Am J Transl Res 2018;10(12):4247-4257
www.ajtr.org /ISSN:1943-8141/AJTR0078083

Original Article

H_2S promotes proliferation of endometrial stromal cells via activating the NF-κB pathway in endometriosis

Shating Lei[1*], Yanling Cao[1*], Jing Sun[1], Mingqing Li[3,4,5], Dong Zhao[2,6]

Departments of [1]Gynecology, [2]Cervical Disease, Shanghai First Maternity and Infant Hospital, Tongji University School of Medicine, Shanghai, People's Republic of China; [3]Laboratory for Reproductive Immunology, Hospital of Obstetrics and Gynecology, Fudan University, Shanghai, People's Republic of China; [4]Key Laboratory of Reproduction Regulation of NPFPC, SIPPR, IRD, Fudan University, Shanghai, People's Republic of China; [5]Shanghai Key Laboratory of Female Reproductive Endocrine Related Diseases, Shanghai, People's Republic of China; [6]Department of Obstetrics and Gynecology, Shanghai Ninth People's Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, People's Republic of China. [*]Equal contributors.

Received April 17, 2018; Accepted November 12, 2018; Epub December 15, 2018; Published December 30, 2018

Abstract: Hydrogen sulfide (H_2S) is substantially converted from cysteine by the enzymes cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE). H_2S can profoundly affect most organ systems in animals and humans by inducing a wide range of physiological functions. However, the roles of H_2S in the progression of endometriosis remain unknown. The aim of the current study was to test the hypothesis that H_2S might play a role in the pathogenesis of endometriosis via modulating the biological behavior of endometrial stromal cells (ESCs). First, we explored the expression level of CBS and CSE in ESCs via immunohistochemistry and immunocytochemistry. Second, cell Count Kit-8 (CCK-8) assays were utilized to investigate the cell viability of human ESCs (HESCs) *in vitro*. Third, we studied the potential effects of H_2S in a rodent model of endometriosis. Both CBS and CSE were overexpressed in endometriotic lesions. Exogenous and endogenous H_2S could promote HESC proliferation *in vitro*. Furthermore, this pro-proliferation effect could be reversed by treating with inhibitors of CBS, CSE, or the NF-κB pathway. *In vivo*, we uncovered that inhibitors of CBS and CSE could remarkably reduce the number and weight of mouse endometriotic lesions. These data suggested that H_2S promotes ESC proliferation via activation of the NF-κB pathway, which provides a scientific basis for the clinical application of blocking H_2S to treat endometriosis.

Keywords: Hydrogen sulfide, CBS, CSE, proliferation, endometriosis

Introduction

Endometriosis, one of the most frequently encountered gynecological disorders affecting up to 10% of the reproductive-aged female population, is characterized by the presence and growth of endometrial tissue outside the uterine cavity [1]. Endometriosis is associated with dysmenorrhea, chronic pelvic pain, dyspareunia, and infertility. Although it is not a malignant disorder, endometriosis exhibits proliferation, migration, neo-angiogenesis, and recrudescence. The underlying mechanisms and pathophysiology of endometriosis remain poorly understood [2]. In recent years, researchers examined the balance of pro-inflammatory factors and anti-inflammatory factors, and dysfunction of immunocytes in the peritoneal microenvironment of endometriosis [3]. Knowledge of those factors is fundamental for developing strategies to prevent and treat endometriosis.

Hydrogen sulfide (H_2S) was identified as the third gas-transmitter along with nitric oxide (NO) and carbon monoxide (CO) [4]. H_2S is produced by a variety of mammalian tissues and regulates multiple physiological functions, such as inflammation, proliferation, apoptosis, angiogenesis, glucose metabolism, and autophagy [5-11]. Endogenous H_2S is substantially synthesized from L-cysteine by two enzymes: cystathionine γ-lyase (CSE) and cystathionine β-synthetase (CBS), both of which are pyridoxal-5-phosphate-dependent and found throughout the body [12, 13]. While CBS is mainly expressed in the nervous system, CSE is distributed primarily in peripheral tissues, such as heart, liver, kidney, pancreas and others. Another H_2S producer is 3-mercaptopyruvate sulfurtransferase (3-MST) located in mitochondria [14].

Previous studies have suggested that disrupted H_2S homeostasis affects cardiovascular, liver,

neuronal, gastrointestinal, respiratory, immune, and endocrine systems by modulation of cellular signaling pathways and sulfhydration of target proteins [15-23]. Accumulating evidence suggests that H_2S is involved in inflammatory diseases. It seems that physiological concentrations of H_2S exhibit anti-inflammatory effects, whereas higher concentrations show pro-inflammatory effects [24, 25]. H_2S exhibits both pro- and anti-cancer effects by regulating cancer biological processes. In colon cancer, CBS-derived H_2S was shown to promote colon HCT-116 cell proliferation, migration, and invasion, and improve angiogenesis and vasorelaxation. Additionally, inhibiting CBS exerts antitumor effects *in vivo* [26]. Another study demonstrated that exogenous H_2S stimulates cell proliferation, migration, angiogenesis, and anti-apoptosis effects in hepatoma cells, and that these H_2S-induced effects are produced via activating the NF-κB pathway [7]. Meanwhile, some investigators showed that exogenous H_2S shows anti-cancer effects through p38 MAPK signaling pathway in C6 glioma cells [27]. In recent years, attention has been drawn to the pharmacological effect of H_2S on cell proliferation [28], yet ESCs are known to possess abnormal proliferation and invasion ability in endometriosis [29]. Nevertheless, few studies have addressed the role of H_2S in endometriosis. Based on previous studies, we hypothesized that endogenous H_2S might take part in the pathogenesis of endometriosis.

The aims of the current study were to investigate the molecular mechanism underlying the interaction between H_2S and endometriosis, illustrate the effect of sodium hydrosulfide (NaHS) (a donor of H_2S) [30] and/or the inhibitors of CBS and CSE on the proliferation of human endometrial stromal cells (HESCs) *in vitro*, and determine the role of H_2S in a rodent model of intraperitoneal endometriosis.

Materials and methods

Subjects and tissue collection

The subjects recruited into the study were women of reproductive age from Shanghai First Maternity and Infant Hospital between January 2016 and January 2017. Prior to sample collection, all participating women signed a written informed consent, and all the tissues were taken for research use only. The study protocol was approved by the Medical and Life Science Ethics Committee of Tongji University. All patients had regular menstrual cycles and were not receiving any hormonal treatment or contraceptives for at least 6 or 3 months, respectively, prior to surgery. Women suffering from neoplastic, endocrine, or infectious diseases were excluded. Samples of ectopic endometrial tissues were obtained from women with endometriosis (n = 21), and normal endometrial tissues from women in the control group (n = 35). Endometriosis was definitively diagnosed during laparoscopic surgery or hysterectomy and was confirmed by histopathological evaluation. For the control group, endometrial tissue samples were collected through curettage from women who exhibited fallopian tube jam but lacked any clinical indication or history of adenomyosis or endometriosis, or samples collected from women who underwent hysterectomy for leiomyoma. Samples were collected only in the proliferative phase of the cycle. All samples collected under sterile conditions were at least 300 mg and divided into two parts: one part was immediately fixed in 4% formalin for immunohistochemical analysis, and the another was transported to the laboratory on ice in Dulbecco's modified Eagle medium (DMEM)/F-12 (Hyclone, USA) for isolating and culturing endometrial stromal cells (ESCs).

Cell isolation and culture

The isolation and culture of ectopic ESCs and normal ESCs were conducted according to a previously reported method [3, 31, 32]. The endometrial tissues were minced into small pieces (1-2 mm^3) and digested with collagenase type IV (0.1%, Sigma, USA) in a 37°C shaking table for 40-60 min. The digested tissues were first filtered through a 100-μm (pore size) nylon mesh to remove debris and then through a 40-μm (pore size) nylon mesh (Falcon cell strainers; Thermo Fisher Scientific, USA). The filtrate was then centrifuged at 1300 rpm for 10 min to collect ESCs. After that, the ESCs were re-suspended in DMEM/F-12 containing 10% fetal bovine serum (FBS, Hyclone, USA), ampicillin (100 IU/mL) and streptomycin (100 IU/mL), then transferred into culture flasks. The adherent ESCs were cultured in a humidified atmosphere with 5% CO_2 at 37°C, and the culture medium was replaced every 2 days. This method provided over 95% vimentin-positive and cytokeratin-7 (CK7)-negative ESCs. The isolated ectopic ESCs and normal ESCs were used for immunocytochemistry.

Human endometrial stromal cells (HESCs, ATCC CRL-4003) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were maintained according to the vendor's instructions.

Immunohistochemistry

Immunohistochemistry was performed on ectopic endometrial tissues and normal endometrial tissues. Paraffin sections (5 μm) were dewaxed in graded ethanol and rehydrated in 1X phosphate-buffered saline (PBS). Endogenous peroxidase activity was eliminated by incubation in 3% hydrogen peroxide and antigen repair was performed in 0.01 M citrate buffer (PH 6.0) for 25 min at 95°C. After cooling to room temperature, slides were blocked for 30 min using 5% bovine serum albumin (BSA, Hyclone, USA). After that, the samples were incubated with a mouse anti-human cystathionase monoclonal antibody (3 mg/ml, Abcam, UK), mouse anti-human CBS monoclonal antibody (1:50, Santa Cruz, USA), or mouse IgG isotype antibody overnight (12-16 h) at 4°C in a humidified chamber. After washing three times with PBS, the sections were overlaid with peroxidase-conjugated goat anti-mouse IgG antibody (Weiao Biotechnology, China), and the reaction was developed with 3,3-diaminobenzidine (DAB, Weiao Biotechnology), and counterstained with hematoxylin. The samples were then observed using an Olympus BX51+DP70 microscope (Olympus Optical, Tokyo, Japan).

Immunocytochemistry

Sterilized polylysine-coated slides were placed into 6-well plates, then normal ESCs and ectopic ESCs suspensions were added into each well and allowed to grow to approximately 70-80% confluence. Then, the cells were fixed with 4% paraformaldehyde for 20 min at room temperature, washed three times with PBS, and permeabilized with 1% Triton X-100 (Sigma) for 10 min. Non-specific binding of antibodies was blocked by incubation with 1% bovine serum albumin (BSA, Hyclone, USA) for 30 min. Next, the slides were incubated with mouse anti-human cystathionase monoclonal antibody (3 mg/ml, Abcam, UK), mouse anti-human CBS monoclonal antibody (1:50, Santa Cruz, USA), mouse anti-human vimentin monoclonal antibody (1:50, Santa Cruz, USA), mouse anti-human CK7 antibody (1:50, Santa Cruz, USA), or mouse IgG isotype antibody overnight at 4°C in a humidified chamber. The subsequent staining steps were the same as those described for immunohistochemistry. All slides were counterstained with hematoxylin and evaluated under an Olympus BX51+DP70 microscope.

In vitro treatment of ESCs

To evaluate the dose effect of H_2S on HESCs, the HESCs were seeded in 96-well plates at a concentration of 1×10^4/ml per well, and incubated at 37°C until the cells adhered to the wall, then cells were treated with different doses of NaHS (50, 100, 200, 300, 400, 500, or 1000 μmol/l) for 24 h or co-treated with 300 μmol/l NaHS and different doses of D,L-propargylglycine (PAG; 1, 3 or 10 mmol/l, MCE, USA), aminooxyacetate (AOAA; 0.1 or 1 mmol/l, MCE, USA) [33], pyrrolidine dithiocarbamate (PDTC; 0, 5, 10, 50, 100, 200, 250, or 500 μmol/l, MCE, USA), or caffeic acid phenethyl ester (CAPE; 0, 1, 2.5, 5, 10, 25, or 50 μmol/l, MCE, USA) for 24 h.

Cell count kit 8 (CCK 8) assay

The cell viability of HESCs was evaluated by CCK8 (Dojindo Company, Japan) assay. HESCs were seeded into 96-well plates at a density of 1×10^4/ml per well. Cells were treated with the indicated treatments for 24 h. After that, 100 μl of 10-fold diluted CCK-8 solution was added into each well, and then the plate was incubated for 40 min in the dark at 37°C. Absorbance of each well was measured at wavelength of 450 nm by a DigiScan Microplate Reader (ASYS Hitech GmbH, Eugendorf, Austria). The optical density (OD) values were normalized to those of the control group, which was set to 1. Each experiment was repeated three times. Each concentration was assayed in duplicate using at least 3 wells.

CSE and CBS overexpression plasmids transfection

DCE-hCBS, a CBS overexpression plasmid, and DCE-hCTH, a CSE overexpression plasmid, were purchased from Bio Link (Shanghai, China). The day before transfection, 5×10^4 HESCs were seeded into each well of 24-well plates, and when the cells reached about 70% confluence, the complete medium was refreshed. Plasmids were diluted with serum-free medium, then mixed with attractene transfection reagent (Qiagen, Germany) according to the manufacturer's

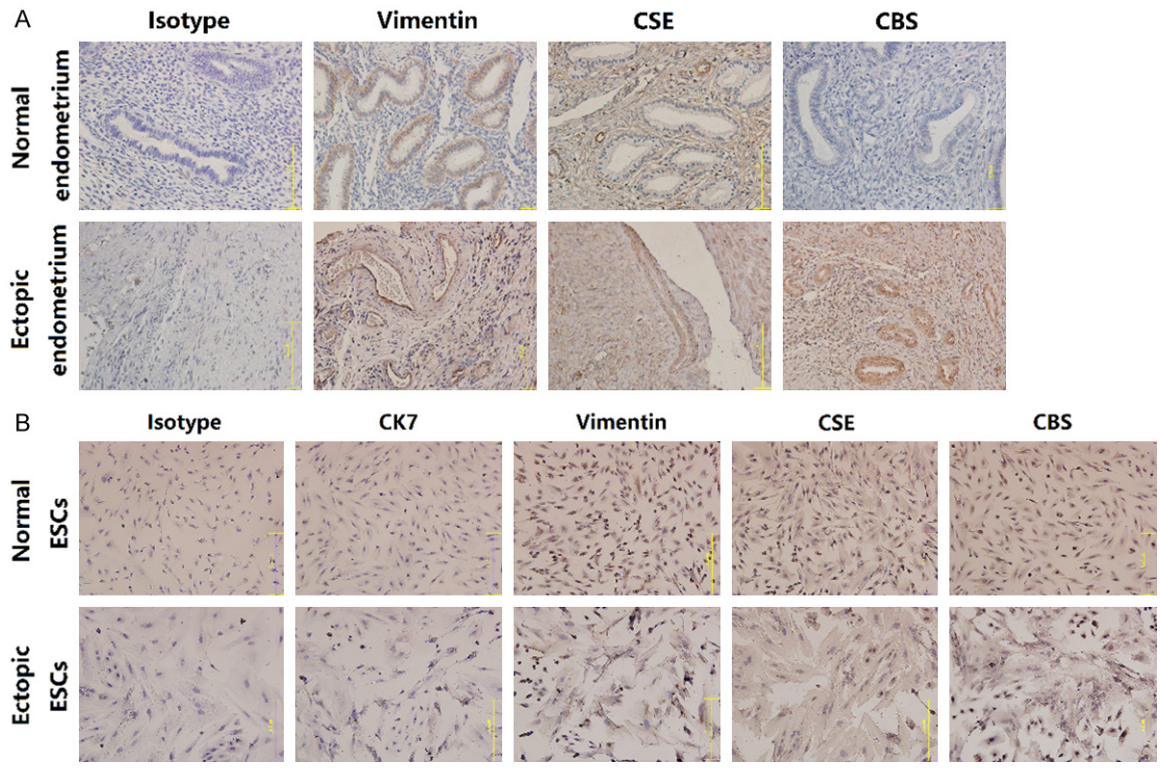

Figure 1. The expression level of cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) in ectopic/eutopic endometrium and endometrial stromal cells (ESCs). A. Representative immunohistochemical staining of CBS and CSE in normal endometrium and ectopic endometrium, respectively. Brown precipitate refers to CBS or CSE expression in glandular epithelium and stroma. B. Expression of CBS and CSE in normal ESCs and ectopic ESCs were tested by immunocytochemistry assay. Isotype antibody and CK7 staining represent the negative control, vimentin staining refers to the positive control (Original magnification, × 200). Normal ESCs refers to endometrial stromal cells from endometrium of the control group, ectopic ESCs refers to ectopic endometrial stromal cells obtained from patients with endometriosis.

instructions. After incubating at room temperature for 15 min, the transfection complexes were added drop-wise onto the cells and incubated at 37°C.

Western blot analysis

Cells were washed twice in PBS, and then lysed with RIPA lysis buffer (Beyotime Biotechnology, China) at 4°C for 30 min. The cell debris was removed by centrifugation at 12,000 rpm at 4°C for 20 min. The protein concentrations were determined by BCA Protein Assay Kit (Beyotime Biotechnology, China). Equal amounts of total protein (30 μg/lane) were loaded onto 10% SDS-PAGE gels. Proteins were then transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, USA), blocked with blocking solution for 1 h, and the membrane was then incubated with mouse anti-human cystathionase monoclonal antibody (2.5 μg/ml, Abcam, UK), mouse anti-human CBS monoclonal antibody (1:500, Santa Cruz, USA), rabbit anti-human GAPDH monoclonal antibody (1:1000, Cell Signaling Technology, USA), rabbit anti-human phospho-NF-κB p65 (Ser536) monoclonal antibody (1:1000, Cell Signaling Technology, USA), or rabbit anti-human phospho-IKKα/β monoclonal antibody (1:1000, Cell Signaling Technology, USA), respectively, at 4°C overnight. Subsequently, the membrane was incubated with HRP-linked anti-mouse IgG (1:3000, Cell Signaling Technology, USA) or HRP-linked anti-rabbit IgG (1:3000, Cell Signaling Technology, USA) at room temperature for 1 h. The membranes were washed by TBST and exposed to ECL (Millipore, USA). Images were captured using a gel imaging analysis system (Bio-Rad, USA).

Intraperitoneal endometriosis model

We constructed an allotransplantation of intraperitoneal endometriosis model according to a

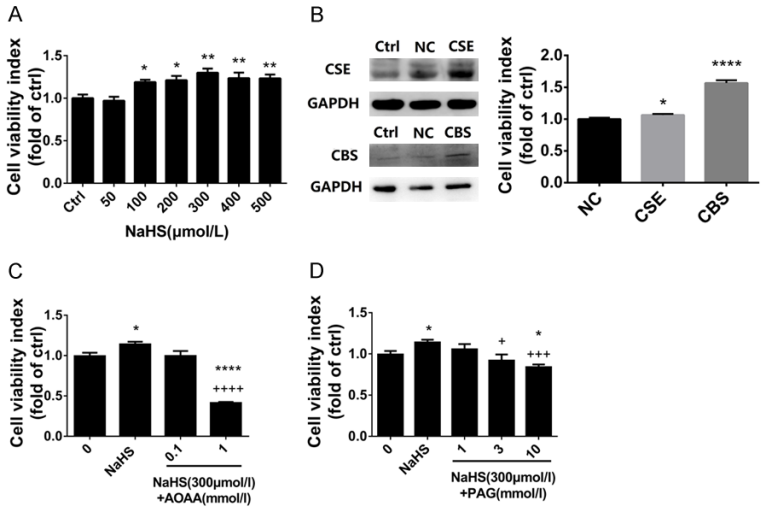

Figure 2. Exogenous and endogenous H_2S could promote proliferation of human endometrial stromal cells (HESCs). A. HESCs were treated with different doses of NaHS (50, 100, 200, 300, 400, and 500 μmol/l) for 24 h. B. Overexpression plasmids of cystathionine β-synthetase (CBS) and cystathionine γ-lyase (CSE) were transfected into HESCs. C. HESCs were co-conditioned with 300 μmol/l NaHS and different doses of AOAA (0.1 and 1 mmol/l) for 24 h. D. HESCs were co-conditioned with 300 μmol/l NaHS and different doses of PAG (1, 3 and 10 mmol/l) for 24 h. Cell viability was tested by Cell Counting Kit-8 (CCK-8). Data were shown as the mean ± S.E.M. *P < 0.05, **P < 0.01 compared with the control group, +P < 0.05, ++P < 0.01 compared with the NaHS group. Ctrl, the control group; NaHS, a donor of H_2S. CBS: HESCs transfected with CBS overexpressed plasmids; CSE: HESCs transfected with CSE overexpressed plasmids. GAPDH: internal control. PAG, D,L-propargylglycine, an inhibitor of CSE; AOAA, aminooxyacetate, an inhibitor of CBS.

previously described method [34] with slight alterations. The Animal Care and Use Committee of Tongji University approved the animal protocols. 18 recipient female BABL/c mice and 9 donor female BABL/c mice were used. The donor mice was treated with estrogen for 7 days, and then sacrificed and their uteruses removed. All the donors' uteruses were minced and intraperitoneally injected into recipient mice on day 0. And the 18 recipient mice were divided into three group after implantation, each group contained 6 mice. Normal saline (control group), PAG (50 mg/kg), and AOAA (30 mg/kg) [33] were intraperitoneally injected into mice in each respective group on day 5. Fourteen days after implantation, all recipient mice were sacrificed, the peritoneal cavities were examined, and the endometriotic lesions were weighed and counted.

Statistical analysis

Values represent means ± S.E.M. Statistical comparisons were performed using GraphPad Prism 6 software. Parametrical data were analyzed by Student's *t*-test for two-group comparisons. One-way ANOVA was used to determine the difference between independent groups. $P < 0.05$ was regarded as statistically significant. For each assessment, at least three independent experiments were performed.

Results

The expression level of CSE and CBS in ectopic endometrium were higher than expression in the normal endometrium

To determine the role of H_2S in endometriosis, the expression levels of CSE and CBS were detected by immunohistochemistry and immunocytochemistry. As shown in **Figure 1A**, both the expression of CSE and CBS were higher in ectopic endometrium from endometriosis samples compared to expression in the normal endometrium. Apparently, CBS and CSE were highly expressed in both the stroma and glandular areas. Immunocytochemistry results (**Figure 1B**) revealed that the expression of CBS and CSE in ectopic ESCs from patients with endometriosis exhibited stronger staining than staining of normal ESCs from the control group. These results suggested that the higher expression of CBS and CSE in ectopic endometrium might be involved in regulating the biological behaviors of ESCs.

H_2S promotes HESC proliferation

To further elucidate the effect of H_2S on HESC proliferation, the administration of exogenous and endogenous H_2S was carried out *in vitro*. First, we performed dose-response experiments in HESCs. Cells were treated with a concentration gradient of NaHS (50, 100, 200, 300, 400, 500 μmol/l) for 24 h. As shown in **Figure 2A**, NaHS administration significantly augmented the cell viability of HESCs and reached a peak at 300 μmol/l, so this NaHS concentration (300 μmol/l) was chosen for use in the subsequent study. To further confirm the

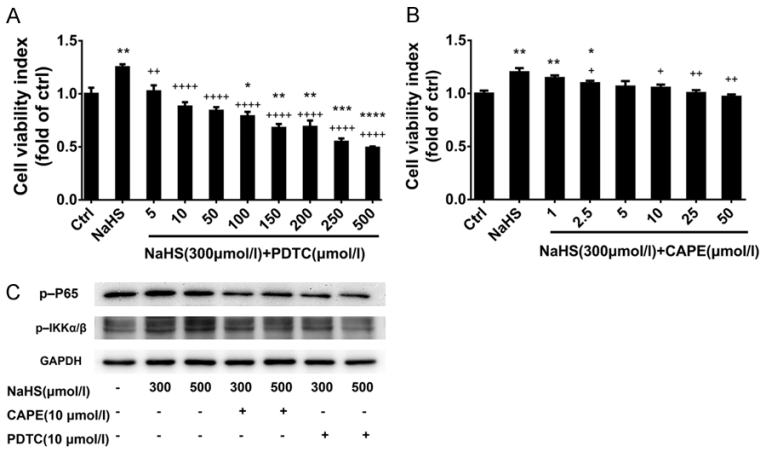

Figure 3. H_2S-induced cell proliferation in human endometrial stromal cells (HESCs) is dependent on the NF-κB signaling pathway. A. HESCs were co-conditioned with 300 μmol/l NaHS and different doses of PDTC (0, 5, 10, 50, 100, 150, 200, 250, and 500 μmol/l) for 24 h. B. HESCs were co-conditioned with 300 μmol/l NaHS and different doses of CAPE (0, 1, 2.5, 5, 10, 25, and 50 μmol/l) for 24 h. Cell viability was tested by Cell Count Kit-8 (CCK-8). C. HESCs were incubated with 300 μmol/l or 500 μmol/l NaHS, and co-conditioned with CAPE (10 μmol/l) or PDTC (10 μmol/l) for 24 h. Western blot analysis of the levels of NF-κB p65 phosphorylation and IKKα/β phosphorylation. Data were shown as the mean ± S.E.M. *P < 0.05, **P < 0.01 compared with the control group. +P < 0.05, ++P < 0.01 compared with the NaHS group. Ctrl, the control group; NaHS, a donor of H_2S. PDTC, pyrrolidine dithiocarbamate, a specific inhibitor of the NF-κB pathway; CAPE, caffeic acid phenethyl ester, a specific inhibitor of the NF-κB pathway.

pro-proliferation effect of H_2S on HESCs, we constructed CBS and CSE overexpression plasmids. As depicted in **Figure 2B**, the expression of CBS and CSE was significantly increased by transfection with overexpression plasmids, which means the transfection efficiency was sufficient for further analysis (Figures S1, S2, S3, S4, S5 and S6). Moreover, cell viability was evidently promoted by overexpression of CBS and CSE. Conversely, this effect was distinctly reduced by treatment with the CBS inhibitor AOAA (1 mmol/l) (**Figure 2C**) or the CSE inhibitor PAG (3 and 10 mmol/l) (**Figure 2D**). Based on the aforementioned results, both exogenous and endogenous H_2S could promote HESC proliferation, while inhibiting CBS and CSE suppressed NaHS-induced proliferation effects.

H_2S-induced HESC proliferation is dependent on NF-κB signaling

To investigate whether the NF-κB pathway was involved in the H_2S-induced proliferation effect on HESCs, we treated cells with PDTC and CAPE, which are specific inhibitors of the NF-κB pathway. As shown in **Figure 3A**, HESCs were co-treated with 300 μmol/l NaHS and different doses of PDTC (0, 5, 10, 50, 100, 150, 200, 250, and 500 μmol/l) for 24 h. Adding 300 μmol/l NaHS alone could significantly augment HESC viability, whereas the administration of PDTC diminished this effect remarkably. The same change was observed when HESCs were co-treated with 300 μmol/l NaHS and different doses of CAPE (0, 1, 2.5, 5, 10, 25, and 50 μmol/l) for 24 h (**Figure 3B**). Meanwhile, upon exposure to 300 μmol/l or 500 μmol/l NaHS, HESCs displayed increased levels of NF-κB p-65 phosphorylation and IKKα/β phosphorylation (**Figures 3C**, S7, S8, S9 and S10). These results proved that H_2S promotes the proliferation of HESCs via the NF-κB pathway.

Inhibiting endogenous H_2S diminished the growth of endometriotic lesions in mice

To further confirm the role of H_2S and its synthetase in the development of endometriosis *in vivo*, we designed intraperitoneal allograft endometriosis model. **Figure 4A** is a schematic diagram showing the model flowchart. Administration of PAG and AOAA *in vivo* led to a distinct decrease in the number of ectopic lesions (**Figure 4B**). Furthermore, the weight of ectopic lesions in PAG and AOAA groups were notably lower compared to that of the control group (**Figure 4B**). Taken together, these data indicated that inhibiting the production of H_2S could obviously suppress the growth and implantation of ectopic lesions.

Discussion

In the present study, we identified that the expression of CBS and CSE was significantly higher in tissues or cells from endometriosis patients in comparison to expression in normal controls. These results suggested that there might be some correlation between the underlying mechanism of endometriosis and H_2S. These two enzymes may be upregulated as a consequence of local chronic inflammation as previously reported [35]. According to previous

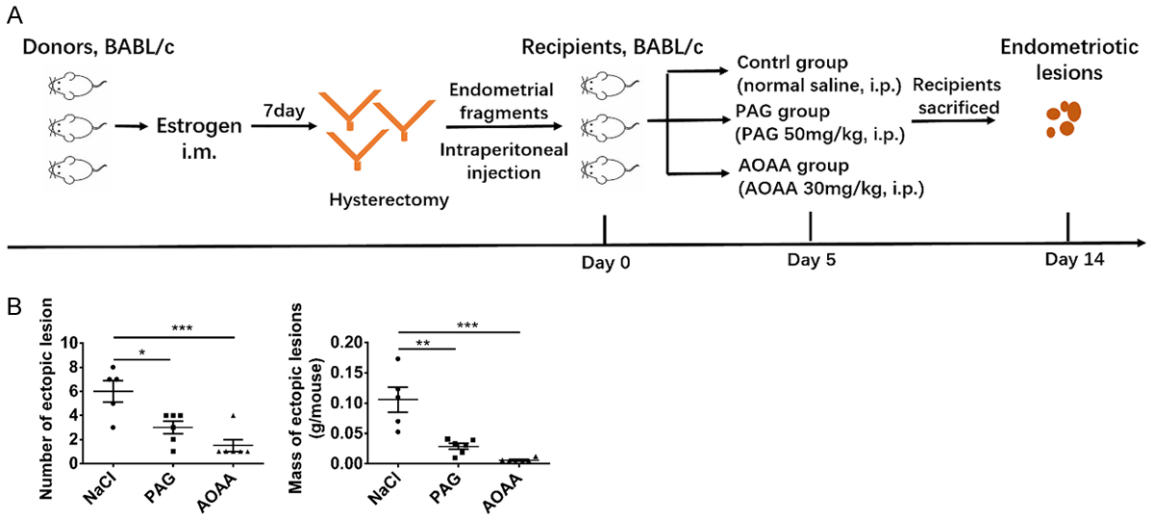

Figure 4. Inhibiting H_2S production *in vivo* diminished the growth of endometriotic lesions in mice. A. Endometriosis-like lesions were induced in BABL/c mice by allotransplantation of endometrial fragments. The mice were treated with Control (normal saline), D,L-propargylglycine (PAG; 50 mg/kg) or aminooxyacetate (AOAA; 30 mg/kg) by intraperitoneal injection. Two weeks after allotransplantation, all the recipients were sacrificed, and all the endometriosis-like lesions were removed. B. The endometriosis-like lesions in mice were calculated and weighed. Results were presented as the mean ± S.E.M. *: $P < 0.05$.

studies, the activity of CBS is up-regulated by S-adenosyl-L-methionine (SAM) and Ca^{2+}-calmodulin, and treatment with specific inhibitors of Ca^{2+} and calmodulin could suppress the activity of CBS [13, 36, 37]. Meanwhile, the expression of the *CALM2* gene (which encodes calmodulin) is increased in endometriotic lesions [38]. Additionally, the expression of the protein caldesmon and its encoding gene *CALD1* are remarkably elevated in ectopic endometrium samples [39]. However, some experts demonstrated that low intracellular concentration of Ca^{2+} could promote CES and the 3-MST pathway to produce H_2S [40]. Further studies are needed to uncover the regulatory mechanism of H_2S expression.

As a gasotransmitter molecule, H_2S can enter cells rapidly without the help of any receptors or transporters. Under physiological pH, H_2S is in fast equilibrium with HS- in aqueous solutions. Therefore, there are two isoforms of H_2S in our body, and proportions of HS- and H_2S are 81% and 19%. For this reason, inorganic sulfide salts (eg. NaHS and Na_2S) are usually used as H_2S donors [41]. To verify the role of H_2S in the progression of endometriosis, we treated HESCs with NaHS *in vitro* to simulate a high endogenous H_2S level in the microenvironment. Our results indicated that NaHS remarkably improved proliferation of HESCs at concentrations ranging from 100 to 500 μmol/l, and the optimal concentration was 300 μmol/l, which lead to the maximal effect on proliferation. Additionally, overexpression of CBS and CSE, which increase endogenous H_2S, could promote HESC survival. Further, CBS and CSE inhibitors neutralized the NaHS-mediated proliferation effect, indicating that the pro-proliferation effect of H_2S could be notably abolished by blocking the endogenous biosynthesis of H_2S. Consistent with our findings, Zheng et al. found that H_2S could induce cell proliferation in multiple myeloma cells via activating the Akt pathway [42], and Zhen proved that NaHS could induce cell proliferation and prevent apoptosis in hepatoma cells by amplifying activation of the NF-κB pathway [7]. However, Wang et al. demonstrated that the CSE/H_2S pathway inhibits the proliferation of vascular smooth muscle cells in high homocysteine conditions [43], and Ding et al. identified that high glucose induced mouse mesangial cell over-proliferation by inhibiting the production of H_2S [11]. Treating H9c2 cardiac cells with NaHS remarkably decreases the nuclear translocation of the NF-κB p65 subunit and attenuates the p38 MAPK/NF-κB pathway-mediated inflammatory responses [44].

The manifestations of endometriosis include up-regulation of cell survival and invasion [45]

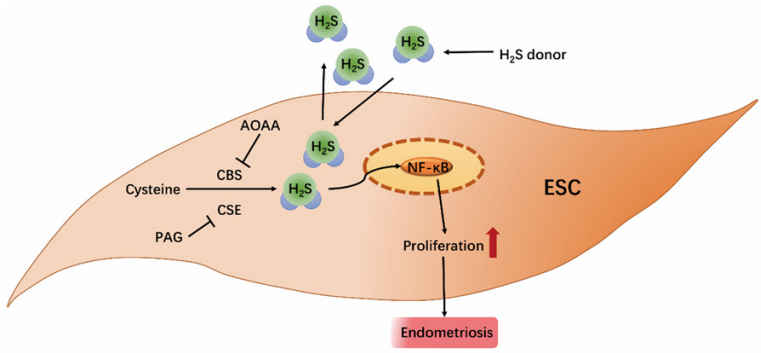

Figure 5. Schematic showing the role of H_2S in endometriosis. Endometrial stromal cells (ESCs) from ectopic tissue of patients with endometriosis express high levels of cystathionine β-synthetase (CBS) and cystathionine γ-lyase (CSE), which catalyze cysteine into H_2S. Endogenous or exogenous H_2S activates the NF-κB signaling pathway, then enhances the survival and proliferation of ESCs, and finally promotes the progression of endometriosis. These effects can be reversed by aminooxyacetate (AOAA) and D, L-propargylglycine (PAG), inhibitors of CBS and CSE, respectively.

and multifactorial inflammation with persistent activation of the NF-κB signaling pathway. According to Gonzalez-Ramos *et al.*, red, active endometriotic lesions possess a higher level of NF-κB pathway activation than that of black, inactive lesions [46]. Moreover, proinflammatory cytokines that are distinctly elevated in the peritoneal fluid of endometriosis patients, such as IL-6, IL-8, and TNF-α, can induce the activation of NF-κB. Meanwhile, many inflammatory cytokines, chemokines, and immune response genes are regulated by activation of the NF-κB pathway. These factors are involved in cellular proliferation, cell adhesion, apoptosis, and invasion. Hence, the NF-κB pathway plays a vital role in the development and progression of endometriosis. LPS promotes ESC proliferation and invasion via activating NF-κB signaling and up-regulating COX-2 and PGE2 expression [47]. Tsai et al. showed that DcR3 is upregulated by activation of the Akt/NF-κB pathway and promotes cell adhesion by enhancing intercellular adhesion molecule 1 and homing cell adhesion molecule [48]. Additionally, numerous reports showed that the NF-κB pathway involved in ESC proliferation, and that inhibiting the NF-κB pathway by PDTC treatment decreases cell viability; thus, inhibitors of the NF-κB pathway are potential therapeutic agents for endometriosis or cancer [7, 32, 49]. Additionally, H_2S was reported to increase IkB-a degradation and thereby up-regulate NF-κB activation in U937 cells [50]. However, under some circumstances, H_2S plays the opposite role. NaHS exhibits an inhibitory effect on LPS-induced NF-κB translocation in astrocytes [50]. Clearly, the relationship between H_2S and the NF-κB pathway still needs to be elucidated. Our results demonstrated that inhibiting the NF-κB pathway by PDTC or CAPE treatment could block NaHS-induced pro-proliferation effects, so H_2S may promote the progression of endometriosis might by activating the NF-κB signaling pathway.

Several pilot studies have tested the therapeutic potential of H_2S in various diseases. Silencing CBS contributed to inhibiting ovarian cancer cell volume and nodule number [51]. Treating mice with AOAA suppressed the growth of patient-derived tumor xenografts (PDTXs) from different colon carcinoma patients [26] and attenuated the growth of MDA-MB-231 breast tumors in athymic mice [52]. Our experiments also obtained similar interesting results: treatment with PAG and AOAA distinctly attenuated the number and weight of endometriotic lesions in a mouse model of endometriosis. Our results pointed out that PAG and AOAA may have therapeutic potential for endometriosis. Further studies are needed to confirm this.

In summary, our study suggests that ectopic endometrium expresses a high level of CBS and CSE. Meanwhile, we identified that exogenous and endogenous H_2S could induce cell proliferation, and this pro-proliferation effect was mediated by activation of NF-κB signaling. To this end, data gathered in this study suggested that administration of PAG and AOAA could significantly suppress the weight and number of endometriotic lesions (**Figure 5**). Furthermore, these findings highlighted H_2S inhibitors as potential candidates for controlling endometriosis progression.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (NSFC81471436 and NSFC81771548) awarded to Dong Zhao.

Disclosure of conflict of interest

None.

Address correspondence to: Dong Zhao, Department of Cervical Disease, Shanghai First Maternity and Infant Hospital, Tongji University School of Medicine, No.2699, West Gaoke Road, Shanghai 201204, People's Republic of China. Tel: 86-21-20261000; E-mail: hendryz@gmail.com

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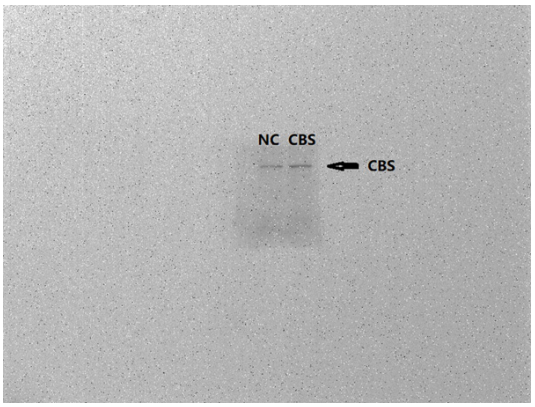

Figure S1. Overexpression plasmids of cystathionine β-synthetase (CBS) was transfected into HESCs. Whole membrane of CBS expression in western blot analysis.

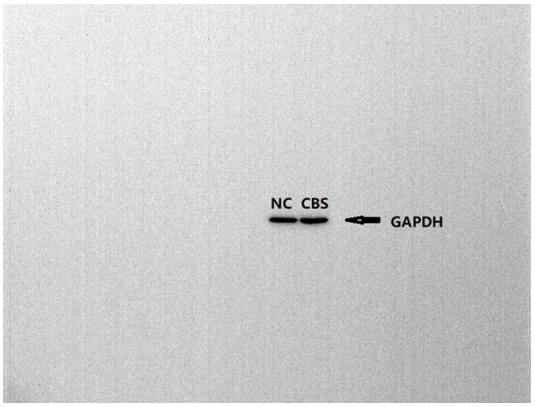

Figure S2. Overexpression plasmids of cystathionine β-synthetase (CBS) was transfected into HESCs. Whole membrane of GAPDH expression in western blot analysis.

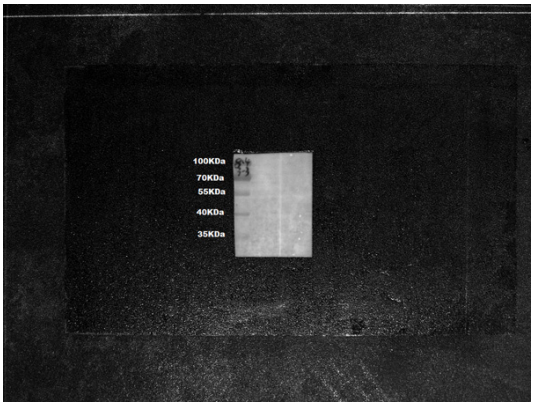

Figure S3. Overexpression plasmids of cystathionine β-synthetase (CBS) was transfected into HESCs. This picture shows the marker of the whole membrane.

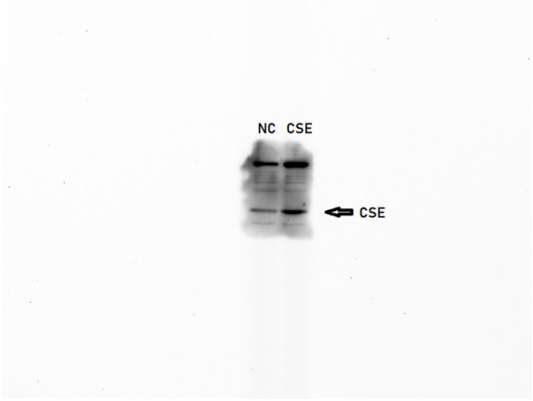

Figure S4. Overexpression plasmids of cystathionine γ-lyase (CSE) was transfected into HESCs. Whole membrane of CSE expression in western blot analysis.

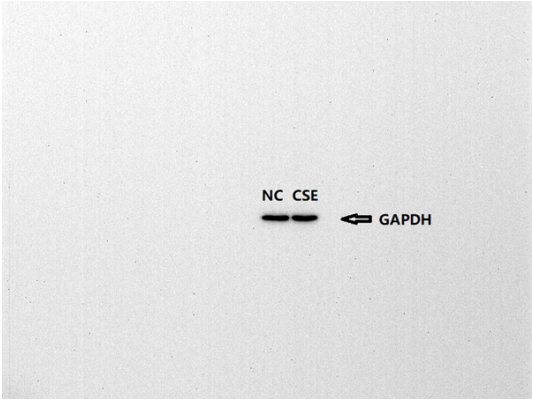

Figure S5. Overexpression plasmids of cystathionine γ-lyase (CSE) was transfected into HESCs. Whole membrane of GAPDH expression in western blot analysis.

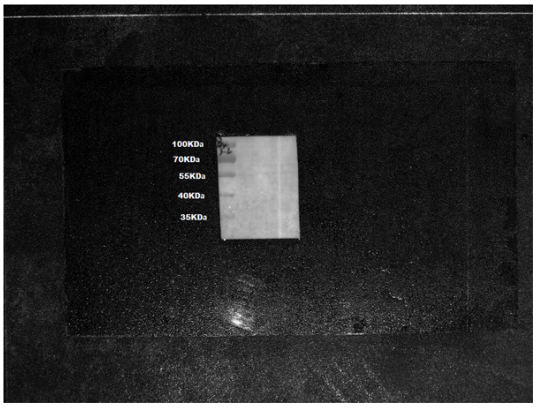

Figure S6. Overexpression plasmids of cystathionine γ-lyase (CSE) was transfected into HESCs. This picture shows the marker of the whole membrane.

Figure S7. HESCs were incubated with 300 μmol/l or 500 μmol/l NaHS, and co-conditioned with CAPE (10 μmol/l) or PDTC (10 μmol/l) for 24 h. Western blot analysis of the levels of NF-κB p65 phosphorylation.

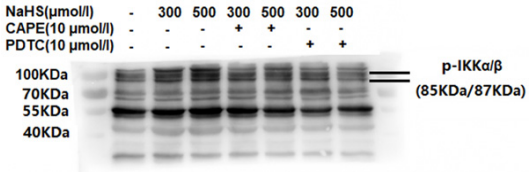

Figure S8. HESCs were incubated with 300 μmol/l or 500 μmol/l NaHS, and co-conditioned with CAPE (10 μmol/l) or PDTC (10 μmol/l) for 24 h. Western blot analysis of the levels of IKKα/β phosphorylation.

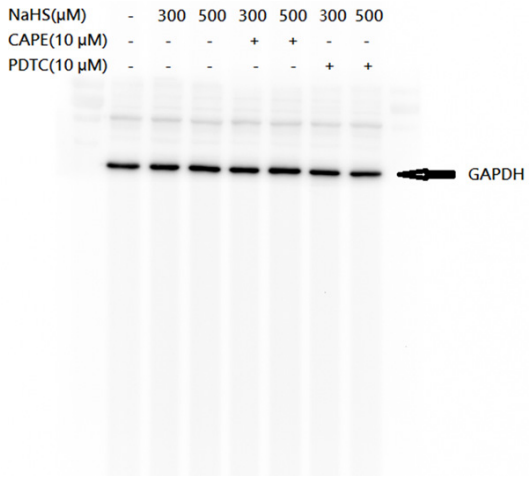

Figure S9. HESCs were incubated with 300 μmol/l or 500 μmol/l NaHS, and co-conditioned with CAPE (10 μmol/l) or PDTC (10 μmol/l) for 24 h. Western blot analysis of the levels of GAPDH.

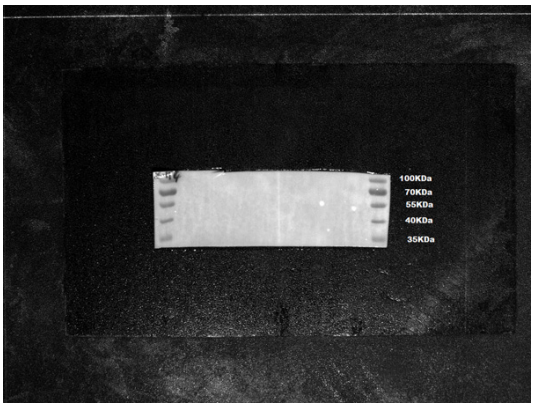

Figure S10. HESCs were incubated with 300 μmol/l or 500 μmol/l NaHS, and co-conditioned with CAPE (10 μmol/l) or PDTC (10 μmol/l) for 24 h. This picture shows the marker of the whole membrane.