Estradiol-mediated hepatocyte growth factor is involved in the implantation of endometriotic cells via the mesothelial-to-mesenchymal transition in the peritoneum

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Ono YJ, Hayashi M, Tanabe A, Hayashi A, Kanemura M, Terai Y, Ohmichi M. Estradiol-mediated hepatocyte growth factor is involved in the implantation of endometriotic cells via the mesothelial-to-mesenchymal transition in the peritoneum. Am J Physiol Endocrinol Metab 308:E950–E959, 2015. First published April 7, 2015; doi:10.1152/ajpendo.00573.2014.—The pathogenesis of endometriosis, a chronic painful gynecological disease characterized by the presence of endometrial tissue located outside the uterus and often adhering to the peritoneum, is known to be estrogen dependent. However, the precise pathophysiology of endometriosis remains elusive. Recent studies indicate that the epithelial-to-mesenchymal transition (EMT) of human endometrial cells is important for the progression of endometriosis, and another previous study has implicated hepatocyte growth factor (HGF) in endometriosis progression. The aim of the present study was to examine the role of estradiol in the regulation of HGF production and progression of peritoneal endometriosis, focusing on the interactions between the peritoneum and endometriotic cells. Consequently, estradiol was found to promote the proliferation, invasion, and migration of immortalized human endometrial epithelial cells (hEECs) via HGF upregulation, and the estradiol-induced direct binding of estrogen receptor-α to the HGF promoter was confirmed on a chromatin immunoprecipitation (ChIP) assay. Estradiol also induced the EMT in hEECs by promoting HGF production. Furthermore, human mesothelial cells were induced to the mesothelial-to-mesenchymal transition (MMT) during culture with estradiol-stimulated hEEC conditioned medium. Importantly, estradiol itself did not induce the MMT, and the estradiol-stimulated hEEC-conditioned medium in the presence of HGF antibodies reversed the MMT process. These results, which were obtained using immortalized hEECs, indicate that estradiol-induced HGF production may play a crucial role in the peritoneal implantation of human endometriotic cells by exerting proliferative and invasive effects via the EMT in hEECs and promoting the MMT in mesothelial cells.

Estradiol, the most active member of the group of hormones known as estrogens, is crucial for the normal function of a broad range of cells and organs. Endometriotic lesions exhibit a high level of estradiol biosynthesis and low level of estradiol inactivation compared with that observed in the endometrium in unaffected patients (5). The progression of endometriosis is widely believed to be estrogen-dependent; therefore, the aim of traditional treatment is to decrease endogenous estradiol production from the ovaries (5, 21).

Recent studies indicate that the epithelial-to-mesenchymal transition (EMT), a developmental process by which epithelial cells reduce intercellular adhesion and acquire fibroblastoid properties, plays important roles in the invasive and metastatic potential associated with cancer progression (26, 32). Another recent report also suggests that the EMT of human endometrial cells is a hallmark of progressive endometriosis (30). Generally, the activation of a wide variety of ligands, including fibroblast growth factor, transforming growth factor-β, epidermal growth factor, vascular endothelial growth factor, and hepatocyte growth factor (HGF) and its receptor MET, upregulates the expression of EMT-regulating transcription factors (26). Among these ligands, many actions of HGF are mirrored by estradiol in the female reproductive tract, and several investigators have reported a relationship between HGF and estradiol in the pathology of female genital disease (8, 31, 36).

The peritoneal cavity is lined by a continuous single layer of mesothelial cells, a unique epithelial-like cell type that covers only peritoneal, pleural, and pericardial serosal surfaces (38). It has been reported that inflammatory mediators as well as a low pH induce peritoneal mesothelial cells to lose certain epithelial characteristics, thus acquiring a fibroblast-like phenotype. This EMT-like process, the so-called mesothelial-to-mesenchymal transition (MMT), serves as a trigger for peritoneal fibrosis and angiogenesis and is considered to be an important potential therapeutic target in patients with sclerotic peritonitis (1).

Endometriosis is characterized by the presence of endometrial cells outside the uterus, in most cases, attached to the peritoneal wall. Recent studies suggest that the phenotype of the pelvic peritoneum plays a part in the establishment and persistence of endometriosis (3, 37); however, little is known about how the peritoneum is affected by and related to the progression of endometriosis under the influence of estradiol.

We herein evaluated the role of estradiol in the progression of endometriosis via HGF production using immortalized human endometrial epithelial cells (hEECs). We also assessed the effects of estradiol-stimulated immortalized hEECs on human mesothelial cells to understand the pathogenesis of peritoneal implantation induced by endometriotic cells.

ENDOMETRIOSIS IS A CHRONIC, BENIGN DISEASE characterized by the presence of endometrium-like tissue outside the uterine cavity primarily on the ovaries or pelvic peritoneum. This condition is a major cause of symptoms, such as pelvic pain, dysmenorrhea, dyspareunia, and infertility, that affect 6–10% of females of reproductive age and at least one-third of women with infertility and often relapses after surgery (9–11). Although endometriosis was first described in 1860 (33), the etiology and pathogenesis of this disorder remain unclarified.
MATERIALS AND METHODS

Reagents and antibodies. Estradiol was purchased from Wako Pure Chemical Industries (Osaka, Japan). The rabbit polyclonal anti-human Met antibodies, rabbit polyclonal anti-human phospho-Met antibodies, rabbit monoclonal anti-human Snail antibodies, rabbit polyclonal anti-human E-cadherin antibodies, rabbit polyclonal anti-human Vimentin antibodies, and rabbit monoclonal anti-human β-actin antibodies used for immunoblotting and immunohistochemistry and the rabbit monoclonal anti-human ERα antibodies (DSHB) used for immunoprecipitation were all purchased from Cell Signaling Technology, (Danvers, MA). The rabbit polyclonal anti-human HGF antibodies (ab24865) and rabbit polyclonal anti-human N-cadherin antibodies used for immunoblotting and immunohistochemistry and the rabbit polyclonal anti-human HGF antibodies (ab10679) used as HGF-neutralizing antibodies were purchased from Abcam (Cambridge, MA). The rabbit polyclonal anti-human pan-cytokeratin antibodies used for immunoblotting and immunohistochemistry were purchased from Thermo Fisher Scientific, (Waltham, MA).

Cell lines and conditioned media. We used immortalized hEECs of ovarian endometrioma, EMOSis-CC/TERT (4), a kind gift from Dr. Satoru Kyo (Kanazawa University, Kanazawa, Japan). Human adult mesothelial cells, MES-F, were purchased from Zen-Bio (Research Triangle Park, NC). The EMOSis-CC/TERT cells were grown in DMEM supplemented with 10% charcoal-stripped fetal bovine serum (FBS) (Equitech-Bio, Kerrville, TX), and the MES-F cells were grown in mesothelial cell growth medium (Zen-Bio) in an atmosphere of 5% CO₂ at 37°C (4). To obtain estradiol-stimulated EMOSis-CC/TERT cell-conditioned medium (E-EMO-CM), the EMOSis-CC/TERT cells were seeded and cultivated until 60% confluence. The medium was then replaced with DMEM supplemented with 10% charcoal-stripped FBS in the presence of 100 nM estradiol, and the supernatants were collected after 48 h of further incubation.

Proliferation assay. The EMOSis-CC/TERT cell proliferation was measured by manual cell counting. Briefly, cells were plated in six-well plates at 2 × 10^4 cells/well in triplicate for 24 h. The cells were then incubated for 48 h in the presence or absence of the vehicle (PBS), 0.1, 1, 10, 100, 1,000, or 10,000 nM estradiol, or 1, 5, 10, 50, or 100 ng/ml of human recombinant HGF with or without 10 μg/ml HGF-neutralizing antibodies. Thereafter, the cells were removed by trypsinization, and the number of viable cells was counted in a hemocytometer. Cell proliferation was additionally measured by using CellTiter 96 AQueous (MTS) One Solution Cell Proliferation Assay (Promega, Tokyo, Japan) according to the manufacturer’s instructions. Briefly, the EMOSis-CC/TERT cells were seeded in 96-well plates at a density of 2 × 10^4 cells/well in DMEM supplemented with 2% charcoal-stripped FBS. The cells were then incubated for 24 h in the presence or absence of the vehicle (PBS), 0.1, 1, 10, 100, 1,000, or 10,000 nM estradiol or 1, 5, 10, 50, or 100 ng/ml human recombinant HGF with or without 10 μg/ml HGF-neutralizing antibodies. CellTiter 96 AQueous (MTS) One Solution reagent (Promega) was added to each well, and the absorbance was recorded at 490 nm using the Corona SH-1000 lab absorbance microplate reader (Corona Electric, Ibaraki, Japan). The sample concentrations were determined via interpolation from the standard curve. The assay was performed three times, and the ratio was expressed as means ± SD.

Chromatin immunoprecipitation assay. The chromatin immunoprecipitation (ChIP) assay was conducted using the ChIP-IT Express Chromatin Immunoprecipitation Kit (Active Motif) according to the manufacturer’s protocol. Briefly, 1 × 10⁷ EMOSis cc/TERT cells were starved for 16 h, after which the vehicle (PBS) or 100 nM estradiol was added to the medium. Following overnight incubation, formaldehyde was added directly to the culture at a final concentration of 1%, and the cells were incubated for an additional 15 min at 37°C to cross-link the protein to the DNA. The cells were subsequently pelleted and resuspended in 600 μl of lysis buffer supplemented with 3 μl of protease inhibitor cocktail and 50 mM phenylmethylsulfonyl fluoride. The nuclear fraction was resuspended in shearing buffer and sonicated with a sonicator. The sheared chromatin solution was used for each ChIP assay with 5 μg of anti-ERα antibodies (DSHB; Cell Signaling Technology) or normal rabbit IgG as a negative control. Reading was performed at 450 nm using a Corona SH-1000 lab absorbance microplate reader (Corona Electric, Ibaraki, Japan). The sample concentrations were determined via interpolation from the standard curve. The assay was performed three times with similar results, and representative results for conventional PCR are shown.

Wound-healing assay. EMOSis-CC/TERT cells were grown in DMEM supplemented with 10% charcoal-stripped FBS until confluence. Subsequently, the cells were starved in DMEM serum-free medium for 18 h, and then one artificial wound per well was scratched into the monolayer using a sterile, plastic, 10-μl pipette tip to generate a uniform wound devoid of adherent cells. After wounding, the cells were incubated in DMEM serum-free medium until confluence. Subsequently, the cells were starved in DMEM serum-free medium with or without 10 μg/ml HGF-neutralizing antibodies. Wound closure was monitored according to the manufacturer’s instructions. After wounding, the cells were incubated in DMEM serum-free medium for an additional 24 h. The wound-healing assay was performed as described previously (14). Briefly, total proteins were prepared using Pierce RIPA Buffer (Thermo Fisher Scientific). The equal amounts of total proteins were separated via SDS polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membranes. The membranes were then blocked in Tris-buffered saline containing 10% bovine serum albumin and incubated with specific primary antibodies. After thorough washing, the membranes were further incubated with the secondary antibody recognizing rabbit immunoglobulin. Finally, the bands were visualized using enhanced chemiluminescence (ECL Plus; GE Healthcare Life Sciences, Pittsburgh, PA).

Cell invasion assay. Changes in the invasive potential of the EMOSis-CC/TERT cells and MES-F cells were assessed using an invasion assay; 5 × 10⁵ cells starved for 16 h were harvested, and 0.2
ml of the EMOsis-CC/TERT cell suspension in serum-free DMEM treated with the vehicle (PBS) or 100 nM estradiol with or without 10 μg/ml HGF-neutralizing antibodies and MES-F cell suspension in DMEM serum-free medium or E-EMO-CM treated with the vehicle (PBS) or 100 nM estradiol with or without 10 μg/ml HGF-neutralizing antibodies were seeded into the upper wells coated with a thin layer of Matrigel. A total of 0.6 ml of DMEM supplemented with 10% charcoal-stripped FBS was added in the lower chamber. Following incubation for 48 h, the chambers were disassembled, and the membranes were stained with hematoxylin. The number of cells penetrating across the membrane was counted under a microscope in five random visual fields. The assay was repeated three times, and the ratio was expressed as means ± SD.

**Morphological analysis.** EMOsis-CC/TERT cells were incubated in DMEM serum-free medium with the vehicle (PBS) or 100 nM estradiol with or without 10 ng/ml HGF-neutralizing antibodies. After 48 h, the cells were analyzed using a light microscope. The experiment was performed in triplicate.

**Patients and sample collection.** Tissue samples of peritoneal implant sites and normal peritoneum were obtained from five patients who underwent surgical treatment for endometrioma at the Department of Obstetrics and Gynecology of Osaka Medical College between January 2002 and July 2013. This retrospective, cross-sectional, case-controlled study of human tissue samples was approved by the Institutional Review Board of Osaka Medical College, and written, informed consent was obtained from all patients participating in the study. The inclusion criteria were as follows: an age > 20 and ≤ 40 yr at the time of the treatment procedure, the presence of regular menstrual cycles (interval of 24–35 days), body mass index of 20–30 kg/m², both ovaries present, no history of ovarian surgery, no evidence of past or recent pelvic inflammatory disease, no evidence of endocrine disorders, and no history of hormonal treatment for ≥ 12 mo prior to surgery. The diagnosis of endometriosis was confirmed histologically. The samples obtained during surgery were immediately treated with estradiol at various concentrations with or without HGF-neutralizing antibodies (10 μg/ml) for 48 h, and the degree of proliferation was measured by direct cell count (top) and CellTiter 96 AQueous (MTS) assay (bottom). B: EMOsis-CC/TERT cells were treated with HGF at various concentrations with or without HGF-neutralizing antibodies (10 μg/ml) for 48 h, and the degree of proliferation was measured by direct cell count (top) and MTS assay (bottom). C: mRNA expression of HGF was determined via semi-RT-PCR of the total RNA obtained from EMOsis-CC/TERT cells cultured with the vehicle (PBS), estradiol with or without HGF-neutralizing antibodies (10 μg/ml), or HGF-neutralizing antibodies alone for 12 h after starvation. β-Actin mRNA expression was used as the loading control. Representative examples of bands from 3 independent experiments are shown. D: EMOsis-CC/TERT cells were harvested and used to prepare cell lysates after stimulation with the vehicle (PBS), estradiol with or without HGF-neutralizing antibodies (10 μg/ml), or HGF-neutralizing antibodies for 24 h after starvation. The cell lysates were analyzed using Western blotting with antibodies to anti-HGF, anti-Met, anti-phospho-Met, and anti-β-actin. Representative examples of bands from 3 independent experiments are shown. E: EMOsis-CC/TERT cells were treated with estradiol at various concentrations for 48 h, and the HGF concentration was measured using an ELISA. The sample concentrations were determined via interpolation from the standard curve. Data are shown as means ± SD (n = 5); *P < 0.05 and **P < 0.01, significant differences compared with the untreated control groups.
fixed in 10% formaldehyde and routinely processed for paraffin embedding for the histological analysis.

**Immunohistochemistry.** The tissue samples were fixed in formalin and embedded in paraffin. Deparaffinized and rehydrated sections (4 μm) were autoclaved in 0.01 mol/l citrate buffer with a pH of 6.0 for 15 min at 121°C for antigen retrieval. The endogenous peroxidase activity was blocked with 0.3% solution hydrogen peroxide in methanol for 30 min, after which the sections were incubated at 4°C for 12 h with anti-E-cadherin antibodies (1:100 dilution), anti-N-cadherin antibodies (1:100 dilution), or anti-pan-cytokeratin antibodies (1:100 dilution). After washing with PBS, the cells were incubated with Histofine simple stain MAX PO (multi; Nichirei) for 30 min at room temperature. Finally, the slides were washed with PBS and incubated with H2O2-diaminobenzidine substrate solution for 5 min. The sections were counterstained with hematoxylin prior to dehydration and mounting. Two independent pathologists who were blinded to the clinicopathological data performed the immunohistochemical evaluations.

**Statistics.** The statistical calculations were made using the StatView statistical software package (SAS Institute, Cary, NC), and the statistical significance of differences was determined according to the Kruskal-Wallis and Mann-Whitney U-test or paired t-test, as appropriate. A P value of <0.05 was considered to be statistically significant.

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**Fig. 2.** Estradiol induced the binding of estrogen receptor-α (ERα) to the estrogen response element (ERE) site on the HGF promoter. **A:** schematic representation of the human HGF gene promoter region showing the locations of potential ERE sites (□). Two pairs of primers (labeled **primer A** and **primer B**) were designed to monitor specific regions in the gene using a chromatin immunoprecipitation (ChIP) analysis. **B:** a ChIP assay was performed using EMOSis-CC/TERT cells with primers specific to the ER-binding site of the HGF promoter. EMOSis-CC/TERT cells treated with PBS or estradiol for 16 h were used for the ChIP assays with IgG and anti-ERα antibodies, as indicated. Following cross-link reversal, the coimmunoprecipitated DNA was amplified via PCR using primers amplifying the HGF ERE-containing **region A** (−1,592/−1,523) and HGF ERE-containing **region B** (−261/−248) and subsequently resolved in 2% agarose gel.

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**Fig. 3.** Effects of estradiol on the migration and invasion of immortalized hEECs dependent on HGF. A wound-healing assay (**A**) and Matrigel invasion assay (**B**) were performed to evaluate the role of estradiol in the migratory and invasive capacity of the cells induced by HGF. **A:** line graph shows the %closure of the original wound from triplicate plates. B: mean no. of invasive cells in 5 random microscope fields is shown. *P < 0.05 and **P < 0.01, significant differences.
RESULTS

Estradiol promotes the proliferation of EMOsis-CC/TERT cells via HGF. The effects of estradiol on the proliferation of the immortalized hEECs, i.e., EMOsis-CC/TERT cells, were examined using proliferation assays. The growth of the immortalized hEECs, as assessed by manual cell counting and MTS assay, was promoted by estradiol in a dose-dependent manner (Fig. 1A). To determine the mitogenic effects of HGF on the immortalized hEECs, we explored the degree of cell proliferation. As shown in Fig. 1B, HGF stimulated the proliferation of the hEECs in a dose-dependent manner. In contrast, these findings were cancelled by the addition of HGF-neutralizing antibodies (Fig. 1A and B). These data suggest that estradiol and HGF possess proliferative effects and that estradiol promotes cell growth by inducing HGF.

Estradiol treatment promotes the expression and secretion of HGF in EMOsis-CC/TERT cells. We verified whether estradiol stimulates the HGF mRNA expression in the immortalized hEECs using a semiquantitative RT-PCR analysis. After 16 h of starvation, EMOsis-CC/TERT cells were treated with the vehicle (PBS), 100 nM estradiol, or 100 nM estradiol in the presence of 10 μg/ml HGF-neutralizing antibodies. The mRNA expression of HGF was increased in the estradiol-treated groups compared with that observed in the control group. The presence of HGF-neutralizing antibodies did not affect the estradiol-induced mRNA upregulation of HGF (Fig. 1C).

Next, we ascertained whether estradiol promotes synthesis of the HGF receptor Met in the hEECs using a Western blot analysis. The protein expression of Met was increased significantly in the estradiol-treated EMOsis-CC/TERT cells compared with that observed in the PBS-treated control cells. Treatment with HGF-neutralizing antibodies alone exerted an insignificant impact on the estradiol-induced Met protein up-regulation. Furthermore, the phosphorylation of Met was increased significantly in the estradiol-treated EMOsis-CC/TERT cells compared with that observed in the PBS-treated control cells and was subsequently inhibited by the addition of HGF-neutralizing antibodies (Fig. 1D). We then examined whether estradiol promotes HGF secretion in the hEECs using HGF-ELISA. The HGF concentration was increased significantly by estradiol administration in a dose-dependent manner (Fig. 1E), suggesting that estradiol promotes HGF secretion in EMOsis-CC/TERT cells.

Estradiol stimulates the binding of ERα to the estrogen response element of the HGF promoter in EMOsis-CC/TERT cells. We next evaluated whether the ER is recruited to the promoter of the HGF gene by performing a ChIP assay. Scanning the HGF gene revealed the presence of a potential estrogen response element (ERE) in the promoter region (nt

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Fig. 4. Estradiol regulated the epithelial-to-mesenchymal transition (EMT) process in the immortalized hEECs via HGF. A: EMOsis-CC/TERT cells were treated with vehicle (PBS) or estradiol either with or without HGF-neutralizing antibodies (10 μg/ml) for 48 h after starvation, and the morphology was viewed under a light microscope at ×10 (top) and ×400 (bottom) magnification. B: EMOsis-CC/TERT cells were harvested and used to prepare cell lysates following stimulation with vehicle (PBS) or estradiol in the presence or absence of HGF-neutralizing antibodies (10 μg/ml) for 24 h after starvation. The cell lysates were analyzed using Western blotting with antibodies to EMT markers. β-Actin was used as the loading control. Representative examples of bands from 3 independent experiments are shown.
tion in the present study, we hypothesized that estrogen promotes the EMT in immortalized hEECs via HGF production. From a morphological point of view, the EMT is characterized by an increase in cell scattering and elongation of the cell shape (20). In this study, the original appearance of the epithelial cells was observed to switch to an elongated and fibroblast-like shape along with enhanced cell scattering under conditions of estradiol treatment, and these morphological changes were reversed by the addition of HGF-neutralizing antibodies (Fig. 4A). These data indicate that estradiol mediates morphological changes that are compatible with the induction of the EMT via HGF in EMOsis-CC/TERT cells. The levels of EMT-related proteins produced in response to estradiol were subsequently evaluated using a Western blot analysis (Fig. 4B). Cadherin switching, which is characterized by the downregulation of E-cadherin and upregulation of N-cadherin, is known to be one of the most pivotal cellular events in the EMT (34). Cadherin switching was clearly observed based on the protein levels measured after estradiol stimulation; this effect was also canceled by the addition of the HGF-neutralizing antibodies. Another basic well-known EMT marker, vimentin, was also increased by estradiol treatment, and its upregulation was canceled by the addition of HGF-neutralizing antibodies. These findings suggest that the estrogen-induced EMT of hEECs is mediated via HGF.

Estradiol promotes the EMT in EMOsis-CC/TERT cells via HGF. It has been reported that estrogen enhances the migration and invasion capacity of immortalized hEECs induced by HGF, a wound-healing assay was performed. As shown in Fig. 3A, the migration of EMOsis-CC/TERT cells by estradiol increased significantly, whereas these estradiol-induced changes were canceled in the presence of the HGF-neutralizing antibodies. We also examined whether estradiol has an impact on the invasion capability of immortalized hEECs induced by HGF. Consequently, a Matrigel invasion assay showed that the invasion of EMOsis-CC/TERT cells was significantly enhanced by estradiol treatment, whereas these estradiol-induced changes were canceled by the addition of the HGF-neutralizing antibodies (Fig. 3B). These data indicate that estradiol enhances the migration and invasion capacity of hEECs via the induction of HGF.

Estradiol enhances EMOsis-CC/TERT cell migration and invasion via HGF. To evaluate whether estradiol affects the migration ability of immortalized hEECs induced by HGF, a wound-healing assay was performed. As shown in Fig. 2B, estradiol induced the binding of the ERα to the ERE site of the HGF promoter in the EMOsis-CC/TERT cells. These results demonstrate that estradiol plays a role in recruiting ERα to the ERE sites on the HGF promoter in EMOsis-CC/TERT cells.

Fig. 5. Conditioned media derived from the immortalized hEECs under conditions of estradiol treatment (E-EMO-CM) induced the mesothelial-to-mesenchymal transition (MMT) process in the mesothelial cells. A: MES-F cells were examined after being cultured with E-EMO-CM in the presence or absence of HGF-neutralizing antibodies for 48 h, and the morphology was viewed under a light microscope at ×400 magnification. B: MES-F cells were cultured for 24 h with conditioned medium (control), estradiol, or E-EMO-CM in the presence or absence of HGF-neutralizing antibodies. Cell lysates were analyzed using Western blotting with antibodies to recognized total and phosphorylated (p-Met). Met and MMT markers. β-Actin was used as the loading control. Representative examples of bands from 3 independent experiments are shown.
with the disruption of intercellular junctions and loss of apical-basolateral polarity of the mesothelium, the cells of which are transformed into fibroblast-like cells with increased migratory, invasive, and fibrogenic features (29, 35). We speculate that the MMT, an event required for peritoneal structural alteration, is involved in the etiology and progression of peritoneal endometriosis. To understand how endometriotic cells are implanted in the peritoneum and whether HGF derived from estrogen plays a role in this implantation, the cellular morphology of human mesothelial MES-F cells was examined after culture with estradiol-treated E-EMO-CM with or without HGF-neutralizing antibodies for 48 h. Cultivation with E-EMO-CM clearly induced the acquisition of a spindle-like morphology in the MES-F cells. However, these spindle-like morphological changes were not induced by estradiol itself or E-EMO-CM in the presence of the HGF-neutralizing antibodies (Fig. 5A). To verify the mesenchymal conversion of the MES-F cells through HGF/Met signaling pathway, the changes in the levels of MMT-related proteins and Met phosphorylation were evaluated using a Western blot analysis in the cells treated with each conditioned medium. As shown in Fig. 5B, phosphorylation of Met was observed in the E-EMO-CM-treated MES-F cells. Furthermore, cadherin switching was clearly observed based on the protein levels measured in the MES-F cells treated for 24 h with E-EMO-CM compared with that seen in the control medium-treated or estradiol-treated MES-F cells. Meanwhile, the addition of HGF-neutralizing antibodies under treatment with E-EMO-CM reversed the phosphorylation of Met and the cadherin switching. The expression of vimentin was also upregulated following cultivation with E-EMO-CM, and this upregulation was inhibited by the addition of the HGF-neutralizing antibodies (Fig. 5B). Basic epithelial marker cytokeratins are usually expressed in mesothelial cells and poorly expressed in fibroblasts (2, 19). A Western blot analysis showed the presence of cytokeratins in both the control and estradiol-treated MES-F cells and that E-EMO-CM treatment repressed the cytokeratin expression in the MES-F cells (Fig. 5B). The decrease in the cytokeratin expression observed under E-EMO-CM treatment was also reversed by the addition of HGF-neutralizing antibodies. These data indicate that HGF derived from the estrogen stimulation of EMOsis-CC/TERT cells mediates morphological changes compatible with the induction of the MMT in MES-F cells.

Conditioned medium obtained from estradiol-treated EMOsis-CC/TERT cells stimulates the invasion of MES-F cells. A Matrigel assay was performed to assess the impact on the invasion capability of the MES-F cells. As a result, the invasion assay showed that the invasion of MES-F cells was significantly enhanced by cultivation with E-EMO-CM but not estradiol treatment alone. Moreover, these changes were canceled by the addition of the HGF-neutralizing antibodies (Fig. 6). An immunohistochemical analysis of the mesothelial specimens also showed strong staining for immunoreactivity to E-cadherin and cytokeratin, with faint N-cadherin staining in the tissue samples of the normal peritoneum (Fig. 7). In contrast, staining for E-cadherin and cytokeratin was markedly decreased at the sites of peritoneal implantation, whereas staining for N-cadherin was enhanced at these locations (Fig. 7). Additionally, phosphorylation of Met was observed in the peritoneal implantation sites of endometriosis. These data indicate that the mesothelial cells were undergoing an MMT process through activation of Met signaling.

DISCUSSION

HGF activates the Met-signaling pathway, which is known to be a very powerful stimulator of the invasive and metastatic potential of cells, subsequently allowing for the survival of cancer cells in the bloodstream in the absence of anchorage (39). HGF is also known to be a potent angiogenic cytokine by which the HGF-Met axis is activated, thus modifying the microenvironment to facilitate cancer progression (39). In the
field of endometriosis, it has been reported that higher levels of immunoreactivity for HGF and its receptor Met are observed in the eutopic endometrium of endometriosis patients compared with that seen in subjects without endometriosis (17). In addition, HGF is found at increased concentrations in the peritoneal fluid of patients with endometriosis (16, 24), thus suggesting that the HGF present in peritoneal fluid is involved in the pathogenesis of endometriosis. A recent report showed that treating uterine fibroblasts with estradiol stimulates HGF production (7). Another previous study reported that the production of HGF in peritoneal macrophages is stimulated by estradiol (18). However, the precise mechanisms regarding such estradiol-induced HGF synthesis in human endometrial cells remain uncertain. In the current study, we added a new aspect in demonstrating direct HGF production by estradiol via recruitment of the ER\(\alpha\)/H9251 to the HGF upstream promoter element in human hEEC, EMOsis-CC/TERT cells. To the best of our knowledge, this is the first report to show that epithelial endometriotic cells exhibit autocrine growth by producing HGF under the influence of estrogen. Moreover, estradiol-induced HGF synthesis in human endometrial cells remain uncertain. In the current study, we added a new aspect in demonstrating direct HGF production by estradiol via recruitment of the ER\(\alpha\) to the HGF upstream promoter element in human hEEC, EMOsis-CC/TERT cells. To the best of our knowledge, this is the first report to show that epithelial endometriotic cells exhibit autocrine growth by producing HGF under the influence of estrogen. Moreover, estradiol-induced HGF promoted the EMT in immortalized hEECs and enhanced the migration and invasion of these cells. These data explain why many actions of HGF are mirrored by estradiol in the female reproductive tract and why the concentrations of HGF in the peritoneal fluid are high in endometriosis patients.

The most widely accepted theory regarding the pathogenesis of endometriosis is “Sampson’s hypothesis,” which suggests that the disorder originates from the retrograde menstruation of endometrial tissue sloughed through patent fallopian tubes into the peritoneal cavity, where it subsequently becomes implanted (10, 28). The retrograde menstruation/implantation theory is further supported by the finding that women with endometriosis have higher volumes of refluxed menstrual blood and endometrial tissue fragments than those without this disorder (12), and they also display more frequent subendometrial myometrial contractile waves than healthy controls with antegrade patterns (27). However, precisely how endometriotic cells invade and survive in the peritoneum remains unclear.

The EMT is important for tumor invasion and metastasis, including that observed in endometriosis (22), and mesothelial cells are thought to be the first line of defense against abdominally metastasizing tumors (13, 15). However, few studies have examined whether the peritoneum plays a role in the establishment and maintenance of endometriosis. Accordingly, we speculate that both the EMT of endometrial cells and transition from a mesothelial to a mesenchymal phenotype (MMT) in peritoneal cells play a role in the pathogenesis of peritoneal endometriosis. As expected, the MMT was observed in the human mesothelial MES-F cells under the influence of the estradiol-mediated activation of HGF produced by human epithelial endometrial EMOsis-CC/TERT cells in this study. These findings suggest that a high concentration of HGF in the peritoneal fluid affects endometriotic cells by not only promoting the EMT of endometriotic cells but also causing mesothelial cells to lose their polarity via the MMT. The MMT in mesothelial cells may provide the basis for the peritoneal invasion of endometrial cells. Although our in vitro data showed that endometriosis-derived transformed cells undergo...
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