quantifying CBS and CSE messenger RNAs (mRNAs) by quantitative real-time polymerase chain reaction (PCR) (run in triplicate) with gene-specific primers as listed in Supplementary Table S3 (33). The real-time PCR was run as follows: 95°C for 10 seconds, followed by 42 cycles of 95°C for 5 seconds and 55°C for 30 seconds. Comparative C_T method ($^{\Delta\Delta}C_T$ method) was used to calculate relative CBS and CSE mRNA levels with ribosomal protein L19 as the internal reference control.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot

Endometrial samples were homogenized in a nondenaturing buffer containing protease cocktail (23). Protein concentrations were determined by using the Pierce BCA Protein Assay Kit. Proteins (15 µg/sample) were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. CBS and CSE proteins were analyzed by immunoblotting with specific antibodies as described previously (35). β-Actin was analyzed for normalizing sample loading. CBS and CSE proteins were quantified by National Institutes of Health ImageJ software and presented as fold changes over control groups.

Immunofluorescence microscopy and image analysis

Tissues were chosen from 4 to 6 women enrolled in each group for CBS and CSE protein expression analysis according to sample availability and convenience. Endometria were paraffin embedded; sections (5 µm) were cut and deparaffinized in xylene and hydrated in graded ethanols and deionized water. The sections were labeled with mouse antihuman CD31, followed by Alexa^{568} antimouse IgG (red); after washing, the sections were incubated with mouse anti-CBS or CSE, followed by Alexa^{488} antimouse IgG. Sections were mounted with Prolong Gold antifade reagent (Invitrogen) containing DAPI for labeling cell nuclei. Samples were examined under a Leica fluorescence microscope. Digital images were acquired using a charge-coupled device camera with *Simple*PCI image analysis software (Hamamatsu Corporation). The images were used to determine relative levels of CBS and CSE proteins by quantifying mean green fluorescence intensity using *Simple*PCI. CBS and CSE levels were averaged from data collected from 5 to 6 images per participant, and 3 or 4 women per group. Epithelial glands, stromal cells, and endothelial cells were outlined using the ROI selection tool. "Mean Green Value" was recorded for a cell. The average "Mean Green Value" from negative controls without primary antibody accounted for autofluorescence and nonspecific background was recorded as background and subtracted from all counts generated from specific antibody-treated samples. CBS and CSE protein levels were presented as fold over POM in the average fluorescence intensity.

For immunocytochemical analysis, ESCs and EMECs were cultured on collagen-coated glass coverslips. The cells were fixed with 4% paraformaldehyde and processed below room temperature. Nonspecific binding was blocked with PBS containing 1% BSA, 1% gelatin, and 0.15% saponin for 30 minutes. The cells were then incubated with antivimentin, anti-CD31, anti-ERα, anti-ERβ, and anti-CBS in PBS containing 0.5% BSA, 0.5% gelatin, and 0.075% saponin for 1 hour, followed by Alexa^{488}/Alexa^{568} antimouse/rabbit IgG. Samples were examined as described earlier.

Cystathionine-β synthase and cystathionine-γ lyase knockdown with specific small interfering RNA

ESCs were transfected with scrambled control small interfering (siRNA), CBS siRNA (Hs-CBS-5 FlexiTube siRNA, S100001316, Qiagen), CSE siRNA (sc-78973, Santa Cruz), or scrambled siRNA (sc-37007, Santa Cruz), by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol as previously described (36). The transfection medium was replaced with DMEM/F12 containing 5% charcoal-striped FBS 6 hours later; cells were harvested at 72 hours to determine CBS and CSE knockdown efficiency by immunoblotting.

Cell proliferation

Cell proliferation was measured by CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) exactly as described previously (16).

Cell migration

A Transwell migration assay using the 24-well multiwell BD Falcon Fluoroblok Insert System (8.0 µm pores; BD

Biosciences) was performed as described previously (16). Briefly, ESCs were seeded into the bottom chamber with DMEM/F12 and cultured to approximately 70% confluence. EMECs (1.5×10^4) in ECM containing 0.5% FBS were seeded in the top insert. For CBS and CSE inhibition experiments, 2 mM of BCA or CHH were added into ESC culture. The systems were cultured to allow EMEC migration for 16 hours and then incubated with calcein acetoxymethyl ester (Calcein-AM, 0.2 μg/mL, Invitrogen) at 37°C for 30 minutes. The insert was examined with an inverted fluorescence microscopy. Migrated cells on the bottom of each insert (6 fields/insert) were averaged for each treatment and data were averaged from 3 women for each group.

Tube formation

EMEC differentiation was determined in an in vitro tube formation assay as described previously (16).

Statistical analysis

All experiments were repeated at least 4 times using cells (in triplicate) from different participants. Data were presented as means ± SEM and analyzed by using SigmaStat (Systat Software Inc). One-way analysis of variance was used to compare 2 or more independent groups for 1 variable and 2-way analysis of variance was used to compare 2 independent variables between groups. Post hoc Bonferroni test was used for multiple comparisons. Two-tailed significance was accepted at P less than .05 unless indicated in the figure legends.

Results

Endometrial angiogenesis during menstrual cycle and pregnancy

The CD31 (an EC marker)-labeled human endometrial vascular network is shown in Fig. 1A. Changes in vessel counts and the average diameter, perimeter, and area of the vessels were calculated as angiogenesis indices to assess endometrial angiogenesis. Endometria of POM women had the fewest CD31-positive vessels, and all the angiogenesis indices were lowest among all groups. All the angiogenesis indices of sPRM endometria remained very low, similar to that in POM endometria. However, compared to POM, the average vessel area increased 2.0 ± 0.5-fold in pPRM and 4.3 ± 0.5-fold in Preg endometria; vessel diameter enlarged 1.8 ± 0.4-fold in pPRM and 2.9 ± 0.2-fold in Preg endometria; vessel perimeter increased 2.4 ± 0.4-fold in pPRM and 3.7 ± 0.3-fold in Preg endometria; vessel counts increased 2.4 ± 0.4-fold in pPRM and 3.7 ± 0.3-fold in Preg endometria ($P < .05$) (Fig. 1B).

Human endometrial cystathionine-β synthase/cystathionine-γ lyase–hydrogen sulfide biosynthesis

Endometrial H_2S production was low in POM women, but increased 13.9 ± 0.4-fold in pPRM ($P < .001$), 4.2 ± 0.2-fold in sPRM ($P < .05$), and 19.8 ± 1.6-fold ($P < .001$) in Preg women. Coincubation with BCA did not significantly inhibit H_2S production in endometria from all 4 groups. Coincubation with CHH did not alter H_2S production in POM endometria, but significantly inhibited H_2S production in pPRM and sPRM as well as Preg endometria, with the greatest inhibition in pPRM and Preg endometria. BCA and CHH combination completely blocked H_2S production in endometria of all groups (Fig. 2A).

Compared to POM endometria, CBS mRNA levels increased 3.2 ± 1.5-fold ($P < .05$) in pPRM, 1.6 ± 0.7-fold ($P > .05$) in sPRM, and 4.6 ± 2.1-fold ($P < .05$) in Preg endometria. CSE mRNA levels did not differ among all groups (Fig. 2B). Consistently, levels of CBS protein increased 1.9 ± 0.4-fold ($P < .05$) in pPRM, 1.2 ± 0.6-fold ($P > .05$) in sPRM, and 2.2 ± 0.4-fold ($P < .05$) in Preg endometria compared to POM. CSE protein levels did not differ among all groups (Fig. 2C). In keeping with data in Fig. 2A, augmented CBS, but not CSE, expression is responsible for augmented endogenous H_2S production in the proliferative phase and pregnant human endometria.

Localization of endometrial cystathionine-β synthase and cystathionine-γ lyase proteins

The immunofluorescence signal of CBS protein was very low in all cell types in POM endometria; CBS immunoreactive signal was detectable in the glands, stroma, and vessels of endometrium in sPRM and pPRM NP and Preg women (Fig. 3A). Quantitative analysis revealed stromal CBS protein expression (relative fluorescence intensity) was increased 2.3 ± 0.2-fold ($P < .05$) in pPRM, 1.5 ± 0.2-fold in sPRM ($P > .05$), and 3.0 ± 0.5-fold ($P < .05$) in Preg compared to POM endometria; vascular and glandular CBS protein expression were also remarkably enhanced in pPRM and Preg compared to POM and sPRM endometria (Fig. 3B). Immunoreactive CSE protein was detected in human endometria; however, levels of CSE protein did not differ in all the cell types in the endometria among all 4 groups (Supplemental Fig. S1) (33).

Estrogens and hydrogen sulfide biosynthesis in endometrial stromal cells in vitro

In culture, ESCs were stained positively with the fibroblast marker vimentin and negatively with the endothelial cell marker CD31. In contrast, EMECs were CD31 positive

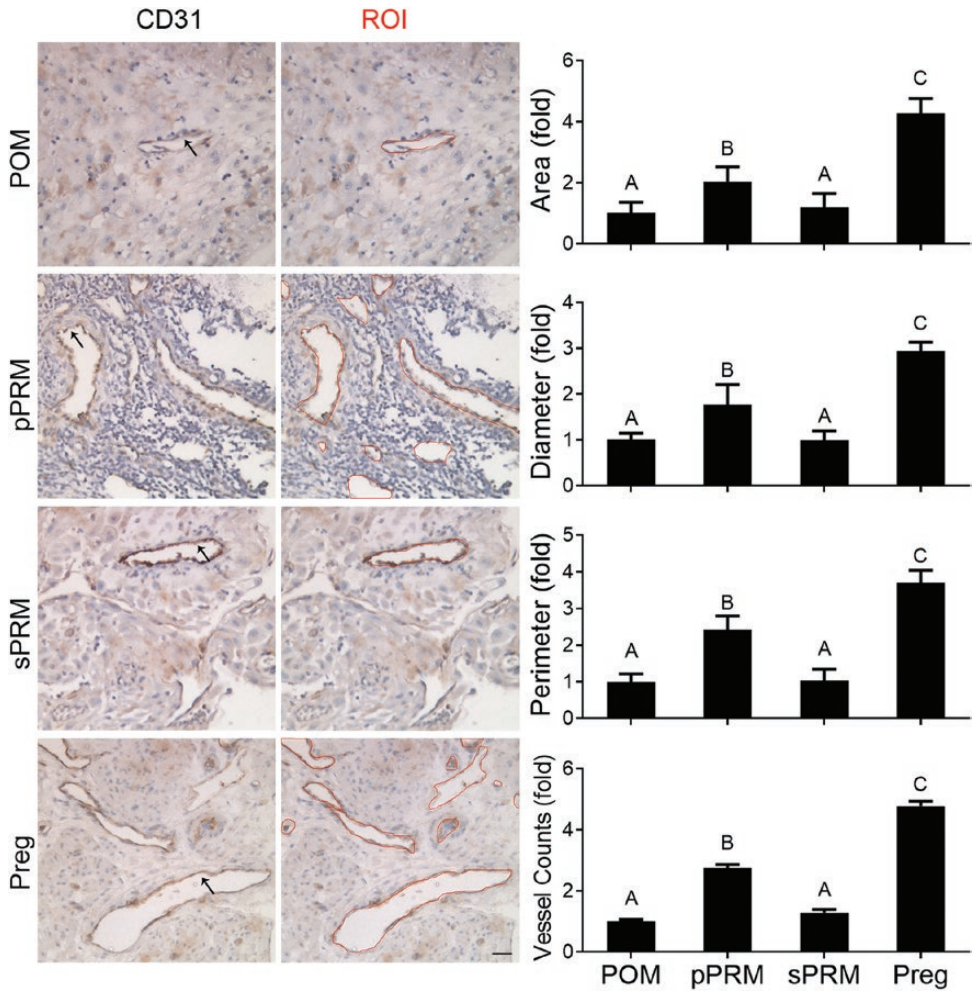

Figure 1. Endometrial angiogenesis in women of different physiological status. A, Uterine endometrium sections were labeled with a specific antihuman CD31 antibody and revealed by diaminobenzidine peroxidase substrate to visualize CD31-labeled vessels. POM (n = 6), postmenopause; pPRM (n = 6), proliferative premenopause; sPRM (n = 4), secretory premenopause; P (n = 6), pregnancy. Scale bar = 100 μm; arrow, endothelial cells. B, Area, diameter, perimeter, and average count of vessels were quantified from 6 randomly fields from Fig. 1A. Data (mean ± SEM) are presented as fold of POM group. Bars with different letters differ significantly among groups ($P < .05$).

and also stained with factor VIII and uptook acetylated low-density lipoprotein (data not shown), like human uterine artery EC (35). Consistent with previous reports (10, 12, 37), ESCs stained positively both with ERα and ERβ, whereas EMECs stained positively with ERβ only (Fig. 4A). These cell models were further validated by immunoblotting of other specific marker proteins; EMECs expressed nitric oxide synthase 3, and ESCs specifically expressed ERα and CBS, whereas both cell types expressed vimentin, CSE, and ERβ (Fig. 4B). Treatment with 10 nM E_2 stimulated CBS protein (1.8 ± 0.3-fold, $P < .05$), but not CSE in pPRM ESCs, whereas treatment with P_4 (1 μM) did not alter CBS and CSE expressions. Coincubation with the ER antagonist ICI 182 780 (ICI, 1 μM) nullified E_2-induced CBS expression (Fig. 4C). Treatment of pPRM ESCs (5×10^5 cells/time point) with 10 nM E_2 stimulated 2.5 ± 0.4-fold ($P < .05$) H_2S production, which was blocked by ICI (Fig. 4D).

Hydrogen sulfide and endometrial microvascular endothelial cell angiogenesis in vitro

ESCs are in close contact with EMECs in vivo. Because endometrial angiogenesis correlates with enhanced endometrial H_2S production and augmented CBS upregulation in ESC during the proliferative phase and pregnancy (Figs. 1-3), we speculated that augmented ESC CBS/H_2S production plays a role in endometrial angiogenesis during the proliferative phase and pregnancy because of the potent proangiogenic activity of H_2S (14-16). Indeed, treatment with an H_2S donor (NaHS, 100 μM) increased EMEC proliferation by 1.6 ± 0.3-fold

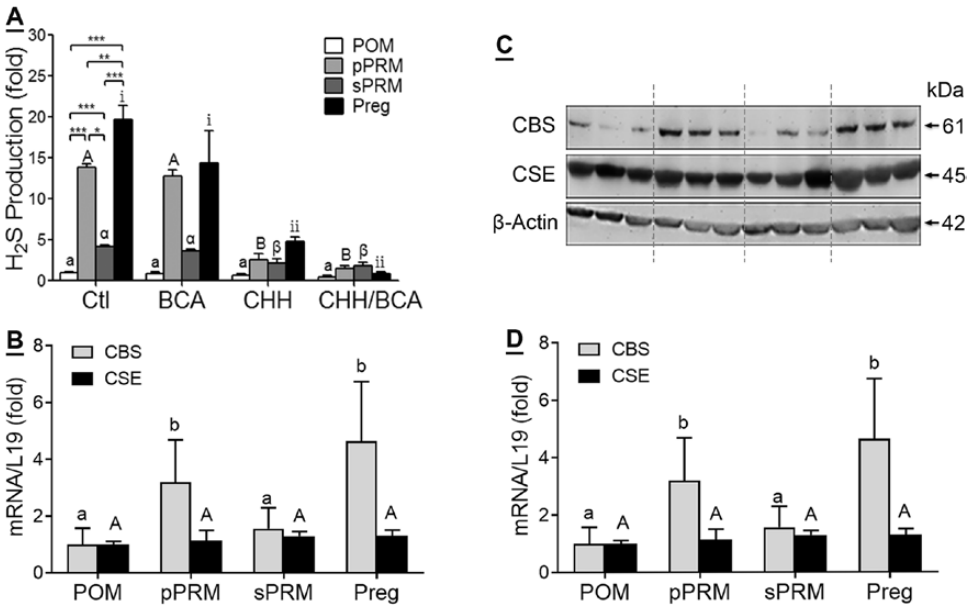

Figure 2. Endometrial hydrogen sulfide (H_2S) biosynthesis in women of different physiological status. A, H_2S production in human endometrium was determined by the methylene blue assay with or without the specific inhibitors of cystathionine-γ lyase (CSE) (β-cyano-L-alanine [BCA]), cystathionine-β synthase (CBS) (O-[carboxymethyl] hydroxylamine hemihydrochloride [CHH]), or BCA plus CHH, respectively. POM (n = 6): postmenopause; pPRM (n = 8), proliferative premenopause; sPRM (n = 4), secretory premenopause; P (n = 8), pregnancy. Data (mean ± SEM) were presented as fold of POM group without any inhibitor. B, Endometrial CBS and CSE messenger RNA were detected by real-time polymerase chain reaction. C, Endometrial CBS and CSE proteins were detected by Western blot. D, Quantitation of CBS and CSE proteins. Data (mean ± SEM) are presented as fold of POM group. Bars with different letters differ significantly among groups: **P* less than .05. ***P* less than .01. ****P* less than .001.

($P < .05$), migration by 3.2 ± 1.1-fold ($P < .05$), and tube formation by 4.1 ± 1.1-fold ($P < .05$) (Fig. 5). We then developed a co-culture trans-well assay to determine whether endogenous H_2S derived from ESCs stimulates EMEC migration. Co-culture with ESCs from pPRM, sPRM, and Preg endometria all significantly stimulated EMEC migration compared with the control; the migrated EMEC number increased 3.6 ± 0.4-fold ($P < .01$) when co-cultured with pPRM ESCs, 1.8 ± 0.4-fold ($P < .05$) with sPRM ESCs, and 4.7 ± 1.2-fold ($P < .01$) with Preg ESCs (Fig. 6A). EMEC migration did not respond to E_2 or ICI treatment in the absence of ESC co-culture (Fig. 6B). However, E_2 increased EMEC migration by 1.7 ± 0.6-fold ($P < .05$) when co-cultured with sPRM ESCs; this effect was abolished by ICI treatment (Fig. 6C). Coincubation with CHH or CHH with BCA, but not BCA alone, significantly inhibited the migration ability of EMECs stimulated by Preg ESCs (Fig. 6D). In Preg ESCs, treatment with the specific CBS siRNA specifically downregulated CBS protein and treatment with specific CSE siRNA specifically downregulated CSE protein. Treatment with CBS siRNA alone or in combination with CSE siRNA inhibited EMEC migration stimulated by co-culture with Preg ESCs; CSE siRNA alone had no effect. Controls transfected with scrambled siRNAs did not affect CBS and CSE expression and ESC-induced EMEC migration (Fig. 6E and 6F).

Discussion

We have shown herein that endometrial CBS mRNA/protein expression and H_2S production are elevated and positively correlated with enhanced endometrial angiogenesis and endogenous estrogens in the proliferative phase of the menstrual cycle and pregnancy in women. CBS and CSE proteins are localized to the stroma, epithelium/glands, and microvessels in the endometrium, with enhanced stromal CBS but not CSE expression in pPRM and Preg endometria. In vitro, treatment with E_2 stimulates specific ER-dependent CBS expression and H_2S production in ESCs and treatment with a H_2S donor potently stimulates EMEC angiogenesis (ie, proliferation, migration, and tube formation) in vitro. Co-culture with primary ESCs significantly stimulates EMEC migration; however, cells isolated from the proliferative and pregnant endometria possess significantly greater potency in stimulating EMEC migration than cells from the secretory endometria. Moreover, the stimulatory effect of pregnant ESCs on EMEC migration is significantly inhibited by CBS but not CSE downregulation. Thus, these findings show that augmented H_2S production

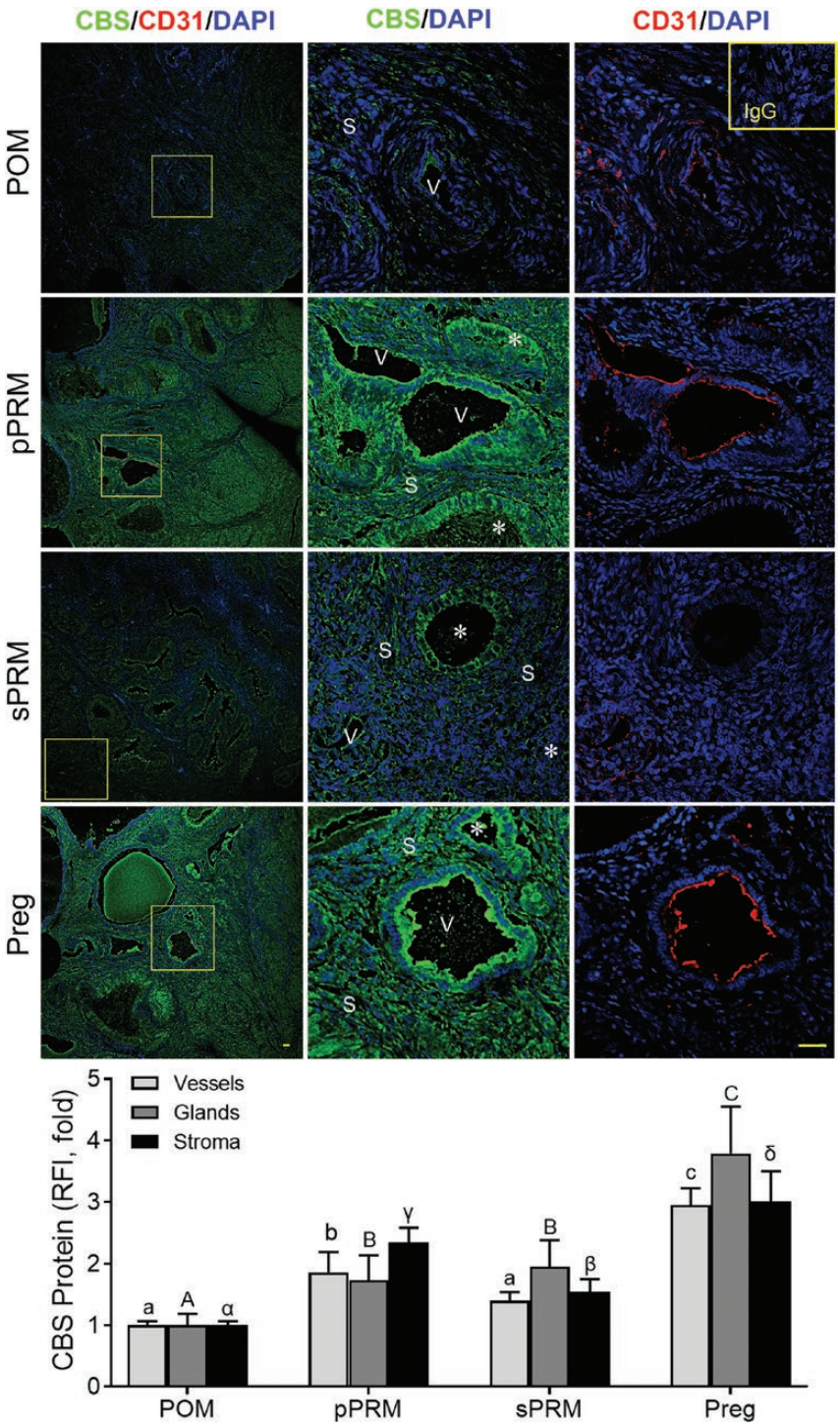

Figure 3. Cellular endometrial cystathionine-β synthase (CBS) protein. Human endometrium sections were labeled with specific antibodies against human CBS or CD31, followed by Alexa^{488} (green), or Alexa^{568} (red)-labeled secondary antibodies; immunoglobulin G was used as a control and cell nuclei were stained with DAPI (4,6-diamidino-2-phenylindole; blue). Immunofluorescence images were used to analyze the relative green fluorescence intensity (RFI) for quantifying CBS protein levels in endometrial stroma, vessels, and glands. POM (n = 5), postmenopause; pPRM (n = 6), proliferative premenopause; sPRM (n = 4), secretory premenopause; P (n = 7), pregnancy; S, stroma; *, gland; V, vessel. The left panel was captured at low magnification and the right 2 panels are images of boxed areas with a higher magnification; scale bar = 100 μm. Data (mean ± SEM) are presented as fold of POM group. Bars with different letters differ significantly among groups ($P < .05$).

via selective CBS upregulation in ESCs stimulates EMEC angiogenesis in human endometrium during the proliferative phase and pregnancy.

The uterine vasculature mostly rests in premenarchal and postmenopausal women because of limited ovarian function; whereas in reproductive-age premenopausal

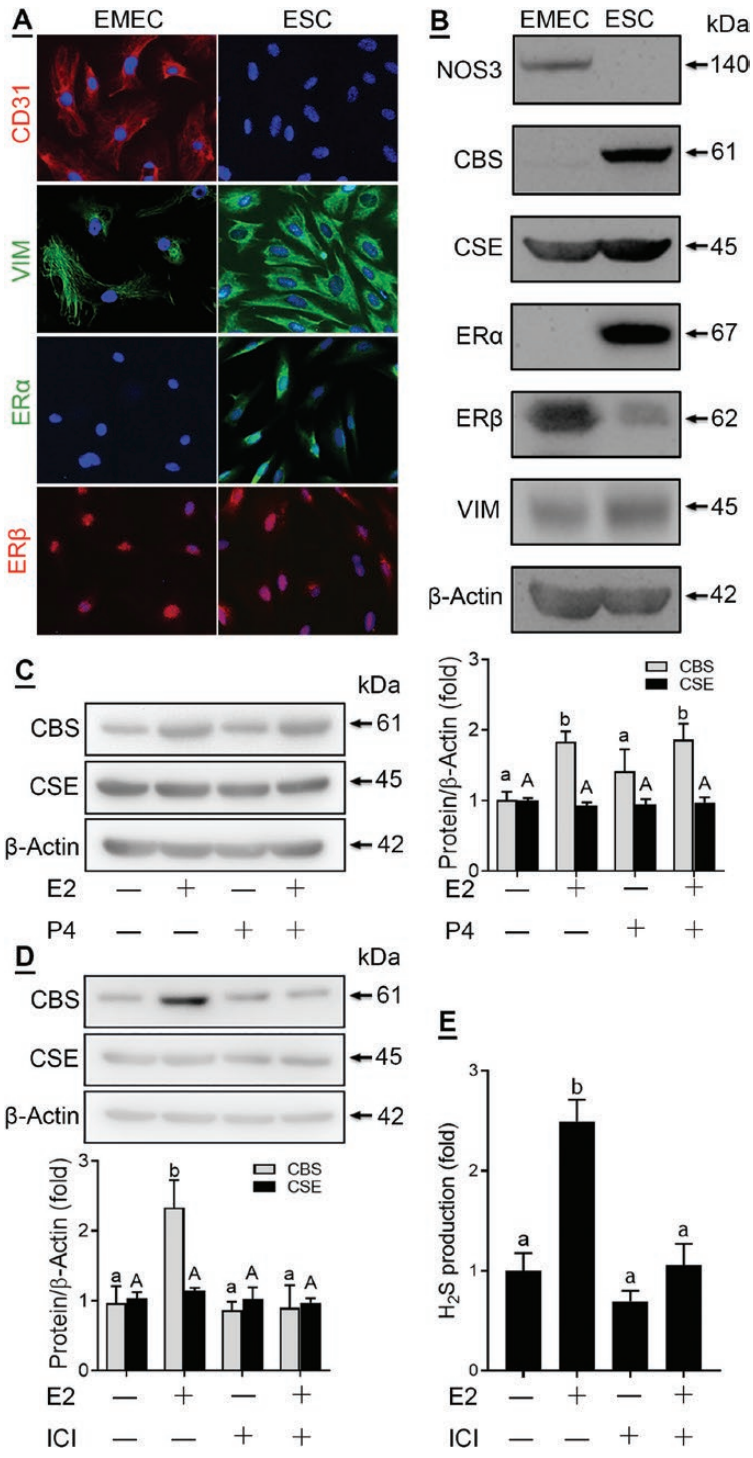

Figure 4. Estrogens and endometrial stromal cell (ESC) cystathionine-β synthase (CBS)/cystathionine-γ lyase (CSE)–hydrogen sulfide (H_2S) system in vitro. A, Primary cultured ESCs from proliferative premenopause (pPRM; n = 3), secretory premenopause (sPRM; n = 2), pregnant (Preg; n = 3) endometria, and endometrial microvascular endothelial cells (EMECs) from Preg endometria (n = 3) were characterized by morphology and immunofluorescence labeling with endothelial cell and fibroblast markers of CD31 and vimentin (VIM), as well as estrogen receptors (ERα and ERβ), followed by Alexa^{488} (green), or Alexa^{568} (red)-labeled secondary antibodies; immunoglobulin G was used as a control and cell nuclei were stained with DAPI (4,6-diamidino-2-phenylindole blue). B, Protein expressions of nitric oxide synthase 3 (NOS3), CBS, CSE, ERα, ERβ, and VIM in ESCs and EMECs were detected by Western blot. C, CBS and CSE protein expression in pPRM ESCs treated by estradiol (E2, 10 nM), progesterone (P_4, 1 μM), or both for 24 hours. D, CBS and CSE protein expression in pPRM ESCs treated by 10 nM E2 with or without 1 μM ICI 182 780 for 24 hours. E, H_2S production in pPRM ESCs treated with 10 nM E2 with or without 1 μM ICI 182 780 for 24 hours. Data (mean ± SEM) are presented as fold of controls. Data (mean ± SEM) obtained from cells of 3 women are presented as fold of controls. Bars with different letters differ significantly among groups. *P* less than .05.

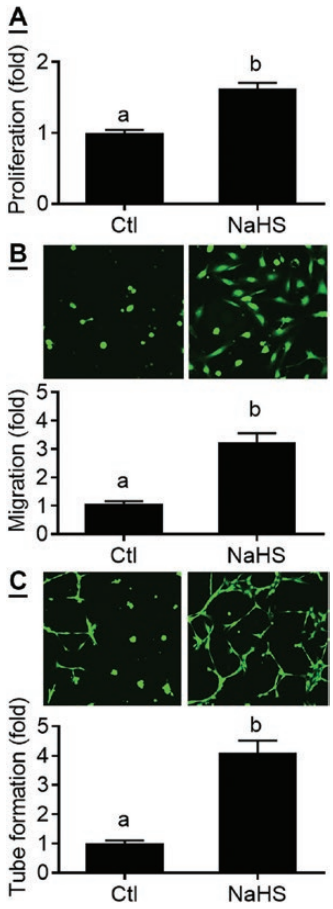

Figure 5. Hydrogen sulfide (H_2S) donor sodium hydrosulfide (NaHS) stimulates endometrial microvascular endothelial cell (EMEC) angiogenesis in vitro. A, EMECs were treated with an H_2S donor (NaHS, 100 μM) for 24 hours, followed by cell proliferation assay. B, EMECs were seeded on the top of inserts in a 24-well Transwell plate and treated with or without NaHS (100 μM). The Transwell system was cultured for 16 hours to allow EMEC migration. The migrated cells were quantified and averaged from 6 random fields. C, EMEC differentiation was determined by Matrigel-based tube formation assay. Data (mean ± SEM) are presented as fold of controls. Bars with different letters differ significantly among groups ($P < .05$).

women, the uterine vasculature undergoes ovarian hormone-dependent cyclic remodeling during the menstrual cycle, allowing for endometrial regeneration in the proliferative phase. Further proliferation and maturation of the uterine vasculature occurs following conception to establish and maintain the bidirectional mother-fetus exchanges during pregnancy. Consistent with previous reports (38, 39), our analysis of the CD31-labeled endometrial vessels in different physiological states shows significantly greater angiogenesis indices, that is, increased number of vessels with larger area, diameter, and perimeter, in the proliferative and pregnant endometria in women, suggesting enhanced angiogenesis in these physiological states of significantly elevated endogenous estrogens. Thus, consistent with previous correlation studies (38, 39), our present study has further strengthened the conclusion that estrogens play a key role in endometrial angiogenesis (40-42).

Genetic mutation studies have shown an essential role of CBS but not CSE in uterine biology. Female mice lacking the *Cbs* gene are infertile because of uterine dysfunction, displaying a shortened and irregular estrus cycle, with decreased estrus and diestrus periods and a prolonged metestrus period, as well as increased P_4 production during pseudopregnancy induction (28). In contrast, female mice lacking the *Cse* gene are fertile and indistinguishable from their wild-type littermates (43). Endogenous estrogen levels rise during the proliferative phase and further increase during pregnancy (44). Significantly greater CBS (but not CSE) mRNA and protein in proliferative and pregnant compared to secretory endometria suggest that endometrial CBS expression is stimulated by endogenous estrogens in women, consistent with our recent reports showing uterine artery endothelium and smooth muscle CBS (but not CSE) expression is upregulated by exogenous estrogens in nonpregnant ovariectomized ewes (21) and positively correlates to endogenous estrogens in the follicular phase and pregnant ewes (22) and in the proliferative phase and pregnant women (23). Higher CBS expression leads to significantly greater endometrial H_2S production in the proliferative phase and pregnancy with elevated estrogens, consistent with our recent report showing positive correlation between endogenous estrogens and H_2S production in uterine artery endothelium and smooth muscle in women (23). CBS but not CSE inhibition results in decreased human endometrial H_2S production, although CSE may contribute to baseline H_2S production. These data show that 1) CBS is the primary enzymatic pathway responsible for augmented endometrial H_2S production during the menstrual cycle and pregnancy and 2) elevated endogenous estrogens during the proliferative phase and pregnancy stimulate endometrial H_2S production via selective upregulation of CBS expression in women.

The mechanisms underlying endometrial angiogenesis have been extensively studied but remain controversial. In keeping with the potent proangiogenic activity of H_2S in vivo and in vitro (14-16), the positive correlations between endometrial angiogenesis indices and H_2S production as revealed by the present study implicates a novel role of H_2S in endometrial angiogenesis. The human endometrium is composed of multiple cell types, including ESCs, EMECs, and epithelium/glands, etc. Baseline CBS and CSE protein expression are detectable in all these cell types. However, augmented CBS but not CSE protein localizes in ESCs and vessels in the proliferative and pregnant endometria, suggesting a potential role of elevated estrogens in ESC CBS expression. The primary human EMECs established in our

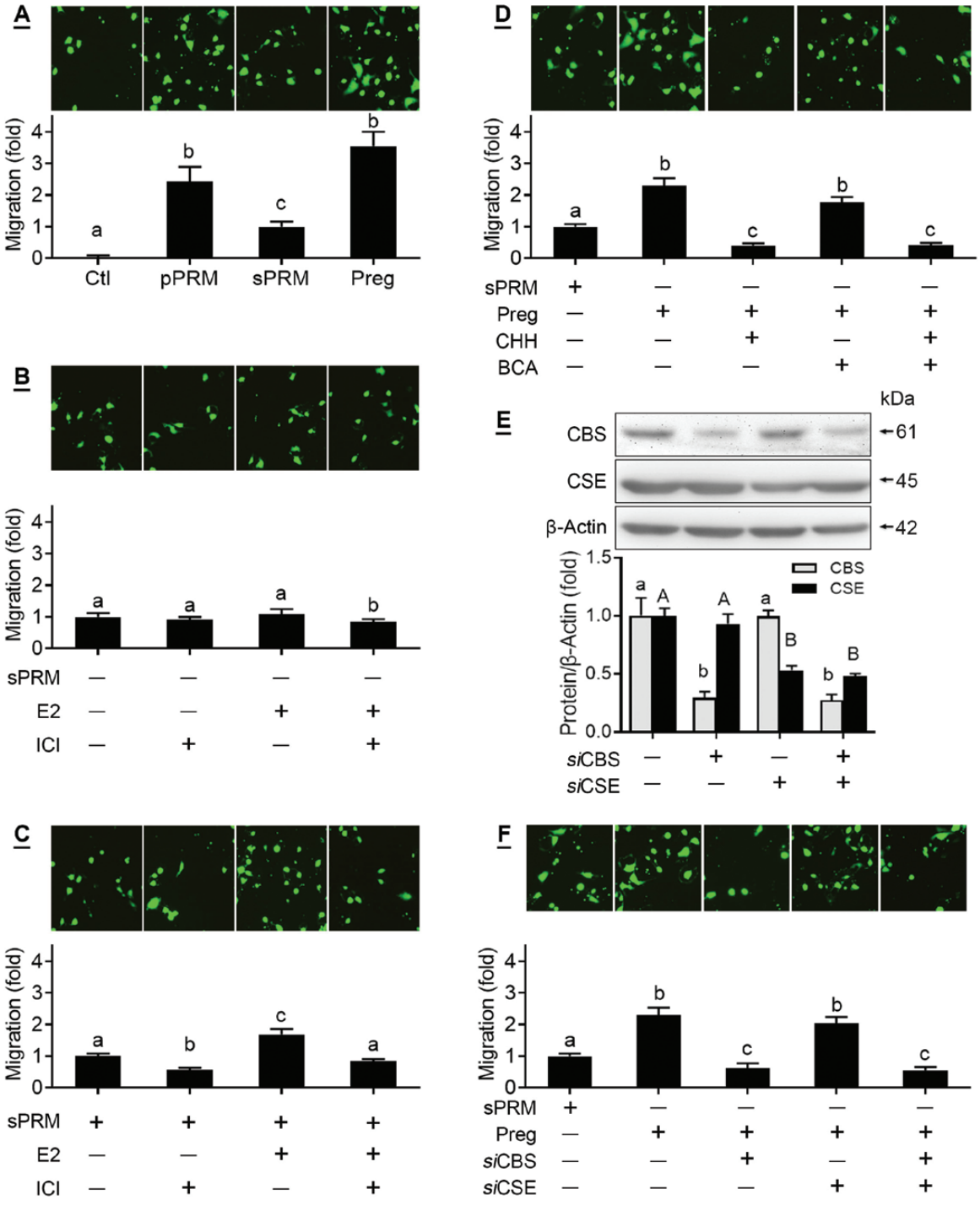

Figure 6. Endometrial stromal cell (ESC)-derived hydrogen sulfide (H_2S) on endometrial microvascular endothelial cell (EMEC) migration. Cell migration was determined by a co-culture Transwell assay with pregnant (Preg) EMECs on the top of the inserts with or without ESCs on the bottom chamber as described in each panel. A, EMECs were seeded on the top of inserts in a 24-well Transwell plate; ESCs isolated from proliferative (pPRM), secretory (sPRM), and pregnant (P) endometria were cultured in the bottom chamber. EMECs alone without ESCs in the bottom chamber was used as a control (Ctl). B, EMECs, without ESCs in the bottom chamber, were treated with estradiol (10 nM; E2), ICI 182 780 (1 μM ICI), or both. C, EMECs in the top insert were co-cultured with sPRM ESCs with 10 nM E2, 1 μM ICI, or both. D, EMECs in the top insert were co-cultured with sPRM ESCs with or without β-cyano-L-alanine (BCA) and O-(carboxymethyl) hydroxylamine hemihydrochloride (CHH). E, Knockdown of cystathionine-β synthase (CBS) and cystathionine-γ lyase (CSE) proteins in ESCs transfected with small interfering (si)CBS or siCSE; controls were transfected with scrambled siRNAs. F, Endogenous ESC H_2S in EMEC migration. EMEC on the top insert were co-cultured with Preg ESCs treated with or without siCBS or siCSE, or control small interfering RNA; nontransfected sPRM ESC was used as a control. In all migration experiments, the Transwell system was cultured for 16 hours to allow EMEC migration. The migrated cells were quantified and averaged from 6 randomly fields. Data (mean ± SEM) are presented as fold of controls. Bars with different letters differ significantly among groups ($P < .05$).

study express ERβ but not ERα, in agreement with previous reports (10, 11). In vivo, E_2 induces endometrial glandular and stromal vascular endothelial growth factor expression and stimulates EMEC proliferation to promote endometrial vascular expansion in the baboon (45). However, neither E_2 nor the ERβ agonist DPN stimulates in vitro human EMEC angiogenesis (ie, proliferation, migration and tube formation); instead, DPN decreases EMEC tube formation in vitro (11). Thus, it is speculated that estrogens regulate endometrial EMEC angiogenesis indirectly through interaction with other cell types; however, this has never been tested. Because EMECs are in close contact with ESCs, we posit that elevated estrogens act on the $ERα^+/ERβ^+$ ESCs to stimulate CBS expression and H_2S production, which in turn plays a paracrine role in stimulating EMEC angiogenesis in the proliferative and pregnant endometria. Indeed, treatment with physiological relevant E_2 but not P_4 stimulates CBS protein and H_2S production in ESCs in vitro; these responses of estrogens are abolished by ICI, implicating specific ER-mediated mechanisms, although the specific role of ERα and ERβ needs to be determined. We then developed a co-culture model for determining EMECs interactions with ESCs. Using EMEC migration as a means for in vitro angiogenesis, we show that EMEC migration is stimulated by co-culture with ESCs from endometria of all physiological states tested; however, ESCs from the proliferative and pregnant endometria possess significantly greater capacity of doing so. E_2 alone is unable to stimulate EMEC migration, consistent with a previous report (11). However, E_2 potentiates EMEC migration by co-culture with ESCs. Furthermore, blockade of H_2S production by using a specific CBS (but not CSE) inhibitor or siRNA blocked Preg ESC-stimulated EMEC migration. These data show that CBS-derived H_2S mediates ESC-stimulation of EMEC migration. The endometrium is composed of many cell types, with the majority being stroma, epithelium/glands, and blood vessels; all of these express CBS and CSE. H_2S derived from endometrial vessels is also expected to play a role in EC angiogenesis when the potent proangiogenic activity of H_2S is taken into consideration (14-16). H_2S derived from endometrial epithelial cells/glands seems not to play a key role in EMEC angiogenesis because co-culture with endometrial epithelial cells decreases EMEC angiogenesis in a 3-dimensional gelatin hydrogel angiogenesis assay (46). Nonetheless, a role of ESCs in endometrial vessel formation revealed in our study agrees with the report using the 3-dimensional gelatin hydrogel of ESC-EMEC co-culture model for EMEC angiogenesis assay (46). Furthermore, our study shows for the first time that CBS-derived H_2S mediates ESC-EMEC interactions in the context of angiogenesis in response to estrogens. Interestingly, a recent study using a co-culture model has shown that prostaglandin E_2 and prostacyclin produced by EMEC on shear stress stimulation can regulate ESC decidualization (47). Thus, cell-cell interactions are bidirectional in endometrial function regulation.

A limitation of this study is the small sample size (n = 4) of the secretory phase endometria surveyed, resulting in a loss of opportunity to correlate changes in the H_2S system with endometrial angiogenesis in the middle secretory phase, when estrogens are temporarily increased with high progesterone, along with enhanced angiogenesis for preparing the endometrium receptive for embryo implantation (48). ESCs express both ERα and ERβ ([12, 40-42], present study), which function as nuclear transcription factors. E_2 stimulates CBS mRNA and protein in ESCs in vitro and in vivo, suggesting transcriptional regulation. Thus, future studies are warranted to determine the specific role of ERα and ERβ in the transcriptional regulation of CBS expression in ESCs. The heterogeneity of the populations of endometria in the other groups surveyed is another potential limitation; however, this is unlikely to significantly change our conclusion. Our findings consistently show that the endometrial CBS/H_2S system increases significantly in the proliferative phase and pregnancy in women; E_2 stimulates ER-dependent selective CBS upregulation to increase endometrial stromal production of H_2S, which in turn promotes EMEC angiogenesis. This pathway correlates to elevated endogenous estrogens and can be stimulated by exogenous estrogens but not progesterone in vitro, providing a novel important mechanism for controlling endometrial vascular remodeling during the menstrual cycle and pregnancy.

Collectively, our present study demonstrates for the first time that endometrial H_2S production is augmented via selective CBS upregulation, positively correlating with enhanced angiogenesis indices and endogenous estrogens in the proliferative phase and pregnancy. Additionally, our mechanistic studies have established a novel paracrine role of ESC CBS/H_2S in mediating estrogen regulation of EMEC angiogenesis, providing novel insight into the understanding of how estrogens regulate human endometrial endothelial cell angiogenesis.

Perspectives

During the menstrual cycle, the endometrial functionalis layer sheds during menstruation and then rapidly regenerates within a 5- to 7-day window under the influence of estrogens from the growing follicles in the proliferative phase. Following ovulation, P_4 from the corpus luteum promotes differentiation of endometrial cells for preparing the uterus to be receptive for the blastocyst to implant in the secretory phase. If the embryo implants successfully,

endometrial cells proliferate and differentiate further to form the maternal placenta. Otherwise, the endometrium is shed to enter the next menstrual cycle following P_4 withdrawal (5). All endometrial events are accompanied by dynamic vasculature remodeling, involving interactions among endometrial epithelial, stromal, and endothelial cells, etc. Endometrial vasculature repairs during its regeneration and grows rapidly via angiogenesis during the proliferative phase, followed by the growth and coiling of the spiral arterioles in the secretory phase (48). Once conceived, placental trophoblasts invade and transform endometrial/decidual spiral arterioles into high-flow and low-resistance vessels (49); these vessels expand further to result in an increase in uterine blood flow that essentially keeps pace with the growth rate of the fetus during pregnancy (49). Endometrial angiogenesis is tightly regulated during the menstrual cycle and pregnancy; either excessive or insufficient endometrial angiogenesis can result in reproductive diseases such as abnormal uterine bleeding, endometriosis, implantation failure and early pregnancy loss, placenta accrete spectrum, preeclampsia, fetal growth restriction, and endometrial cancer. (5, 6). It is unknown whether the H_2S system is dysregulated in these reproductive diseases; however, dysregulated H_2S has been recently found in endometriosis characterized by excessive angiogenesis (31). Thus, future studies are warranted to further explore whether the endometrial CBS/CSE-H_2S system is dysregulated in these reproductive disorders and whether H_2S may serve as a therapeutic target for these reproductive diseases.

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