ORIGINAL ARTICLE: ENDOMETRIOSIS

Estrogen stabilizes hypoxia-inducible factor 1α through G protein-coupled estrogen receptor 1 in eutopic endometrium of endometriosis

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Objective: To investigate whether G protein-coupled estrogen receptor (GPER, also known as GPR30 and GPER1) stabilizes hypoxia-inducible factor 1α (HIF-1α) in eutopic endometrium (EuEM) of endometriosis.
Design: Immunohistochemical analysis and experimental in vitro study.
Setting: University hospital.
Patient(s): Patients with or without endometriosis.
Intervention(s): The EuEM and normal control endometrium (CoEM) were obtained by curettage. Primary cultured endometrial stromal cells (ESCs) were treated with 17β-E_2, G1, or G15.
Main Outcome Measure(s): The EuEM and CoEM were collected for immunohistochemistry. Western blot, polymerase chain reaction, ELISA, and dual luciferase experiments were used to detect expression of GPER, HIF-1α, vascular endothelial growth factor (VEGF), and matrix metalloproteinase 9 (MMP9) in ESCs. Estradiol and G1 were used as agonists of GPER, G15 as an antagonist. Migration of ESCs and endothelial tube formation of human umbilical vein endothelial cells cultured in medium collected from ESCs were measured.
Result(s): Protein levels of GPER and HIF-1α were higher in EuEM than in CoEM. Protein levels of HIF-1α but not HIF-1α mRNA levels increased concurrently with GPER after E_2 and G1 treatment. Furthermore, expression and activity of VEGF and MMP9 increased under E_2 and G1 stimulation. However, these effects disappeared when GPER was blocked.
Conclusion(s): G protein-coupled estrogen receptor stabilizes HIF-1α and thus promotes HIF-1α–induced VEGF and MMP9 in ESCs, which play critical roles in endometriosis. (Fertil Steril® 2016;■:■–■. ©2016 by American Society for Reproductive Medicine.)
Key Words: Endometriosis, Estrogen, G protein-coupled estrogen receptor (GPER), hypoxia-inducible factor 1α (HIF-1α)

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Endometriosis, defined as the presence of endometrial-like tissue outside the uterus, is a common benign gynecologic disease affecting 6%–10% of the general female population (1). Endometriosis causes pelvic pain and infertility and has been associated with several types of cancer and other chronic diseases (2). Visual inspection of the pelvis at laparoscopy is the gold standard investigation for diagnosis, but it is invasive and results in long delays (3). Current therapeutic success is often unsatisfactory because of limited insight into disease mechanisms. The most widely accepted theory, retrograde menstruation, is insufficient to explain why most women have retrograde menstruation but only some of them develop endometriosis (4). Recent studies have

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focused on eutopic endometrium (EuEM) of endometriosis, which is possible to be collected simply and comfortably, because it appears to be biochemically, functionally, and genetically different compared with normal endometrium (CoEM) (5–7). It is possible that the EuEM may therefore play a key role in the pathogenesis of endometriosis.

Although a benign disease, endometriosis shares some similar features with malignancy, such as angiogenesis and metastasis (8). Recent studies suggested that hypoxia is vital for tumor formation and that hypoxia-inducible factor 1α (HIF-1α) plays a key role in tumor progression by up-regulating genes that control angiogenesis and metastasis (9, 10). Under normoxic conditions, HIF-1α is bound by the von Hippel-Lindau protein for proteasomal degradation. While under hypoxic conditions, the hydroxylation reaction is inhibited, allowing HIF-1α to escape degradation and increasing HIF-1α stability. Stabilized HIF-1α enters into the nucleus and initiates the transcription of target genes (11). In fact, vascular endothelial growth factor (*VEGF*) and matrix metalloproteinase 9 (*MMP9*) are target genes of HIF-1α (12, 13). Even though the presence and function of hypoxia and HIF-1α in menstrual physiology remain controversial (14), increasing evidence validated that hypoxia played vital roles in endometriosis and that HIF-1α was up-regulated with the development of endometriosis (15–18). In our previous studies, we discovered that expression of HIF-1α in ectopic endometrium (EcEM) was higher than that in CoEM (19), which was consistent with the results of others. In fact, EuEM shares changes with EcEM that were distinct from CoEM, and the view that a primary defect in endometriosis is to be found in EuEM has advanced (20, 21). So we compared EuEM and CoEM and found that EuEM also showed higher HIF-1α than CoEM. Additionally, we found that expression levels of VEGF and MMP9 were increased in EuEM (22, 23). Therefore, we hypothesized that high levels of HIF-1α in EuEM may increase VEGF and MMP9 expression, which are involved in the formation of endometriosis. However, in the same microenvironment, what causes the different expression of HIF-1α in EuEM and CoEM? The underlying mechanism remains unknown.

As we all know, estrogen (E) is one of the admitted factors of endometriosis (1). G protein-coupled E receptor (GPER, also known as GPR30 and GPER1), a seven transmembrane-domain G protein-coupled receptor, was identified as a novel E receptor that mediates the balance between nongenomic and genomic activity in response to 17β-E_2 (24). Research has proven the pathologic roles of GPER in a diverse array of disorders, and GPER is emerging as a novel therapeutic target and prognostic indicator (24). In endometriosis, GPER expression in EuEM has been demonstrated to be relatively higher than in CoEM (25–27). However, there is no report to explore its follow-up effects after activation by E_2 or other ligands. The actual role elicited by GPER in endometriosis is still controversial. However, in cancer research GPER has been found to play important roles in activating signaling mediated by HIF-1α (28, 29). Following the background information above, we hypothesized that GPER may be involved in the pathogenesis of endometriosis through acting on HIF-1α.

The aim of this study was to determine whether expression levels of GPER and HIF-1α were different between EuEM and CoEM; and whether HIF-1α was activated by GPER. First we investigated localization and protein levels of GPER and HIF-1α in CoEM and EuEM. Then we examined the correlation between GPER and HIF-1α in the primary endometrial stromal cells (ESCs) under E_2 and G1 stimulation. To be more convincing, we next examined the expression of HIF-1α target genes VEGF and MMP9 simultaneously. Finally, we examined the effect of blocking GPER on VEGF and MMP9 expression. Our studies suggested that GPER stabilized HIF-1α in EuEM and plays a key role in endometriosis angiogenesis and metastasis.

MATERIALS AND METHODS

Patients and Tissues

Ethics approval was obtained from the local Ethics Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology. Written, informed consent was obtained from all participants. Human tissues were obtained in accordance with the guidelines of the Declaration of Helsinki. Twenty samples of EuEM (age [mean $\pm$ SD], 26 $\pm$ 5 years) were curetted from patients with endometriosis in stages III and IV diagnosed by both pathology and laparoscopic findings according to the revised classification of the American Fertility Society (30). Sufficient CoEM (age, 28 $\pm$ 4 years) were available from 72 patients with tube infertility (no previous history of pelvic inflammatory disease, chronic pelvic pain, dysmenorrhea, or dyspareunia) and confirmed without endometriosis by laparoscopy. None of them had received hormonal treatments or sex steroids, and none used intrauterine contraception for at least 6 months before surgery. Recruited patients had regular menstrual cycles (between 26 and 32 days), with confirmation of their menstrual history. At the time of tissue collection all patients were in the early proliferative phase of the menstrual cycle. All samples of EuEM and 20 samples of CoEM were fixed in 4% buffered formalin for immunohistochemistry evaluations. The remaining 52 CoEM biopsies were collected and transported to the laboratory for ESCs culture establishment.

Immunohistochemistry

Paraffin-embedded endometrial sections were subjected to immunohistochemistry as described previously using rabbit anti-human GPER (1:50 ab39742; Abcam) and HIF-1α (1:150 AF1009; Affinity) antibodies (22). The stained slides were evaluated by light microscope and digitally scanned images by two independent pathologists. All scoring was performed blind to patient outcome. The immunohistochemical scores were calculated by positive rate and staining intensity of cells reactive with antibodies. Positive rate was categorized as 0 (no positive cells), 0 (<10% positive cells), 1 (10%–25% positive cells), 2 (26%–50% positive cells), 3 (50%–75% positive cells), or 4 (76%–100% positive cells), and staining intensity was categorized as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). The scoring pattern for staining was multiplied to give a total immunohistochemical score, and

immunohistochemical score ranged from 0 to 12. Scores of 0–2 points were considered as negative (0); 3–5 points as weak staining (+); 6–8 points as intermediate (++); and 9–12 points as strong staining (+++).

Cell Culture

Endometrial stromal cells were isolated from CoEM as previously described (23). Briefly, fragments were minced, digested with collagenase II (0.1%; Sigma), filtered through 150- and 37.4-μm sieves, centrifuged, and suspended in Red Blood Cell Lysis Buffer (C3702, Beyotime). After a second centrifugation, ESCs were resuspended in full medium. Then cells were seeded on 25-cm^2 culture flasks and maintained in a humidified 5% CO_2 incubator at 37°C. When ESCs were nearly confluent, cells were regularly digested and plated in six-well plates (1×10^6 cells per well) for Western blot and in 24-well plates (2×10^5 cells per well) for ELISA. In each experiment, cells were divided into three groups. Cells in group 1 were stimulated with 10 nM E_2 (E-2758, Sigma-Aldrich) for different times (0, 5, 10, 15, 30, 60, and 120 minutes); cells in group 2 were stimulated with 100 nM G1 (CAS 881639-98-1, Cayman) at the same time points. After identifying the most effective stimulation time, cells in group 3 were treated with 10 nM E_2 or 100 nM G1 for the most effective stimulation time, with or without pretreatment with 100 nM GPER inhibitor G15 (CAS 1161002-05-6, Cayman) for 30 minutes. The supernatant was collected after stimulation and stored at −80°C until ELISA and in vitro human umbilical vein endothelial cell (HUVEC) tube formation assay. The cells were washed twice with phosphate-buffered saline and extracted for messenger RNA (mRNA) and protein assay. Each experiment was repeated at least three times with different cell preparations. The >95% purity of ESCs was confirmed by positive staining for Vimentin (1:100; Cell Signaling Technology) and negative staining for E-cadherin (1:150; Cell Signaling Technology) in immunocytochemistry.

Human umbilical vein endothelial cells were purchased from ATCC and cultured at 37°C in a humidified atmosphere of 5% CO_2 in air.

Quantitative Real-time Reverse Transcription Polymerase Chain Reaction

Ribonucleic acid was isolated from the tissues using RNAiso Plus (9109, Takara) according to the manufacturer's protocol. Reverse transcription and amplification for complementary DNA were carried out as described previously (22). The melting curve was analyzed after the reactions to check for primer dimer formation and nonspecific product amplification. The $2^{-\triangle\triangle CT}$ method was used for the determination of relative transcript abundance.

Western Blot Analysis

Protein concentrations from cultured ESCs were quantified using the bicinchoninic acid (BCA) protein assay kit (P0010S, Beyotime). Equal amounts of protein (30 μg) were subjected to 12% sodium dodecylsulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (0.45 mm, Millipore). After blocking for 1 hour at room temperature with blocking buffer (0.1% Tris-buffered saline with Tween (TBST) with 5% fat-free dried milk powder), the blots were incubated with primary antibodies against GPER (1:500; ab39742, Abcam) or HIF-1α (1:1,000; AF1009, Affinity) at 4°C overnight. The target proteins were visualized by the electrochemiluminescence Western blotting detection system (Millipore) after incubation with a secondary antibody (1:5,000 diluted with 5% fat-free dried milk powder in 0.1% TBST).

Enzyme-linked Immunosorbent Assay

Endometrial stromal cells were cultured and divided into three groups as described previously. Expression levels of VEGF and MMP9 secreted into the conditioned media derived from treated and untreated cells were determined according to manufacturer guidelines using VEGF (DVE00) and MMP9 ELISA kits (DMP900) from R&D Systems. All samples were assayed in duplicate. The amount of protein secreted was determined as an optical density value using a microplate reader at a wavelength of 450 nm, with the correction wavelength set at 570 nm. A standard-curve analysis was included on each plate, and protein secretion was compared against this curve.

Immunofluorescence

Endometrial stromal cells were seeded on glass coverslips sitting on the bottom of six-well plates. Fresh medium was provided to the cells 24 hours before the experiment. The cells were pretreated or not with 100 nM G15 for 30 minutes, followed by treatment with E_2 or G1 for 15 minutes. The cells were then fixed (4% paraformaldehyde, 20 minutes, room temperature), permeabilized (0.1% Triton X-100 in phosphate-buffered saline, 20 minutes, room temperature), blocked (5% bovine serum albumin, 1 hour, room temperature), and incubated (overnight, 4°C) with primary antibody against GPER (1:100; ab39742, Abcam). A secondary antibody conjugated with Cy3 (1:10; Google Biological Technology) was used to visualize GPER. The cells were counterstained with 6-diamino-2-phenylindole stain (Sigma) to visualize nuclei. Sections were examined with an Olympus FV1000 laser scanning confocal microscope.

Dual Luciferase Experiments

The 2,050-bp (−2,000 to 50 bp) sequence of wild-type *VEGF* promoter and the 2,020-bp (−1,900 to 119) of wild-type *MMP9* promoter were cloned from human genomic DNA and subcloned into pcDNA3.0 basic vectors. Endometrial stromal cells were seeded into 24-well plates the night before transfection. Cells were always co-transfected with the internal control plasmid pRL-SV40 (Promega) containing the Renilla luciferase gene for 24 hours. Then cells were pretreated or not with 100nM G15 for 30 minutes, followed by treated with E_2 or G1 for 120 minutes. After cells harvested, firefly and Renilla luciferase activities were measured using the dual luciferase assay system kit (Promega).

In Vitro Migration Assays

In vitro migration assays were performed using a transwell insert (Corning Costar) with 8-μm pore membrane filters. Briefly, Matrigel (Sigma-Aldrich) was precoated, 10^4/mL of ESCs were plated in the upper chamber in a low serum medium (5%), and the units were transferred to a serum gradient (20%) in the lower chamber for 16 hours. Then ESCs were treated with E_2 or G1 for 120 minutes, with or without pretreatment with G15 for 30 minutes. The noninvasive cells and Matrigel on the upper side were removed with a cotton swab. The membrane was then fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The number of migrating cells was determined using light microscopy (Olympus) on each membrane in five random fields. The values reported were the averages of triplicate experiments. Duplicate wells were used per condition in each independent experiment.

In Vitro HUVEC Tube Formation Assay

A 96-well plate was evenly loaded with Matrigel (0.05 mL per well) (Sigma-Aldrich) and incubated at 37°C for 30 minutes before seeding the HUVECs (5×10^4 cells per well). The HUVECs were cultured using conditioned medium from ESCs previously treated with E_2 or G1 for 120 minutes, with or without pretreatment with G15 for 30 minutes and incubated under normal condition. Tube formation was quantified 18–20 hours later and photographed using light microscopy (Olympus). Tube formations were measured blind on three randomly chosen microscopic fields per well by an independent observer, giving [1] the total length of tube-like cells; and [2] the number of junctions or joint forming cell–cell networks. Experiments performed for the analysis of tubular formation were repeated at least three times.

Statistical Analysis

Each experiment was performed in triplicate or quadruplicate. Statistical analysis was performed by GraphPad Prism 5, and results were expressed as mean ± SEM. Wilcoxon's matched pairs test was used for the comparison of quantitative differences in the staining of GPER and HIF-1α between CoEM and EuEM. One-way analysis of variance (ANOVA), followed by the Newman-Keuls test, was used for mean comparisons between groups. Pearson correlation was used to investigate the correlation between GPER and HIF-1α protein levels in ESCs under different time points of E_2 or G1. A P value of $<.05$ was considered statistically significant.

RESULTS

GPER and HIF-1α Expression in CoEM and EuEM

Expression and localization of GPER and HIF-1α were studied by immunohistochemistry staining in 20 CoEM and 20 EuEM. Both endometrium epithelial cells (EECs) and ESCs were analyzed. Representative staining examples are shown in Figure 1, and staining intensities are depicted in Supplemental Table 1 (available online). Generally, GPER staining was more intense in EECs than in ESCs. G protein-coupled estrogen receptor was detected as predominantly cytoplasmic in most EECs (Fig. 1A, 1B, 1E, and 1F), and the intensity was significantly higher in EuEM when compared with CoEM (P=.009; Fig. 1I). Staining was rarely seen in most of ESCs from CoEM (Fig. 1A and B), but was predominantly detected in the cytoplasm of ESCs from EuEM (Fig. 1E and F) (P=.0047; Fig. 1J). Hypoxia-inducible factor 1α was predominantly localized in the nuclear and was observed in both EECs and ESCs (Fig. 1C, 1D, 1G, and 1H). There was no significant difference between CoEM and EuEM in EECs (P=.3746; Fig. 1K), whereas in ESCs, the HIF-1α expression level was significant higher in EuEM than in CoEM (P=.02179; Fig. 1L).

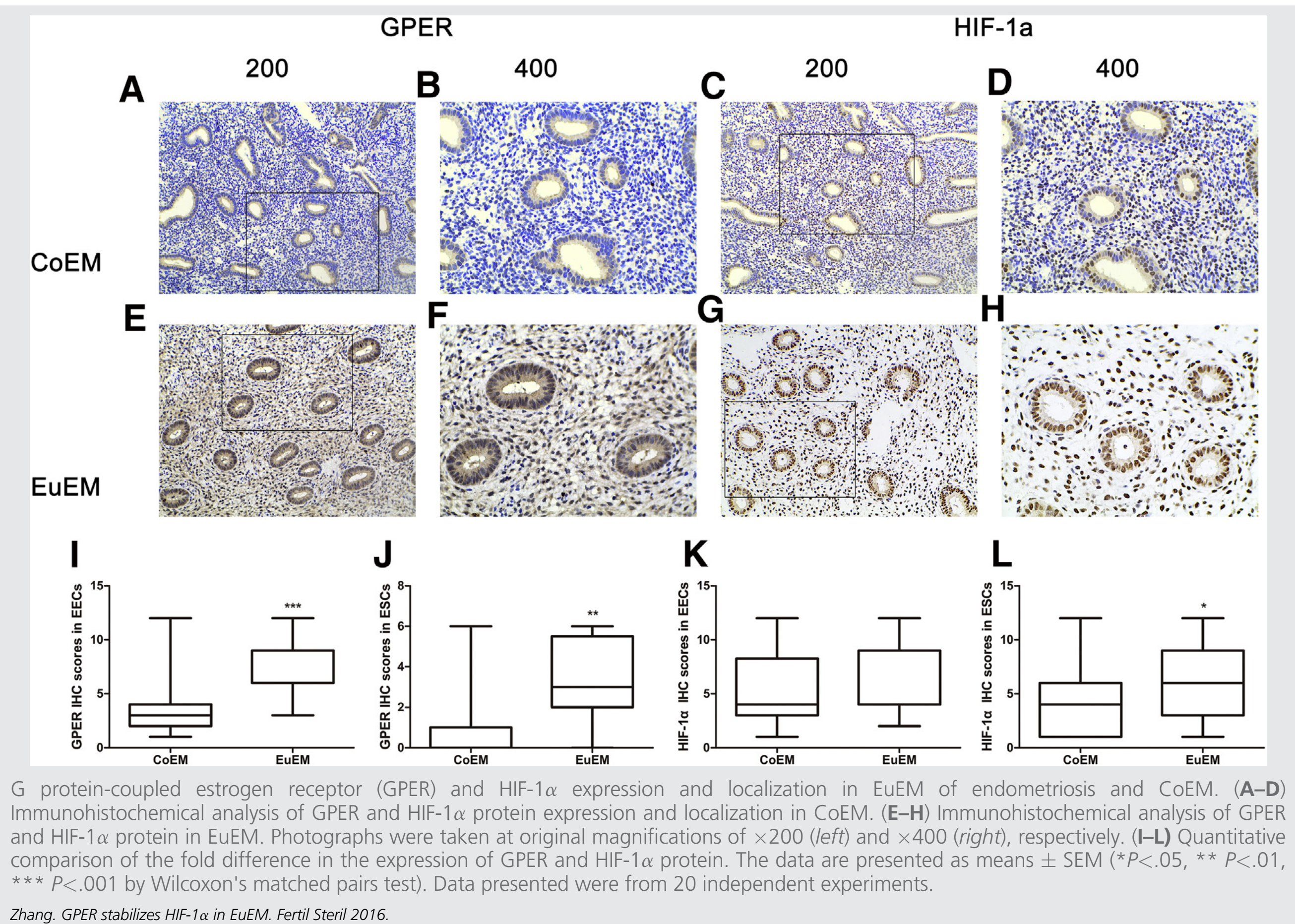
E_2 and G1 Induce GPER, HIF-1α, and HIF-1α Target Genes VEGF and MMP9 Expression in ESCs

Because E_2 is a major factor in the pathogenesis of endometriosis, we hypothesized that it was E_2 that induced higher levels of GPER and HIF-1α in ESCs from EuEM. To determine the effects of E_2 on GPER and HIF-1α level, we analyzed ESCs incubated with 10 nM E_2 for increasing time points (0, 5, 10, 15, 30, 60, and 120 minutes). In polymerase chain reaction assay, GPER mRNA was increased significantly after treatment with E_2 for 10 and 15 minutes (Fig. 2A), whereas HIF-1α mRNA was similar at all the time points tested (Fig. 2B). In Western blot analysis, there was a time-dependent increase in GPER and HIF-1α protein levels, and both reached peak effect at 15 minutes (Fig. 2E–2G). Furthermore, HIF-1α increased coincidently with GPER (Fig. 2H). These results suggested that the elevated level of GPER protein was transcription-dependent, whereas HIF-1α protein was independent of HIF-1a transcription. We further examined expression of HIF-1α target genes VEGF and MMP9 in ESCs under E_2 stimulation. Analysis with ELISA demonstrated that E_2 significantly increased VEGF and MMP9 secretion and reached peak effect at 120 minutes (Fig. 2M and N). As E_2 caused GPER-specific stimulation is difficult for the cross-reactivity of other E receptors (ERs). We repeated the previously mentioned experiments with G1, the first specific agonist of GPER, to exclude the interference of other ERs (31). The results were consistent with the ones stimulated with E_2 (Fig. 2C, 2D, 2I–L, 2O, and 2P). The findings above suggest that E_2 and G1 simultaneously promote protein levels of GPER, HIF-1α, and HIF-1α target genes VEGF and MMP9 in cultured ESCs.

E_2 and G1 Induce HIF-1α–mediated VEGF and MMP9 Expression through GPER

To further determine the role of GPER in E_2- and G1-mediated HIF-1α expression, we used G15, an antagonist of GPER activity. Immunofluorescence staining with GPER antibody revealed an intracellular pattern for GPER in ESCs. Its expression was significantly increased under the stimulation of E_2 or G1 for 15 minutes but was significantly decreased when fore-stimulated with G15 for 30 minutes (Fig. 3A). Furthermore, to rule out the possibility that the protein level of HIF-1α was affected by GPER, we performed Western

FIGURE 1



G protein-coupled estrogen receptor (GPER) and HIF-1α expression and localization in EuEM of endometriosis and CoEM. (**A–D**) Immunohistochemical analysis of GPER and HIF-1α protein expression and localization in CoEM. (**E–H**) Immunohistochemical analysis of GPER and HIF-1α protein in EuEM. Photographs were taken at original magnifications of $\times$200 (*left*) and $\times$400 (*right*), respectively. (**I–L**) Quantitative comparison of the fold difference in the expression of GPER and HIF-1α protein. The data are presented as means $\pm$ SEM (**P*$<$.05, ** *P*$<$.01, *** *P*$<$.001 by Wilcoxon's matched pairs test). Data presented were from 20 independent experiments.

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blotting analysis to examine the HIF-1α protein levels in different treatments of ESCs. Notably, Figure 3B–3D shows that E_2 and G1 up-regulated the HIF-1α expression level, whereas the effect disappeared when blocking GPER. Furthermore, ELISA revealed that stimulation of HIF-1α target genes VEGF and MMP9 in the medium was also dependent on GPER expression (Fig. 3E and F). Accordingly, G1 and E_2 transactivated *VEGF* and *MMP9* promoter constructs (Fig. 3G and H) through GPER, as the luciferase activity was repressed when fore-stimulated with G15. All of the above suggested that E_2 mediates HIF-1α activity in ESCs in a GPER-dependent manner.

GPER is Involved in VEGF-mediated Tube Formation

Previous results suggest that GPER mediates HIF-1α–induced up-regulation of VEGF secretion of ESCs. The influence of GPER can be also observed in an assay much closer to the in vivo situation of angiogenesis—in vitro tube formation of HUVECs. The HUVECs were cultured using conditioned medium from ESCs previously treated with E_2 or G1 for 120 minutes, with or without pretreatment with G15 for 30 minutes. Interestingly, a ramified network of tubules was generated in HUVECs grown in medium from ESCs treated with E_2 and G1; however, there were no effects when knocking down the expression of GPER by G15. These results, recapitulated in Figure 4A and B, indicate that VEGF may be considered as a target of the estrogenic GPER-mediated signaling toward new blood vessels formation.

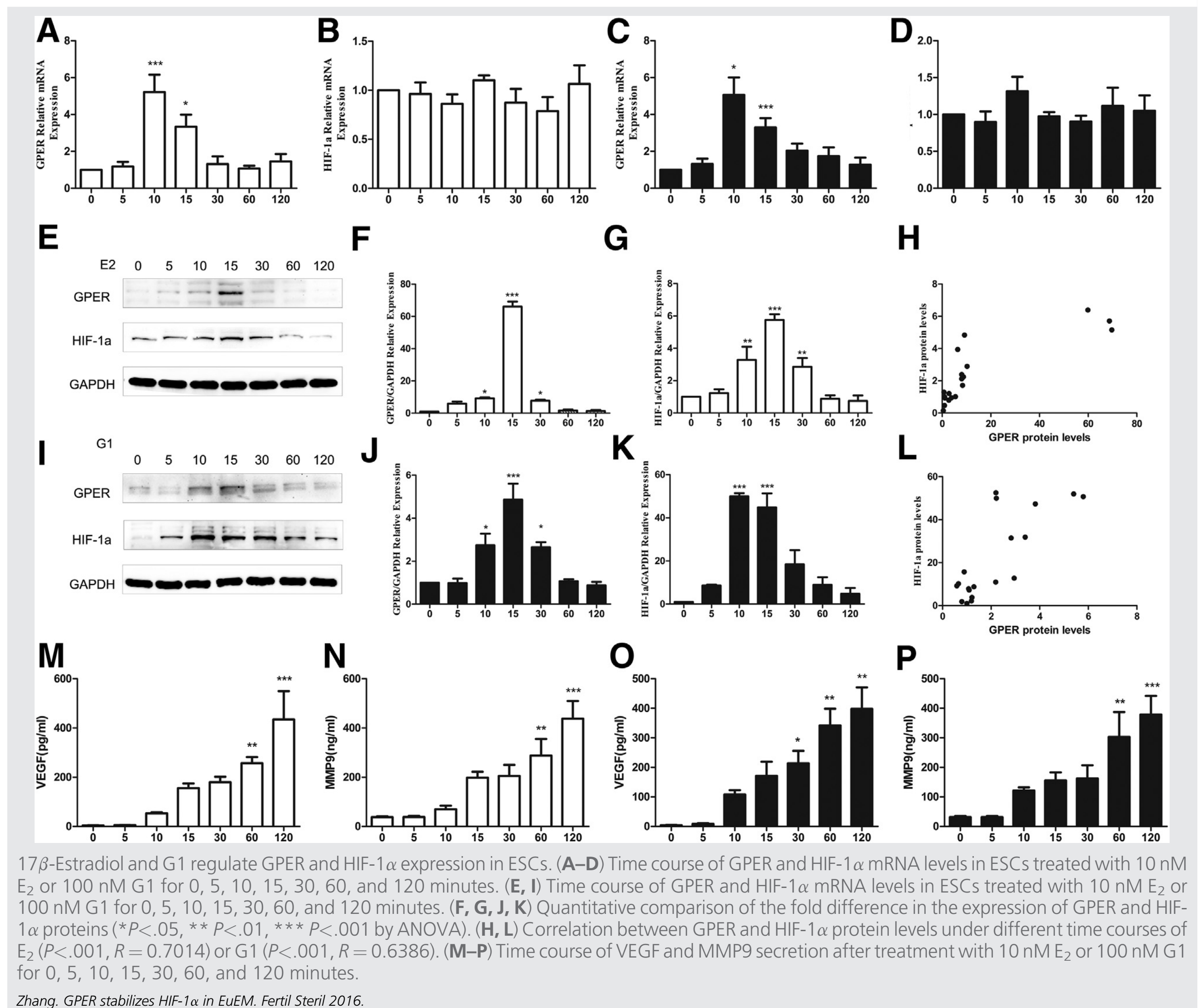
GPER is Involved in MMP9-mediated Cell Migration

To determine whether GPER participates in HIF-1α–mediated ESCs migration, ESCs were stimulated under different conditions. As shown in Figure 4C and D, E_2 and G1 significantly increased ESCs migration ($P<.05$, respectively). However, preincubation of ESCs with G15 abolished the effect, leading to a significant decrease in the number of migrated cells ($P<.05$, respectively). Taken together, these data indicate that HIF-1α may function as a downstream factor in GPER-mediated promotion of ESCs migration.

DISCUSSION

The present study demonstrated that GPER and HIF-1α expression in ESCs was higher in EuEM than in CoEM.

FIGURE 2



17β-Estradiol and G1 regulate GPER and HIF-1α expression in ESCs. (**A–D**) Time course of GPER and HIF-1α mRNA levels in ESCs treated with 10 nM E_2 or 100 nM G1 for 0, 5, 10, 15, 30, 60, and 120 minutes. (**E, I**) Time course of GPER and HIF-1α mRNA levels in ESCs treated with 10 nM E_2 or 100 nM G1 for 0, 5, 10, 15, 30, 60, and 120 minutes. (**F, G, J, K**) Quantitative comparison of the fold difference in the expression of GPER and HIF-1α proteins (*$P<.05$, ** $P<.01$, *** $P<.001$ by ANOVA). (**H, L**) Correlation between GPER and HIF-1α protein levels under different time courses of E_2 ($P<.001$, $R=0.7014$) or G1 ($P<.001$, $R=0.6386$). (**M–P**) Time course of VEGF and MMP9 secretion after treatment with 10 nM E_2 or 100 nM G1 for 0, 5, 10, 15, 30, 60, and 120 minutes.

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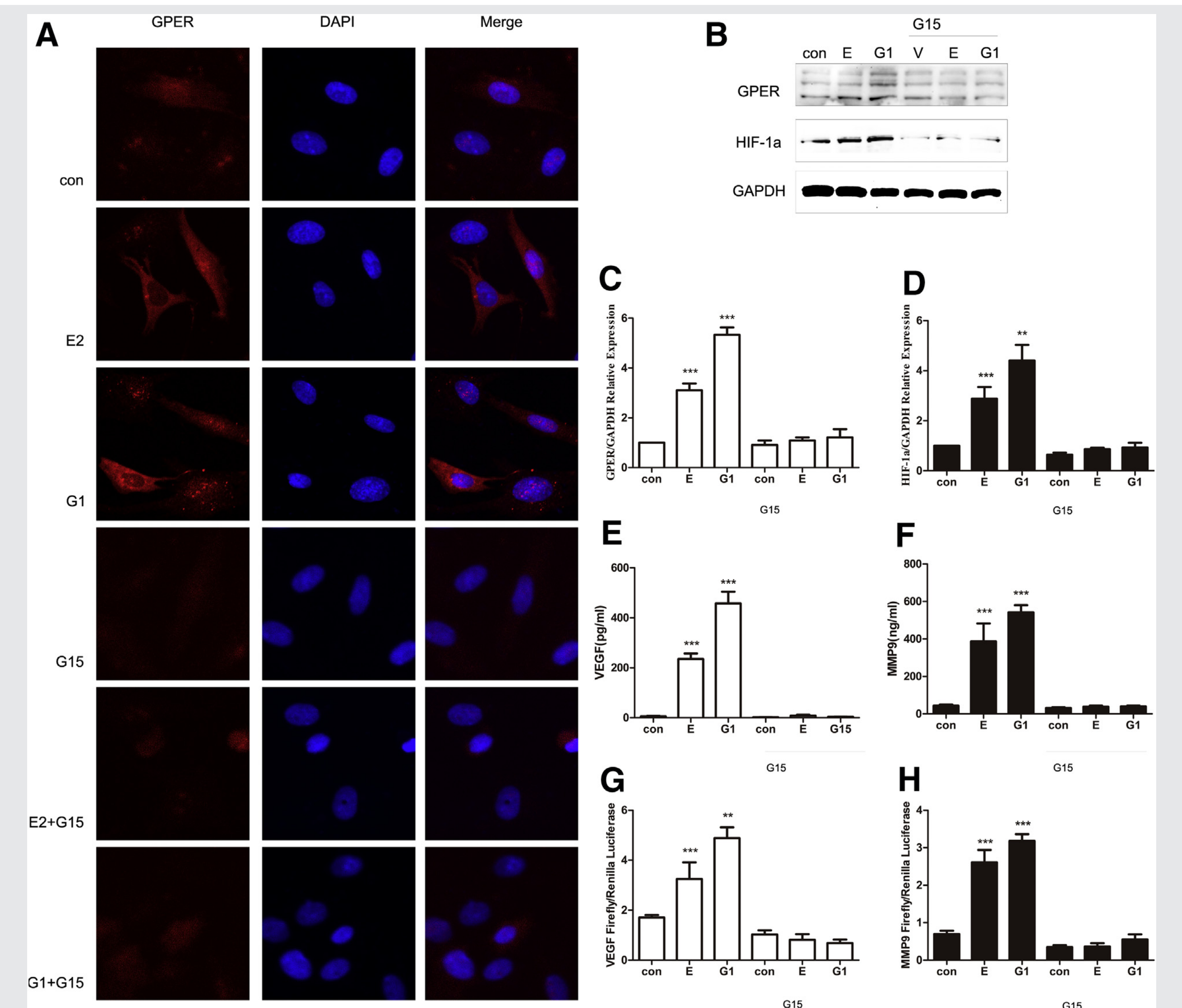
Furthermore, we found that E_2 and G1 promoted HIF-1α expression in a GPER-dependent manner. As a biological counterpart, we have evidence showing that GPER-promoted HIF-1α mediates migration of ESCs and endothelial tube formation of HUVECs cultured in medium from ESCs. The present findings provide novel insight into the potential role of GPER in endometriosis angiogenesis and migration mediated through HIF-1α.

Multiple factors contribute to angiogenesis and migration of endometriosis. Changes in the expression of HIF-1α could be involved in these processes. Recently more researchers studied the role of HIF-1α in endometriosis, and several groups reported up-regulation of HIF-1α in EcEM (15, 17). In our study we focused on EuEM and compared HIF-1α expression between EuEM and CoEM to exclude interference of the peritoneal fluid environment. Most previous studies were designed to clarify how HIF-1α induced expression of downstream genes that regulate proliferation, angiogenesis, and metastasis of endometriosis (15–17,32). However, mechanisms responsible for aberrant expression of HIF-1α remain enigmatic. In fact, cytokines, growth factors, and hormones beyond hypoxia were shown to upregulate HIF-1α expression (33). Because endometriosis is an E-dependent disease, we hypothesized that high E_2 stimulation resulted in increased expression of HIF-1α in EuEM.

The endogenous HIF-1α protein level mainly depends on the rate of protein translation and degradation (34). Under normoxia, HIF-1α is posttranslationally modified by a mechanism that involves ubiquitylation by the Hippel-Lindau tumor suppressor E3 ligase complex and rapid degradation. Conversely, under hypoxia, this process is inhibited by hypoxia, allowing stabilized HIF-1α accumulation and transcriptional activation (35). Recently regulation of HIF-1α protein levels by E_2 has been reported. Estradiol triggers multiple biological responses, mainly through the specific receptors ERα

FIGURE 3



G protein-coupled estrogen receptor (GPER) mediates upregulation of HIF-1α and HIF-1α target gene expression induced by E_2 and G1. Endometrial stromal cells were treated with E_2 or G1, with or without pretreatment with G15 for 30 minutes. (**A**) Evaluation of GPER protein expression by immunofluorescent microscopy in treated or untreated ESCs. (**B**) Immunoblots showing GPER and HIF-1α protein expression in ESCs under different treatment. (**C, D**) Quantitative comparison of the fold difference in GPER and HIF-1α protein expression. (**E, F**) ELISA showing VEGF and MMP9 secretion from ESCs of different conditions. (**G, H**) The transactivation of the VEGF and MMP9 promoters in ESCs by different treatment (*$P<.05$, **$P<.01$, *** $P<.001$ by ANOVA).

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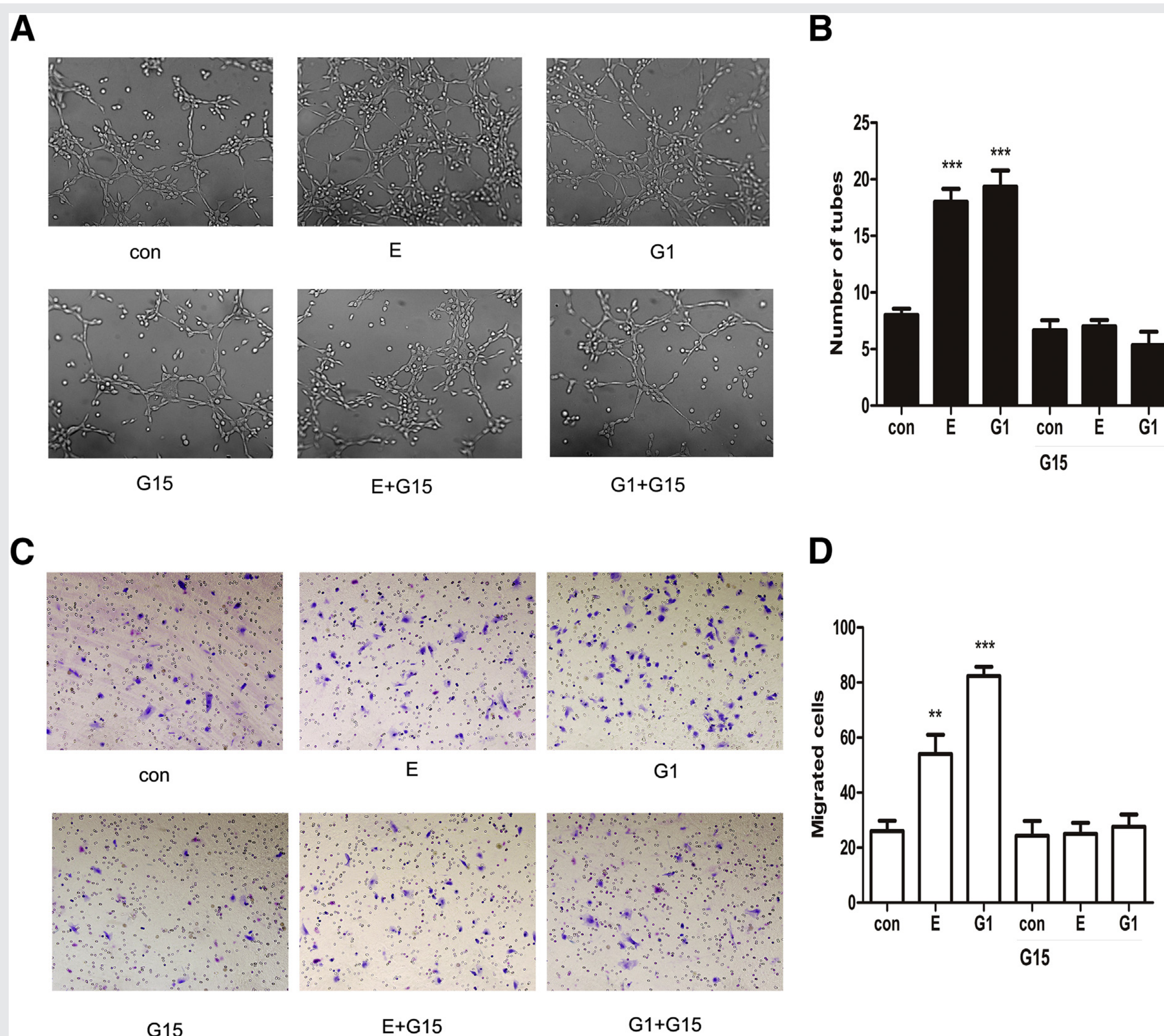
and ERβ (36). In this study we focused on GPER, a newly found receptor that is sensitive to E. Plante et al. (25) demonstrated cycle-regulated expression of GPER in normal human endometrium, with maximal expression in the proliferative phase, and in EuEM and EcEM GPER was overexpressed. Our immunohistochemical results showed that EuEM expressed higher GPER and HIF-1α. Previous studies clarify the relationship between GPER and HIF-1α. Recchia et al. (37) and Ren et al. (38) found that GPER was up-regulated by HIF-1α, whereas Rigiracciolo et al. (29) and De Francesco et al. (39) certified that HIF-1α was up-regulated by GPER. Eutopic endometrium and CoEM exist under the same environment, and the striking difference is that EuEM produces higher E_2. Thus, we hypothesized that GPER promotes HIF-1α expression in EuEM. To prove this, we isolated and cultured ESCs and stimulated with E_2, G1, and G15 and then examined HIF-1α and HIF-1α target genes VEGF and MMP9 expression. The results showed that E_2 and G1 could increase HIF-1α protein expression in a transcriptional independent manner and enhance migration and angiogenesis of the cells, whereas G15 could block these effects.

Even as a common disease, the pathogenesis of endometriosis is still ambiguous. High incidence and recurrence rate and lack of convenient and effective diagnosis and

FIGURE 4



Estradiol and G1 enhance endothelial tube formation and ESCs migration through GPER. For endothelial tube formation, ESCs were treated with E_2 or G1 for 120 minutes, with or without pretreatment with GPER inhibitor G15 for 30 minutes. Tube formation was evaluated in HUVECs cultured for 16–18 hours in medium collected from ESCs. (**A**) Representative photomicrographs of tube formation under different medium. (**B**) Quantified results of tube formation assay. For migration assay, ESCs were cultured in the upper chamber for 16 hours and then treated with E_2 or G1 for 120 minutes, with or without pretreatment with GPER inhibitor G15 for 30 minutes. (**C**) Representative photomicrographs of ESCs migration under different conditions. (**D**) Quantified results of migration assay (*$P<.05$, ** $P<.01$, *** $P<.001$ by ANOVA).

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treatment methods make endometriosis a major problem in gynecology. Early noninvasive diagnosis and efficient treatment are needed. In this study we found that both GPER and HIF-1α were up-regulated in EuEM. Furthermore, we demonstrated that E_2 stabilizes HIF-1α by GPER to promote ESCs invasion and angiogenesis. In summary, these findings provide new etiological insight into the development of endometriosis and shed light on the design of new diagnostic and therapeutic strategies. However, we did not clarify the regulation mechanism of E on GPER and the mechanism of how GPER stabilizes HIF-1α protein. These need to be studied further.

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