Macrophages protect endometriotic cells against oxidative damage through a cross talk mechanism

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Running title:
Cross talk between endometriosis and macrophages.

Key words: Co-culture; Endometriosis; Heme oxygenase-1; Macrophages, Transforming growth factor-beta.

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Abstract

This aim of this study was to investigate whether macrophages protect endometriotic cells from oxidative injury and to elucidate the underlying mechanisms of any protection. Endometriotic cells cultured with or without differentiated macrophages (dTHP-1 cells) were treated with hydrogen peroxide (H_2O_2) or methemoglobin, a major component of hemoglobin species in endometriotic cyst fluid. Co-culture experiments, microarray analysis, screening and validation of differentially expressed genes (DEGs), cell proliferation and viability assays, and experiments using a specific inhibitor were conducted to investigate the functional cross-talk between endometriotic cells and macrophages. Microarray analysis revealed that endometriotic cells co-cultured with dTHP-1 differentially express several genes compared with monoculture. Quantitative Enzyme-linked immunosorbent assay (ELISA) and western blotting analysis identified TGF-β1 as a promising candidate gene expressed in endometriotic cells co-cultured with dTHP-1 cells. TGF-β1 stimulated the expression of heme oxygenase-1 (HO-1) in dTHP-1 cells. HO-1 expression was increased in dTHP-1 cells co-cultured with endometriotic cells compared with the dTHP-1 monoculture. Both H_2O_2 and methemoglobin upregulated the expression of the HO-1 protein in the dTHP-1 monoculture; moreover, co-culture with endometriotic cells further enhanced HO-1 production. The co-culture with dTHP-1 protected endometriotic cells against oxidative injury. Blockade of HO-1 abolished the protective effects of macrophages. In an oxidative stress environment, TGF-β1 produced by endometriotic cells may protect against oxidative injury through the upregulation of macrophage-derived HO-1. The cross-talk between endometriotic cells and macrophages may contribute to the progression and pathogenesis of endometriosis.

Key words: Co-culture; Endometriosis; Heme oxygenase-1; Macrophages, Transforming growth factor-beta.

Introduction

Endometriosis is a common, hormone-dependent gynecological disorder that leads to chronic inflammation and oxidative stress (1,2). Redox balance (i.e., the oxidant/antioxidant balance) due to abnormal hemorrhage is a hallmark event in the pathophysiology of endometriosis (1). Hemoglobin, heme, and free iron are released from damaged erythrocytes into the endometriotic cyst fluid (2). Hemoglobin and its related compounds are well known inducers of oxidative stress via autoxidation and the Fenton reaction (2). The superoxide anion (O_2^-) (3) and hydroxyl radical (·OH) (4), which are representative reactive oxygen species (ROS), are generated by autoxidation and the Fenton reaction, respectively. The accumulation of ROS can ultimately cause the tissue damage of endometriosis (3). Given that endometriosis can persist in an oxidative stress environment, endometriotic cells may have a mechanism to avoid oxidative stress-dependent cell damage.

The endometriosis microenvironment facilitates important cellular interactions between endometriotic cells and the recruited inflammatory cell populations. Substantial infiltration of inflammatory and immune cells is observed in endometriotic lesions (5). Macrophages are the major immune cell population in endometriotic lesions (5); they sense changes in their environment and exhibit plasticity and diverse phenotypes (6). Historically, macrophages have been classified as either M1 or M2 cells: the classical M1 macrophages promote tissue damage by secreting multiple cytokines for inflammatory response; whereas the alternative M2 macrophages exhibit cytoprotective properties by producing anti-inflammatory cytokines (7-9). M2 macrophages also aid the development of lesions by promoting neovascularization (10). In addition, activated macrophages are involved in multiple biological activities: the degradation of damaged erythrocytes, iron uptake and detoxification, and suppression of iron-induced oxidative stress (11). Macrophages are believed to be implicated in all stages of endometriosis development, including the initiation and progression of lesions (12,13). We previously reported that the stress-inducing protein heme oxygenase-1 (HO-1) is induced by various oxidative signals and is highly expressed in M2 macrophages in endometriotic lesions (8). However, the details of the mechanism by which macrophages protect endometrial cells against oxidative injury remains unclear.

This study aimed to investigate the potential role of macrophages in the protection of endometriotic cells from oxidative injury. Our co-culture experimental model provides the first evidence that cross-talk between endometriotic cells and macrophages may contribute to endometriotic cell proliferation and survival under conditions of oxidative stress. Understanding the mechanisms by which macrophages protect endometriotic cells against oxidative damage may provide new insight into potential treatments.

Materials and methods

Ethical statement.

The study was approved by the Institutional Review Board and the Research and Ethical Committee of

Nara Medical University Graduate School of Medicine, Kashihara, Japan (reference no. 1587). Written informed consent was obtained from each patient prior to the start of the study.

Cell culture of primary human ectopic endometrial stromal cells (hEESCs).

Endometriotic tissue specimens were collected from patients who underwent surgical resections for endometriosis at Nara Medical University Hospital between January 2015 and December 2018. The inclusion criteria were: 1) reproductive-age Japanese women; 2) patients undergoing surgery involving lesion removal for histological evaluation; and 3) patients with histologically confirmed ovarian endometriosis. The exclusion criteria were: 1) patients under 20 years of age; 2) patients who underwent hormonal therapy prior to surgical resection or for at least 3 months before surgery; and 3) patients with comorbidities, including other benign and malignant ovarian tumors. The cyst walls of ovarian endometrioma were collected from 14 women (average age, 32 years; range, 25–38 years) undergoing surgery for American Society for Reproductive Medicine (ASRM) stage III (n = 10) and stage IV (n = 4) endometriosis. Primary hEESCs were prepared in accordance with a previously published protocol (14). Briefly, the fresh tissue was minced, incubated with collagenase (3.15 mg/mL, Nordmark Arzneimittel, Utersen, Germany) at 37°C for 60 min, and filtered (15). After enzymatic digestion, the tissue residues were removed using a 70 μm pore size mesh nylon filter (BD Falcon, Franklin Lakes, NJ, USA). The flow-through fraction was collected and centrifuged. The cell suspension was then passed through a 40 μm nylon mesh filter (BD Falcon), centrifuged, and the pellet reconstituted. The cells were cultured in DMEM-F12 without phenol red supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic–antimycotic solution (Fujifilm, Tokyo, Japan), as previously described (16). The culture medium was changed every 2 days. The experiment was started when the cell confluence reached 80%. Fresh-unfrozen primary hEESC cultures up to the third passage were used for each experiment. The same stromal cell donor was used for each experiment. The purity and phenotypic characteristics of freshly isolated hEESCs were verified by immunocytochemistry (17). hEESCs grown on 8-well chamber slides (BD Biosciences, Franklin Lakes, NJ, USA) were fixed with 4% paraformaldehyde, blocked for endogenous peroxidase activity (0.75% H_2O_2 in methanol for 10 min at room temperature), and then immunostained with mouse monoclonal antibodies to vimentin (Clone V9, 1:1,000) (Dako, Hamburg, Germany) or cytokeratin-7 (CK7) (Clone OV-TL, 1:200) (Dako). Immunoreactivity was visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Dako). The immunocytochemical characterization showed that hEESCs ubiquitously expressed vimentin, but not CK7 (data not shown).

Culture of human immortalized endometriotic epithelial cells (hIEECs).

hIEEC were used because human eutopic or ectopic endometrial glandular epithelial cells are difficult to subculture. The cells were kindly provided by Professor Dr. Satoru Kyo (Department of Obstetrics and Gynecology, Shimane University Faculty of Medicine, Shimane, Japan). The hIEECs were established from

an ovarian endometrioma by Bono et al. and the characteristics of this cell line have been previously described (18). Primary human endometriotic epithelial cells were immortalized by transfection with human cyclin D1, cyclin-dependent kinase 4 (CDK4), and human telomerase reverse transcriptase (hTERT) genes to establish hIEECs (18). Subsequent passages from a single batch of immortalized cells were used in the experiment. In this study, hIEECs and hEESCs were separately cultured as endometriotic cells.

Human monocyte THP-1 cell culture and differentiation.

The THP-1 human monocyte cell line was purchased from the American Type Culture Collection and cultured using RPMI 1640 medium supplemented with non-heat-treated 10% fetal bovine serum (FBS; FujiFilm), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere with 5% CO_2. THP-1 monocytes (6×10^5 cells per insert) were plated on Transwell® inserts (6.5 mm inserter, 0.4 μm polycarbonate membrane, Corning, New York, USA) and differentiated into macrophages (dTHP-1 cells) by the addition of 10 ng/mL phorbol 12-myristate 13-acetate (PMA, Abcam, Cambridge, UK) for 48 h.

Co-culture experiments.

Endometriotic cells (hIEECs [4×10^5 cells per well in the lower chamber] or hEESCs [2×10^5 cells per well in the lower chamber]) were cultured in the bottom of a multiwell plate separated by a porous Transwell® membrane insert seeded with dTHP-1 cells (6×10^5 cells per insert). This design avoids any direct cell–cell contact while allowing the exchange of soluble factors produced by cells. We examined the effects of the ROS-generating agents hydrogen peroxide (H_2O_2, Fujifilm) and methemoglobin (Metallogenics, Chiba, Japan). Methemoglobin is a major component of hemoglobin species in endometriotic cyst fluid (3) and promotes the peroxidase reaction to generate H_2O_2 (19). Endometriotic cells co-cultured with (referred to as the co-culture) or without (referred to as the monoculture) dTHP-1 cells were treated with H_2O_2 (0–500 μM) or methemoglobin (0–150 μM) in a dose- and time-dependent manner. After the cells were treated with H_2O_2 or methemoglobin, the co-culture was stopped, the culture supernatant and cells were removed, and the collected materials were stored at −80°C until further analysis. Each experiment was performed in triplicate.

Screening and validation of differentially expressed genes (DEGs).

Tissue samples were collected from three separate patients (30 years, ASRM stage III; 32 years, stage IV; and 34 years, stage III). To identify the soluble factors excreted by endometriotic cells when co-cultured with macrophages after H_2O_2 challenge, hIEECs or hEESCs were monocultured or co-cultured with dTHP-1 in response to 50 μM H_2O_2 for 24 h. In total, two pairs of cell combinations, hIEECs monoculture treated with H_2O_2 and hIEECs and dTHP-1 co-culture treated with H_2O_2, were prepared for microarray analysis. Likewise, hEESCs obtained from the same patient were classified into two groups: hEESCs monoculture

treated with H_2O_2 and hEESCs and dTHP-1 co-culture treated with H_2O_2. To identify DEGs, we selected a human gene-expressing oligonucleotide microarray (Genopal™, Mitsubishi Chemical Holdings, Tokyo, Japan) that could specifically detect 215 human genes, including oxidative stress- and antioxidant-related genes. After background correction, quantile normalization, and probe summarization for variation, the gene expression matrix was obtained. For each significant DEG, a fold-change of >2.0 in gene expression (with a p-value < 0.05) was defined as differentially expressed between two conditions. The experiment was repeated three times.

Western blotting.

Protein expression was validated by immunoblotting. Whole cell lysate was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (BIO-RAD, California, USA). The membrane was blocked, probed with specific primary antibodies at 4°C, and then with peroxidase-conjugated secondary antibodies in accordance with the manufacturer's instructions. Antibodies against the following proteins were used: TGF-β1 (ab64715, 1:5000; Abcam Cambridge, UK), HO-1 (ab13248, 1:1000; Abcam), IL-6 (ab9324, 1:5000; Abcam), IL-10 (ab33471, 1:500; Abcam), and β-actin (ab8227, 1:5000; Abcam). The blot was also probed for β-actin as a loading control. Finally, protein bands were visualized with a detection reagent (GE Healthcare, Chicago, USA).

Enzyme-linked immunosorbent assay (ELISA).

We examined whether TGF-β1, IL-6, and IL-10 proteins were produced in endometriotic cells after H_2O_2 stimulation when co-cultured with macrophages. The concentrations of cytokines in the samples (the culture supernatants of hIEECs monoculture and co-culture; the culture supernatants of hEESCs monoculture and co-culture) were determined using sensitive ELISA kits. ELISAs for TGF-β, IL-6, and IL-10 were purchased from R&D Systems (Minneapolis, USA). Assays were performed in triplicate.

Regulation of heme oxgenase-1 (HO-1) expression in dTHP-1 by soluble factors secreted by endometriotic cells.

hIEECs (4×10^6 cells/well) or hEESCs (2×10^6 cells/well) were monocultured in a plate for 24 h, and each culture supernatant was collected and stored until use. To examine whether the culture supernatant promoted HO-1 expression in macrophages, dTHP-1 cells (6×10^5 cells/well) were cultured in medium with or without the culture supernatant of hIEECs or hEESCs. After culture for the indicated times, dTHP-1 cells were collected and the cell lysate was analyzed for HO-1 expression by western blotting.

Cell viability assay.

In monoculture systems, the cell viability was determined using the MTT assay (Roche, Basel, Switzerland), as described previously (20). Endometriotic cells (hIEECs [1×10^4 cells/well] or hEESCs [5×10^3

cells/well]) and dTHP-1 (1×10^4 cells/well) were seeded in 96-well plates and incubated in the presence or absence of H_2O_2 for the indicated time period. To quantify the adherent living cells, the supernatant was removed, and then 50 µL of 1 mg/mL working solution of MTT was added to each well and incubated at 37°C for 4 h. After incubation, the MTT solution was carefully aspirated. Then, 100 µL of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA) was added to each well to solubilize the formazan product and the absorbance at 470 nm was recorded using a spectrophotometer (Biotek Elx800 - Winooski, VT, USA).

IncuCyte ZOOM™ image capture and cell proliferation analyses.

The IncuCyte ZOOM™ Live-Cell Imaging system (Essen BioScience, Ann Arbor, MI, USA) was used for kinetic monitoring of cell proliferation and cytotoxicity, as described previously (21). IncuCyte image assays quantify how rapidly the proportion of the area covered by cells increases with time, which is a function of cell proliferation rate (21). In the co-culture system, 24-well Transwell plates were used; endometriotic cells (hIEECs [5×10^4 cells per well in the lower chamber] or hEESCs [1.5×10^4 cells per well in the lower chamber]) were seeded in the lower chamber and dTHP-1 cells (1×10^5 cells/well) were placed in the upper compartments. The co-culture system was placed inside the IncuCyte ZOOM™ at 37°C in a humidified incubator, and imaged for 3 days. The medium with or without H_2O_2 was changed daily. The percentage of cell proliferation was calculated by the IncuCyte ZOOM software (Essen BioScience). We confirmed that the cell viability measured by the MTT assay and IncuCyte ZOOM™ was almost the same.

Protection of endometriotic cells against oxidative injury by macrophage co-culture.

To determine whether H_2O_2 was cytotoxic to hIEECs and hEESCs, cell viability and growth were determined after exposure to H_2O_2 by the MTT assay and the IncuCyte ZOOM™ Live-Cell Imaging system, respectively. Next, the co-culture of endometriotic cells and dTHP-1 was treated with a selective HO-1 inhibitor, tin protoporphyrin IX (SnPP; 30 µM, Fujifilm) for up to 72 h to investigate the effect on endometriotic cell proliferation.

Statistical analysis

Statistical analyses were performed using SPSS Statistics version 25 (IBM Japan Inc., Japan). The experiments were repeated three times to ensure reproducibility. All experiments were conducted in triplicate for statistical analysis. Data were expressed as the mean ± SD. Some figures show examples of representative data. Significant differences for individual pairs of means were determined using Student's *t*-test. For datasets comparing more than three groups, one-way ANOVA was used followed by the Kruskal–Wallis test or post hoc Dunnett's multiple comparison test. A *P*-value of <0.05 was considered to indicate a statistically significant difference.

Results

1. DEGs in endometriotic cells co-cultured with macrophages in response to hydrogen peroxide.

We conducted a human gene expression oligonucleotide microarray analysis (Genopal^{R}) of endometrial cells in monoculture or dTHPl-1 co-culture in the presence of 50 μM of H_2O_2 for 24 h (**Figure 1A**). In total, 29 DEGs (absolute fold-change >2 or <0.5) were identified in hIEECs: 15 were upregulated (**Figure 1B**) and 14 were downregulated. In the parallel experiment using hEESCs, 12 upregulated genes (**Figure 1C**) and 11 downregulated genes were identified in the co-culture compared with the monoculture. Of the 15 and 12 genes upregulated in **Figures 1B** and **1C**, seven genes were identical, yielding a total of 20 DEGs. The PubMed database, a public repository, showed that of these 20 genes, the following 12 genes were involved in the pathophysiology of endometriosis or the activation and polarization of macrophages: IL-10 (interleukin-10) (22), TGFB1 (transforming growth factor-1 beta) (23), IL-6 (24,25), GSTO1 (glutathione S-transferase omega 1) (2), KIT (KIT proto-oncogene) (26), NGFR (nerve growth factor receptor) (27), and SERPINE1 (serpin family E member 1) (28) were overexpressed in endometriosis, whereas HSD11B1 (hydroxysteroid 11-beta dehydrogenase 1) (29), ALOX15B (arachidonate 15-lipoxygenase type B) (30), EDN1 (endothelin 1) (31), ICAM1 (intercellular adhesion molecule 1) (32), and AREG (amphiregulin) were mainly activated in macrophages (33). Given that inflammation and oxidative stress participate in the pathogenesis of endometriosis, three of the 12 candidate genes, TGF-β (34), IL-6 (35), and IL-10 (36), were further selected to validate their possible role. The remaining nine genes need to be verified by future experiments.

2. TGF-β1 protein is upregulated in endometriotic cells co-cultured with macrophages.

The results of microarray analysis were validated by quantitative ELISA and western blotting. Different cells and their supernatants were utilized for the validation approach. Human endometriotic cells (hIEECs or hEESCs) were cultured alone or in combination with dTHP-1 in the presence or absence of H_2O_2 using a Transwell cell contact-independent cell culture system. First, the effect of 100 μM H_2O_2 on TGF-β1 expression in endometriotic cell monocultures, dTHP-1 monocultures, and endometriotic cells co-cultured with dTHP-1 was examined by ELISA and western blotting. The culture supernatants and cell lysates were analyzed for TGF-β1 expression by ELISA (**Figure 2A**) and western blotting (**Figure 2B**), respectively. The exposure of hIEECs or hEESCs to H_2O_2 did not increase the concentrations of TGF-β1 protein in the culture supernatant (**Figure 2A**, **lane 1 vs. lane 2** and **lane 5 vs. lane 6**) and cell lysates (**Figure 2B**, **lane 1 vs. lane 2** and **lane 5 vs. lane 6**). In addition, H_2O_2 treatment failed to stimulate TGF-β1 protein release from dTHP-1 cells (**Figure 2A**, **lane 9 vs. lane 10**). Co-culture with dTHP-1 resulted in a significant increase in the secretion of TGF-β1 in hIEECs (**Figure 2A**, **lane 3 vs. lane 1**). Similar to hIEECs, co-culture enhanced TGF-β1 secretion from hEESCs compared with hEESCs monoculture (**Figure 2A**, **lane 7 vs. lane 5**). Furthermore, TGF-β1 expression in cell lysates was significantly upregulated in hIEECs co-culture with dTHP-1 compared with monoculture conditions (**Figure 2B**, **lane 3 vs. lane 1**), whereas TGF-β1

expression tended to be increased in the cell lysates of hEESCs co-cultured with dTHP-1, but the difference was not significant (**Figure 2B, lane 7 vs. lane 5**). The experiments were repeated three times with the same results. The properties of TGF-β1 production and secretion differed between the two cell types, but substantial extracellular secretion of TGF-β1 occurred in endometriotic cells co-cultured with macrophages. Second, IL-6 and IL-10 concentrations in supernatants and cell lysates were determined in both the monocultures and co-cultures of endometriotic cells and macrophages treated with H_2O_2. After exposure to H_2O_2 for 24 h, the culture supernatants were collected and IL-6 and IL-10 were quantified using commercially available ELISA kits. Neither monoculture and nor co-culture conditions in the presence or absence of H_2O_2 increased the concentrations of IL-6 and IL-10 in the culture supernatants (data not shown). As the concentrations of IL-6 and IL-10 were below the detection limit in all supernatant samples, it was unclear whether H_2O_2 addition or co-culture increased the secretion of IL-6 and IL-10 in endometriotic cells. Therefore, we then determined the IL-6 and IL-10 concentrations in the cell lysates using western blotting analysis. The IL-6 concentration in cell lysates was higher in hIEECs than in hEESCs (**Supplemental Figure 1, lane 1 vs. lane 5**). When co-cultured with macrophages, the IL-6 concentration in hIEECs cell lysates was increased significantly (**Supplemental Figure 1, lane 3 vs. lane 1**), but of H_2O_2 addition was not observed to have an effect (**Supplemental Figure 1, lane 4 vs. lane 3**). IL-6 concentration in cell lysates also increased in hEESCs co-cultured with macrophages (**Supplemental Figure 1, lane 7 vs. lane 5**). Similar to hIEECs, the addition of H_2O_2 did not increase the IL-6 concentration in the hEESCs cell lysates. In contrast, the effect of co-culture on IL-10 production was limited. The IL-10 concentration in cell lysates increased in hIEECs co-cultured with macrophages, but not in hEESCs co-cultured with macrophages (**Supplemental Figure 2**). The IL-10 production in hEESCs monoculture and co-culture was negligible. Furthermore, H_2O_2 challenge failed to induce IL-10 expression in endometriotic cell monoculture or the co-culture with dTHP-1 cells. Among the three candidate proteins, the production and secretion of TGF-β1 from endometriotic cells were enhanced by co-culture with macrophages. Collectively, the results show that endometriotic cell-derived TGF-β1 may be a unique soluble factor for molecular communication between endometriotic cells and macrophages.

3. Macrophages co-cultured with endometriotic cells stimulate HO-1 expression in response to oxidative stress.

We focused on the HO-1 protein, which provides protection against oxidative injury. Based on cell type and culture conditions, we investigated which type of cells produced HO-1 protein in an oxidative stress environment and whether HO-1 expression was enhanced in the co-culture compared with monoculture. We initially examined the effect of macrophage co-culture on HO-1 expression by endometriotic cells (**Figure 3A, lanes 1–8**). The HO-1 expression levels were quantified in the endometriotic cell lysates using western blotting analysis. Co-culture with dTHP-1 did not increase the expression of the HO-1 protein in hIEECs (**Figure 3A, lane 2 vs. lane 1**) or hEESCs (**Figure 3A, lane 6 vs. lane 5**). Further, H_2O_2 (100 μM)

did not induce the expression of the HO-1 protein in endometriotic cells in either the monoculture (hIEECs [**Figure 3A, lane 3 vs. lane 1**] and hEESCs [**Figure 3A, lane 7 vs. lane 5**]) or co-culture conditions (hIEECs [**Figure 3A, lane 4 vs. lane 2**] and hEESCs [**Figure 3A, lane 8 vs. lane 6**]).

Next, we examined whether the HO-1 protein was produced by macrophages co-cultured with endometriotic cells in response to H_2O_2 (100 μM) (**Figure 3A, lanes 9–14**). The expression of the HO-1 protein was significantly upregulated in dTHP-1 cells co-cultured with endometriotic cells, hIEECs (**Figure 3A, lane 10 vs. lane 9**), or hEESCs (**Figure 3A, lane 11 vs. lane 9**). There was also a significant increase in HO-1 protein expression in dTHP-1 monoculture (**Figure 3A, lane 12 vs. lane 9**) after H_2O_2 treatment. HO-1 expression levels in dTHP-1 cells co-cultured with endometriotic cells were increased ~2.0-fold (**Figure 3A, lane 14 vs. lane 12**) and 1.5-fold (**Figure 3A, lane 13 vs. lane 12**), respectively, after a 24-hour H_2O_2 challenge as compared with monoculture conditions. Therefore, HO-1 is produced by macrophages co-cultured with endometriotic cells in response to H_2O_2.

In basic research, H_2O_2 is often used to induce oxidative stress. Methemoglobin is a superoxide anion-generating hemoglobin species that is abundant in endometriotic cyst fluids (3). In the next experiment, methemoglobin was used instead of H_2O_2. We investigated HO-1 protein expression in macrophages co-cultured with endometriotic cells after methemoglobin challenge. The co-culture of dTHP-1 with endometriotic cells (hIEECs [**Figure 3B, lane 2 vs. lane 1**] and hEESCs [**Figure 3B, lane 3 vs. lane 1**]) enhanced HO-1 production, as compared with dTHP-1 monoculture. Methemoglobin exposure significantly induced HO-1 protein expression in dTHP-1 monoculture (**Figure 3B, lane 4 vs. lane 1**). In addition, methemoglobin challenge markedly induced HO-1 protein expression in dTHP-1 cells co-cultured with hIEECs (**Figure 3B, lane 5 vs. lane 2**) and hEESCs (**Figure 3B, lane 6 vs. lane 3**). Collectively, these results show that H_2O_2 or methemoglobin challenge stimulated HO-1 protein expression in macrophages co-cultured with endometriotic cells.

4. Endometriotic cell-derived TGF-β1 promotes macrophage-dependent HO-1 protein expression.

We investigated whether supernatants collected from monocultures of endometriotic cells (hIEECs and hEESCs) induced HO-1 production in dTHP-1 cells. The concentration of the HO-1 protein in the cell lysate of dTHP-1 cells treated with the culture supernatant of hIEECs (**Figure 4A, lane 2 vs. lane 1**) and hEESCs (**Figure 4A, lane 3 vs. lane 1**) was 2.1-fold and 1.6-fold higher, respectively, than that of the untreated cells. We subsequently investigated whether recombinant TGF-β1 stimulated the production of HO-1 protein in dTHP-1 monoculture. dTHP-1 monoculture was challenged with different TGF-β1 concentrations (10, 50, and 100 nM) and different exposure times (12 and 24 h), and the concentrations of HO-1 protein were quantified by western blotting. HO-1 production in macrophages was induced after exposure to TGF-β1 ≥10 nM for 24 h (**Figure 4B**). The cytotoxic effects of TGF-β1 were determined using the trypan blue exclusion assay. No cytotoxic effects were observed after exposure to 100 nM TGF-β1 for 24 h (data not shown).

5. Macrophages protect endometriotic cells against oxidative injury.

The cell growth kinetics was monitored using the IncuCyte ZOOM™ time-lapse microscopy system. Endometriotic cells were treated with H_2O_2 (0–500 μM) for up to 72 h to induce oxidative damage. In parallel experiments, cell viability was also determined via a conventional MTT assay. Thus, cell proliferation pattern was simultaneously monitored by an IncuCyte-based analysis system and MTT assay. H_2O_2 inhibited the growth of hIEECs and hEESCs in a time- and dose-dependent manner (**Supplemental Figure 3**). Both analysis methods (IncuCyte and MTT) returned almost the same results.

First, we investigated the effects of H_2O_2 exposure on endometrial cell proliferation. To assess the effect of H_2O_2 exposure on hIEEC and hEESC proliferation, these cells were treated with varying concentrations of H_2O_2 (0–500 μM) for up to 72 h. H_2O_2 suppressed the proliferation of hIEECs (**Supplemental Figure 4A**) and hEESCs (**Supplemental Figure 4B**) in a dose- and time-dependent manner. Endometriotic stromal cells may be more resistant to H_2O_2 than endometriotic epithelial cells.

Next, we investigated whether co-culture with macrophages conferred protective effects against H_2O_2-induced cytotoxicity in endometriotic cells. As shown in **Figure 5**, real-time image analysis of endometriotic cell monoculture demonstrated that H_2O_2 (100 μM for hIEECs (**A**) and 200 μM for hEESCs (**B**)) inhibited cell growth and reduced cell confluence in a time-dependent manner. Co-culture with dTHP-1 had a significant protective role against the H_2O_2-induced inhibition of endometriotic cell growth.

Finally, we investigated whether HO-1 inhibition abolished the protective effect of macrophages on the H_2O_2-induced inhibition of endometriotic cell proliferation. SnPP is a HO-1 small-molecule inhibitor of tin protoporphyrin IX. In **Supplemental Figure 5**, it is shown that exposure of hIEECs to 30 μM SnPP for 72 h did not affect cell proliferation. To assess the protective effect of HO-1, hIEECs and hEESCs co-cultured with dTHP-1 were treated with SnPP (30 μM) for up to 72 h. HO-1 blockade completely negated the protective effects of macrophages on hIEECs (**Figure 6A**) and hEESCs (**Figure 6B**). Therefore, macrophages may protect endometriotic cells against oxidative injury through the upregulation of HO-1 expression.

Discussion

This study was conducted to identify the soluble factors derived from endometriotic cells and macrophages that can protect endometriotic cells against oxidative injury. To explore the molecular evidence supporting the presence of cross-talk between endometriotic cells and macrophages, we performed monoculture and co-culture experiments, screening and validation of DEGs, cell growth experiments using IncuCyte ZOOM™ images and the MTT assay, and experiments using a specific HO-1 inhibitor. Our major findings include: 1) high-resolution microarray analysis identified that several genes, including TGF-β1, are potential protective genes upregulated in endometriotic cells when co-cultured with macrophages; 2) culture supernatants collected from endometriotic cells or recombinant TGF-β1 stimulated the expression

of the HO-1 protein in macrophage monoculture; 3) HO-1 protein expression was induced in macrophages co-cultured with endometriotic cells or in response to H_2O_2 or methemoglobin (a hemoglobin species actually contained in endometriosis cyst fluid); 4) macrophages exerted a beneficial effect on the protection of endometriotic cells against oxidative injury via HO-1 overexpression; and 5) blockade of HO-1 abolished the protective effects of macrophages. Cross-talk between endometriotic cells and macrophages contributes to the protection of endometriotic cells from oxidative damage through the production of TGF-β1 and HO-1, respectively (**Figure 7**). Macrophages may play a beneficial role in the progression of endometriosis in an oxidative stress microenvironment.

It is known that macrophage recruitment, activation, differentiation, and polarization within endometriotic lesions play a role in regulating the growth, development, progression, angiogenesis, and innervation of endometriosis (37). However, the dynamic cross-talk between endometriotic cells and macrophages in an oxidative stress environment has not yet been clarified. In this study, 29 and 23 genes were upregulated in hIEECs and hEESCs, respectively, after endometriotic cells co-cultured with macrophages were exposed to H_2O_2 (**Figure 1B**, **1C**). Among the DEGs, we focused on 12 genes with expression profiles published in a public repository. Previous studies have shown that TGF-β induces the differentiation of T helper cells and upregulates the expression of IL-10 in endometriosis (38); IL-10 promotes the growth of endometrial lesions via the suppression of excessive immune and pro-inflammatory responses (22,39); and natural killer cells play an essential role in eliminating aberrant cells. However, after co-culture with macrophages, these cells suppress cytotoxic function in endometriotic cells by stimulating the secretion of the anti-inflammatory cytokines TGF-β and IL-10 (40). Our study revealed that endometriotic cells were when co-cultured with macrophages, TGF-β1 was secreted into the culture medium by the endometriotic cells (**Figure 2A**) and was significantly overexpressed, especially in epithelial cells (**Figure 2B**). However, the expression of the IL-6 (**Supplemental Figure 1**) and IL-10 (**Supplemental Figure 2**) proteins in endometriotic cell lysates was clearly detected by the western blotting analysis, although their concentrations in the culture medium were below the detection limit of the ELISA. Therefore, among the candidate genes, TGF-β1 was selected for further study.

In addition, the study identified several genes that may contribute to cross-talk between endometriotic cells and macrophages, including HSD11B1 (41), IL-6 (25), ALOX15B (30), EDN1 (31), and GSTO1 (42) (**Figure 1**). HSD11B1 is a key enzyme in steroid hormone metabolism. IL-6 and ALOX15B are related to inflammation. EDN1 is involved in endothelial mitochondrial oxidative damage. GSTO1 is implicated in the regulation of oxidative stress and inflammation. These genes are strongly expressed in macrophages and may be related to the high functional connectivity between oxidative stress and antioxidants. Further molecular studies are needed to validate the novel candidate genes involved in the endometriosis and macrophage cross-talk and to understand their roles in endometriosis development.

In addition, we found that HO-1 expression was induced in macrophages co-cultured with endometriotic cells, and that this induction was further enhanced in an oxidative stress environment (**Figure**

3). The production of HO-1 by macrophages is independent of direct cell contact and is therefore due to soluble factors secreted by endometriotic cells. The addition of culture supernatant from endometriotic cells to macrophage monoculture induced the expression of HO-1 protein in cell lysates (**Figure 4A**). Recombinant TGF-β1 significantly increased HO-1 production in macrophages (**Figure 4B**), suggesting that TGF-β1 secreted from endometriotic cells may contribute to HO-1 production in macrophages. To date, no study has shown that H_2O_2 stimulation induces TGF-β1 expression in endometriotic cells. To verify the possible link between HO-1 expression and oxidative stress, we explored the impact of H_2O_2 and methemoglobin on HO-1 expression in the target cells. This is the first experiment using methemoglobin as an oxidative stress inducer (43). Methemoglobin is a pathological form of oxidized hemoglobin and is accumulated in endometriotic cyst fluid due to the repeated episodes of hemorrhage that occur in endometriosis (3,43). Methemoglobin in endometriotic cysts causes distortion in the homeostatic balance between oxidants and antioxidants, known as a redox imbalance, and leads to the production of excess ROS (2,43,44). As expected, the HO-1 protein was produced by macrophages in response to methemoglobin or H_2O_2, but not by endometrial cells (**Figures 3A** and **3B**). The production of the HO-1 protein in macrophages was dependent on both TGF-β1 secretion by endometrial cells and methemoglobin. HO-1 is known to be regulated by several cytokines, including TGF-β (34), IL-10 (36), and IL-6 (35). Therefore, the TGF-β1 and HO-1 signaling pathways can be considered the key players in the complex microenvironment of endometriosis (**Figure 7**).

Third, we showed that macrophages protect endometriotic cells against oxidative injury through the upregulation of HO-1 expression and promote cell survival. An increase in ROS induces oxidative stress and may contribute to endometriotic cell death (2,3,44,45). As shown in **Supplemental Figure 4,** hEESCs may be more resistant to H_2O_2 than hIEECs, although the underlying mechanism remains to be elucidated. Here we have reported the cytoprotective effect of macrophage-derived HO-1 against H_2O_2-induced endometriotic cell damage (**Figure 5**). HO-1 blockade completely abolished the protective effects of macrophages (**Figure 6**), suggesting that HO-1-dependent cross-talk plays a major role in the ectopic milieu. HO-1 has been reported to protect a variety of cells from ROS-induced oxidative damage and inflammation (45-47). For example, HO-1 is beneficial for cancer cell growth by overcoming increased oxidative stress (48). Both macrophage recruitment and interaction with endometriotic cells have been considered to play a pivotal role in the initiation and progression of endometriosis through the overexpression of TGF-β1 and HO-1 proteins.

Finally, in response to environmental stimuli, macrophages display opposing phenotypes: the classically activated, pro-inflammatory phenotype 1 (M1) and the alternatively activated, anti-inflammatory phenotype 2 (M2) (49). M2 macrophages, with high plasticity and the ability to adapt to environmental changes, may be a key regulator of the resolution of inflammation, tissue repair and fibrogenesis after injury, tissue homeostasis, and the growth of endometriotic lesions (49). Biological factors, including TGF-β, IL-4, IL-10, and IL-13, can promote the polarization of M2 macrophages (50).

Increased accumulation of M2 macrophages expressing HO-1 has been described in endometriotic cysts (8). Our results showing that methemoglobin-induced the expression of the HO-1 protein in macrophages suggest that the polarity was converted to the M2 phenotype. These findings support the hypothesis that methemoglobin converts macrophage polarity to the M2 phenotype and protects endometrial cells against oxidative injury through HO-1 expression. There are at least two distinct phases of protection against oxidative injury in endometriosis: the initial wave of HO-1 expression in macrophages by methemoglobin, followed by the second wave of HO-1 induction through the upregulation of TGF-β1 expression in endometriotic cells (**Figure 7**).

The limitations of this study are as follows: Recruited macrophages or peripheral blood mononuclear cells play an important role in mediating the inflammation, angiogenesis, anti-apoptosis, and fibrogenesis in patients with endometriosis (51). Many factors contribute to the dynamic cross-talk between endometriosis and macrophages. For example, peritoneal macrophages and immune cells have been reported to possess a very limited capacity for the elimination of endometriotic cells; hence, allowing endometriotic cells are allowed to survive and grow (51). Understanding the function of endometriosis-associated macrophages will help elucidate their pathogenesis and design new therapeutic strategies. Therefore, this limitation stems from the lack of consideration of factors other than oxidative stress. Second, the underlying molecular mechanism through which HO-1 is induced in macrophages has not been elucidated. It is well known that hydrogen peroxide modulates the activity of nuclear factor-erythroid 2-related factor 2 (Nrf2) (52) and that the Nrf2 signaling pathway regulates the expression of several antioxidant enzymes, including HO-1, catalase, superoxide dismutase (SOD), gamma-glutamyl cysteine synthetase (γ-GCS), and NAD(P)H:quinone oxidoreductase 1 (NQO1) (53,54). Further research is needed to investigate whether Nrf2 is involved in this cross-talk. Furthermore, we used immortalized endometriotic epithelial cells (18) instead of normal ectopic epithelial cells. As these cells have acquired replicative immortality, they retain responsiveness to sex steroids, but may exhibit different properties to normal cells. The final limitation was the lack of animal experiments to quantify endometriosis macrophage cross-talk.

Clinical implications and future directions

The pathogenesis of endometriosis remains incompletely understood, but genetic, hormonal, inflammatory, immunological, environmental, and oxidative stress factors are thought to be involved (5). The accumulated evidence demonstrates that oxidative stress is currently a major focus of, and a challenge to, basic and clinical scientific research (1,2). Oxidative stress promotes both tissue damage and repair (2). An increase in ROS in the peritoneal microenvironment alters some genes associated with the maintenance of cell proliferation, invasion, and survival, thus promoting disease progression (51). Indeed, urokinase-plasminogen activator (uPA) and matrix metalloproteinases (MMPs) overexpressed by oxidative stress can promote endometriotic cell invasion through the activation of the nuclear factor-kappaB (NF-kB) and mitogen-activated protein kinases signaling pathways (55). In addition, oxidative stress may accelerate the

development of endometriosis by enhancing the production of various pro-inflammatory chemokines (e.g., monocyte chemoattractant protein-1 [MCP-1]) from macrophages in the peritoneal cavity (56). In contrast, macrophage activation triggers greater ROS generation, which participates in oxidative stress. Therefore, oxidative stress and macrophage activation can create a "vicious cycle" in the development of endometriosis. As macrophages are a key mediator of cellular cross-talk in the endometriosis microenvironment with diverse supportive functions, targeting macrophages is an important therapeutic strategy. This study has provided the first demonstration that macrophages activated in response to oxidative stress contribute to the survival of endometriotic cells through the TGF-β–HO-1 pathway. The inhibition of HO-1 production from macrophages leads to the suppression of endometrial cell survival. Once these signaling molecules involved in HO-1 production have been elucidated, potential inhibitor candidates with an innovative mechanism of action can be developed to prevent dynamic cross-talk between endometriosis and macrophages. The study of oxidative stress is therefore critical in supporting therapeutic strategies for endometriosis.

In conclusion, dynamic cross-talk between endometriotic cells and macrophages may affect the progression of endometriosis through the upregulation of TGF-β1 and HO-1 expression. Macrophage-derived HO-1 protects endometriotic cells from oxidative injury. HO-1 may be a therapeutic target for endometriosis.

Acknowledgment:
We thank Mrs. Toyomi Kobayashi for creating the figure.

Patient consent for publication

Written informed consent was obtained from each patient. Human rights statements and informed consent: All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and its later amendments.

Disclosures

Conflict of interest: The authors declare no conflict of interest. Human and Animal Rights: The study was conducted under the guidelines that had been approved by the medical ethics committee of the Nara Medical University (reference no. 1587). This article does not contain any animal studies that have been performed by any of the authors.

Declaration

The research comprises several basic and clinical study designs. This manuscript conforms to the

EQUATOR network guidelines. Our study meets checklists of the STROBE Statement (http://www.strobe-statement.org/).

Funding Sources

This work was supported by Japan Society for Promotion of Science Japan (grant nos. 20K09604, 20K09647 and 20K09648).

Author's contribution

Hiroshi Kobayashi contributed to the study conception and design. Kenji Ogawa was involved in all the experiments in this study. Naoki Kawahara and Tingting Liu helped with cell culture experiments and western blotting. The data analysis was performed by Kenji Ogawa and Naoki Kawahara. The first draft of the manuscript was written by Hiroshi Kobayashi. The final version of the manuscript has been read and approved by all authors.

Availability of data and material

The datasets generated during the current study are available from Hiroshi Kobayashi.

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Figure 1. Differentially expressed genes (DEGs) in endometriotic cells co-cultured with macrophages (dTHP-1) in response to H_2O_2.

A, hEESCs were isolated from patient-derived endometriotic tissue samples. The upper and lower chambers were first cultured separately; the co-culture experiment was started when each cell layer reached 80% confluence. hIEECs and hEESCs (lower chamber) were separately cultured for 24 h (monoculture) or co-cultured with dTHP-1 (upper chamber) in the presence of hydrogen peroxide (H_2O_2) in a two-chamber co-culture system without cell–cell contact. H_2O_2 was added to the upper chamber.

B, hIEECs co-cultured with dTHP-1 cells vs. hIEECs monoculture (Venn diagram). Total RNA was used for microarray expression analysis (Genopal^R), and differential gene expression was assessed based on cell type (hIEECs vs. hEESCs) and culture condition (monoculture vs. co-culture) in response to H_2O_2. In total, 29 transcripts were identified to be differentially expressed between the two groups (hIEECs co-culture with dTHP-1 vs. hIEECs monoculture), 15 of which were upregulated (>2-fold) and 14 were downregulated (<0.5-fold). The 15 genes specifically upregulated in hIEECs when co-cultured with dTHP-1 are listed on the left of the Venn diagram in **Figure 1B**. hEESCs co-culture with dTHP-1 vs. hEESCs monoculture (Venn diagram). In total, 23 transcripts were identified to be differentially expressed between the two groups (hEESCs co-cultured with dTHP-1 vs. hEESCs monoculture); 12 were upregulated and 11 were

downregulated. The 12 overexpressed genes are listed on the left of the Venn diagram in **Figure 1C**. Three genes shown in bold letters, TGF-β, IL-6, and IL-10, were selected for further experiments.

Figure 2. Endometriotic cells co-cultured with macrophages have increased expression of TGF-β1 protein.

hIEEC, hEESC, and dTHP-1 are indicated by blue, green, and black bars, respectively. In the monoculture system, the color of the bars represents the cells used in the experiment; in the co-culture system, the color of the bars represents the cells cultured in the lower chamber. When co-cultured with dTHP-1 cells, "+dTHP-1" is displayed on the bar.

A, TGF-β1 expression in the culture supernatants of the monoculture and co-culture conditions in the presence or absence of H_2O_2 was measured using ELISA. Data represent the mean ± SD. a vs. b, $p < 0.05$. **B**, The expression of the TGF-β1 protein in the cell lysates of the monoculture and co-culture conditions in the presence or absence of H_2O_2 was detected by western blotting analysis. The fold-change in band intensity of TGF-β1 was normalized to β-actin expression. Fold-change vs. lane 5 was calculated. a vs. b, $p < 0.01$; a vs. c, $p < 0.05$; b vs. c, $p < 0.01$; and b vs. d, $p < 0.01$. Western blotting results are representative of three independent experiments.

Figure 3. HO-1 protein is upregulated in macrophages co-cultured with endometriotic cells under oxidative stress challenge.

We examined the effects of monoculture and co-culture on HO-1 expression in endometriotic cells and macrophages in response to H_2O_2 or methemoglobin. Cultured cells were challenged by H_2O_2 (**A**, 100 μM) or methemoglobin (**B**, 50 μM), respectively, for 24 h in monoculture or co-culture conditions. The expression levels of HO-1 protein were assessed based on cell type (hIEECs vs. hEESCs vs. dTHP-1) and culture condition (monoculture vs. co-culture). The "+" in the bar indicates "+dTHP-1." HO-1 protein levels in hIEECs (**A**, **lanes 1–4**), hEESCs (**A**, **lanes 5–8**), and dTHP-1 (**A**, **lanes 9–14** and **B**, **lanes 1–6**) were quantified by western blotting. The results were normalized to the expression of β-actin. Figure 3A: a vs. b, $p < 0.05$; a vs. c, $p < 0.05$; a vs. d, $p < 0.05$; a vs. e, $p < 0.01$; b vs. c, $p < 0.05$; b vs. d, $p < 0.05$; b vs. e, $p < 0.01$; c vs. d, $p < 0.05$; c vs. e, $p < 0.05$; and d vs. e, $p < 0.01$. Figure 3B: a vs. b, $p < 0.05$; a vs. c, $p < 0.05$; a vs. d, $p < 0.01$; b vs. c, $p < 0.05$; b vs. d, $p < 0.01$; and c vs. d, $p < 0.05$.

Figure 4. Addition of endometriotic cell culture supernatants (A) or recombinant TGF-β1 (B) stimulates the expression of HO-1 protein in macrophage monoculture.

A, The addition of culture supernatants of endometriotic cells increased HO-1 production in macrophages. We investigate whether cultured supernatant (CS) collected from hIEECs or hEESCs induced HO-1 expression in dTHP-1 cell lysates. The relative HO-1 expression was determined by western blotting, with β-actin used as the loading control.

B, Recombinant TGF-β1 stimulates the expression of HO-1 protein in macrophage monoculture.
TGF-β1 induced HO-1 protein expression in a time- and dose-dependent manner.
Figure 4A: a vs. b, $p < 0.01$; a vs. c, $p < 0.05$; and b vs. c, $p < 0.05$. Figure 4B: a vs. b, $p < 0.05$.

Figure 5. Macrophage co-culture rescues H_2O_2-induced inhibition of endometriotic cell proliferation.
Cell proliferation was measured by the IncuCyte ZOOM™ real-time imaging system. Treatment of endometriotic cells with H_2O_2 (100 μM for hIEECs (**A**) and 200 μM for hEESCs (**B**)) for up to 72 h resulted in a time-dependent decrease in cell proliferation. Visualization of individual growth curves for the data from the IncuCyte assay: x-axis, elapsed time in h; and y-axis, the cell confluence at the start of cell culture (indicated as 1.0).
A, Treatments are: hIEECs monoculture (blue line), hIEECs monoculture + 100 μM H_2O_2 (orange line), and hIEECs co-cultured with dTHP-1 + 100 μM H_2O_2 (gray line).
B, Treatments are: hEESCs monoculture (blue line), hEESCs monoculture + 200 μM H_2O_2 (orange line), and hEESCs co-cultured with dTHP-1 + 200 μM H_2O_2 (gray line).
a vs. b, $p < 0.05$; a vs. c, $p < 0.01$; and b vs. c, $p < 0.05$.

Figure 6. HO-1 inhibition abolishes the protective effects of macrophages on H_2O_2-induced oxidative injury of endometriotic cells.
hIEECs (**A**) or hEESCs (**B**) were monocultured or co-cultured with dTHP-1 and exposed to H_2O_2 (100 μM for hIEECs and 200 μM for hIEECs) in the presence or absence of the HO-1 inhibitor, SnPP (30 μM), for up to 72 h. Representative images from three similar experiments are shown using the IncuCyte imaging system. a vs. b, $p < 0.01$; a vs. c, $p < 0.05$; and b vs. c, $p < 0.05$.
A, hIEECs; yellow line, H_2O_2 0 μM (control); gray line, H_2O_2 100 μM; orange line, H_2O_2 100 μM + dTHP-1 co-culture; and blue line, H_2O_2 100 μM + SnPP 30 μM + dTHP-1 co-culture.
B, hEESCs; yellow line, H_2O_2 0 μM (control); gray line, H_2O_2 200 μM; orange line, H_2O_2 200 μM + dTHP-1 co-culture; and blue line, H_2O_2 200 μM + SnPP 30 μM + dTHP-1 co-culture.

Figure 7. Dynamic cross-talk between endometriotic cells (producing TGF-β1) and macrophages (producing HO-1).
Macrophages may protect endometrial cells from methemoglobin-induced oxidative injury through a paracrine mechanism. Methemoglobin, a main component of hemoglobin in endometriotic cyst fluids, is known as an inducer of oxidative stress (e.g., superoxide anion). This figure illustrates that (i) TGF-β1 is secreted by endometriotic cells when co-cultured with macrophages; (ii) TGF-β1 stimulates HO-1 production in macrophages; (iii) HO-1 is upregulated in macrophages co-cultured with endometriotic cells in response to oxidative stress; and (iv) macrophage-derived HO-1 protects endometriotic cells from oxidative injury.

Supplemental figures 1 and 2.

Western blotting was performed with the indicated antibodies (IL-6 [**Supplemental Figure 1**] and IL-10 [**Supplemental Figure 2**]) using whole cell lysate from hIEECs and hEESCs monoculture or those co-cultured with dTHP-1 cells. A relative fold-change in band intensity of target proteins was normalized to the level of β-actin. Fold-change vs. lane 5 was calculated. Different letters (a, b, c, and d) represent statistical differences ($p < 0.05$).

Supplemental Figure 3. Comparison of IncuCyte-based analysis system and the MTT assay for the assessment of endometriotic cell growth kinetics.

A, hIEECs; and **B**, hEESCs.

The H_2O_2 (50 and 100 μM)-induced growth inhibition was simultaneously determined by IncuCyte-based and MTT assays. Cell viability was measured by MTT assay every 24 h and by IncuCyte every 3 h. Different letters (a and b) represent statistical differences ($p < 0.05$).

Supplemental Figure 4. Effect of hydrogen peroxide on endometriotic cell proliferation

Visualization of individual growth curves for the data in the IncuCyte assay: x-axis, elapsed time in hours; and y-axis, the cell confluence at the start of cell culture is indicated as 1.0.

A, hIEECs; yellow line, H_2O_2 0 μM; gray line, H_2O_2 50 μM; orange line, H_2O_2 100 μM; and blue line, H_2O_2 200 μM.

B, hEESCs; yellow line, H_2O_2 0 μM; gray line, H_2O_2 100 μM; orange line, H_2O_2 200 μM; and blue line, H_2O_2 500 μM.

Representative imaging from three similar experiments is shown. Different letters (a, b, and c) represent statistical differences ($p < 0.05$).

Supplemental Figure 5. Toxic effects of SnPP on endometriotic cells.

The IncuCyte imaging system was used to assess cell growth/cytotoxicity caused by SnPP in hIEECs. 6% DMSO induced no cytotoxicity. Exposure of 30 μM SnPP for up to 72 h to hIEECs resulted in no cytotoxicity. Representative results from two similar experiments are shown.