0021-972X/06/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 91(6):2358–2365

doi: 10.1210/jc.2005-0601

Extracellular Matrix Metalloproteinase Inducer Regulates Metalloproteinases in Human Uterine Endometrium

A. G. Braundmeier, A. T. Fazleabas, B. A. Lessey, H. Guo, B. P. Toole, and R. A. Nowak

Department of Animal Sciences (A.G.B., R.A.N.), Urbana, Illinois 61801; Department of Obstetrics and Gynecology, University of Illinois (A.T.F.), Chicago, Illinois 60612; Department of Anatomy and Cell Biology, Tufts University (H.G.), Boston, Massachusetts 02111; Department of Cell Biology and Anatomy, Medical University of South Carolina (B.P.T.), Charleston, South Carolina 29425; and Department of Reproductive Endocrinology, Greenville Hospital System (B.A.L.), Greenville, South Carolina 29605

Context: Endometrial remodeling occurs during each menstrual cycle in women and also during the establishment of endometriosis. Both processes involve the production of metalloproteinases (MMPs) by uterine endometrial cells.

Objective: The objective of this study was to determine whether tissue remodeling and endometrial invasion involve activation of MMPs by extracellular matrix metalloproteinase inducer (EMMPRIN).

Main Outcome Measures: EMMPRIN expression was examined by immunohistochemistry and real-time PCR in ectopic and eutopic endometria. For functional assays, human uterine fibroblasts were treated in the absence or presence of IL-1β (10 ng/ml) or purified native EMMPRIN (0.5 or 1 μg/ml) for 24 h. Cellular RNA and conditioned medium were assayed by real-time PCR or immunoblotting.

Results: EMMPRIN protein localized to epithelial and fibroblast cells of eutopic and ectopic endometria. The pattern of localization was regulated by ovarian hormones. EMMPRIN mRNA levels varied throughout the menstrual cycle in parallel with the cyclic changes in estradiol. EMMPRIN treatment (0.5 μg/ml) of human uterine fibroblast cells stimulated MMP-1 (5.23-fold) and MMP-2 (8.55-fold), but not MMP-3, mRNA levels over levels in control cells ($P < 0.05$). EMMPRIN treatment (1 μg/ml) stimulated endogenous EMMPRIN (1.6-fold) mRNA levels ($P > 0.05$). IL-1β stimulated MMP-1 (5.6-fold), MMP-2 (2.8-fold), and MMP-3 (75-fold) gene expression, but not EMMPRIN, over levels in control cells ($P < 0.05$). Both EMMPRIN and IL-1β treatments stimulated MMP-1, -2, and -3, but not EMMPRIN protein secretion, with 0.5 μg/ml producing the greatest response.

Conclusions: The ability of EMMPRIN to stimulate MMP secretion by endometrial fibroblasts indicates its potential role in uterine remodeling and the pathogenesis of endometriosis. (***J Clin Endocrinol Metab* 91: 2358–2365, 2006**)

HUMAN UTERINE ENDOMETRIUM consists of three different layers: the stratum basalis, the stratum spongiosum, and the stratum compactum. During menstruation, the last two layers, commonly called the functionalis layer, are degraded and sloughed off, leading to uterine bleeding. As estrogen and progesterone levels fall before menstruation, the endometrium shows a significant increase in the expression of a group of proteinases called matrix metalloproteinases (MMPs) (1–4), and these regulate the breakdown and remodeling of the endometrium. This cycle of remodeling is necessary for preparation of the endometrium for potential implantation and pregnancy. However, menstrual shedding is also hypothesized to be a major causative factor for the establishment of the disease endometriosis.

Endometriosis is the adherence and growth of components of the functionalis layer of the endometrium outside the uterine cavity. The most common theory for the displacement of endometrial tissue in the peritoneal cavity is retrograde menstruation. The endometrial fragments dispersed throughout the peritoneal cavity remain responsive to ovarian hormone regulation (5, 6). The pathogenesis of endometriosis is poorly understood; however, for endometrial fragments to adhere, invade, and develop, there must be extensive remodeling of the peritoneal mesothelial layer. As with normal menstruation, this remodeling requires activation of MMPs. Several MMPs are expressed in the endometrium, and their expression patterns change throughout the menstrual cycle (reviewed in Ref. 7). With the exception of MMP-2, which is expressed constitutively throughout the cycle, most MMPs are up-regulated when estrogen levels are rising and are down-regulated once progesterone levels increase. The activity of MMPs is controlled by growth factors, hormones, cytokines, immune cells, and tissue inhibitors of metalloproteinases (8–11). Within the last decade, a major inducer of MMPs has been characterized and identified as extracellular matrix metalloproteinase inducer (EMMPRIN).

EMMPRIN is a member of the Ig superfamily, which includes T cell receptors, neural cell adhesion molecules, and major histocompatibility complex antigens. This glycoprotein was first identified as tumor cell collagenase stimulatory factor, a tumor cell surface molecule that stimulates nearby fibroblasts to produce MMPs (12–15). EMMPRIN has been

First Published Online March 7, 2006

Abbreviations: EMMPRIN, Extracellular matrix metalloproteinase inducer; ER, estrogen receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HUF, human uterine fibroblast; MMP, matrix metalloproteinase; PR, progesterone receptor.

JCEM is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.

shown to play a role in monocarboxylic acid transport (16) and reproduction (17). Glycosylation of EMMPRIN is important for its effects on MMP production (18). Most EMMPRIN is located on the cell surface; however, 2–3% can be isolated as a soluble form (19) through microvesicle shedding (20). A recent study by Noguchi *et al.* (21) reported that EMMPRIN protein is expressed in the human endometrium throughout the menstrual cycle. However, they did not examine all stages of the menstrual cycle, such as the menstrual phase.

The objectives of this study were: 1) to examine more closely the pattern of EMMPRIN protein localization and mRNA expression in human eutopic endometrium throughout the menstrual cycle; 2) to determine the effect of purified native EMMPRIN on endogenous EMMPRIN and MMP-1, -2 and -3 mRNA expression and protein secretion by primary cultures of human uterine fibroblast cells; and 3) to determine whether EMMPRIN protein and mRNA are expressed in ectopic endometrium (*i.e.* in endometriotic lesions). The MMPs chosen were representative of each class of MMPs (MMP-1, collagenase-1; MMP-2, gelatinase A; and MMP-3, stromelysin-1).

Patients and Methods

Acquisition of human uterine endometrium

Human endometrial tissues processed for immunohistochemistry were obtained from premenopausal patients undergoing hysterectomy for leiomyomas, who did not have endometriosis, under an approved institutional review board protocol for consent to use discarded tissue for research purposes (Brigham and Women's Hospital, Boston, MA). Human endometrial tissues processed for real-time PCR were obtained from the Specialized Cooperative Centers Program in Reproduction Research Reproductive Tissue Sample Repository (Chapel Hill, NC). These were also women who had not been diagnosed with endometriosis. The stage of the menstrual cycle was determined by the hospital obstetric/gynecology pathologists using the endometrial dating method of Noyes *et al*. (22) or an LH-monitoring test. Ectopic and eutopic endometria for real-time PCR analysis were obtained through the Specialized Cooperative Centers Program in Reproduction Research Reproductive Tissue Sample Repository from five patients undergoing removal of endometriotic lesions by laparoscopy. None of the patients was receiving any type of drug therapy at the time of surgery, and all were cycling. Slides of endometriotic lesions for immunohistochemical analysis were obtained from Brigham and Women's Hospital. The pathologists were able to determine from the records that these patients had not been receiving any hormonal therapy.

Cell isolation, culture, and treatments

Purified EMMPRIN was isolated from LX-1 human lung carcinoma cell membrane extracts by immunoaffinity chromatography as previously described (13). Human uterine fibroblast (HUF) cells were obtained from the Cell Biology Core Laboratory of the Center for Women's Reproductive Health at University of Illinois-Chicago College of Medicine under an approved institutional review board protocol. The cells were isolated and processed as described previously (23) and were received in our laboratory at passage 2. HUF cells have been used by other investigators as a model cell line to study the regulation of decidualization. These cells are nondecidualized fibroblasts isolated from term deciduas, and they can undergo decidualization in culture in the presence of estrogen, progesterone, and either cAMP or IL-1β (24–26). Because of the ability of these cells to undergo decidualization in the presence of steroid hormones, hormones were excluded from our cell-conditioned medium. HUF cells were grown to confluence in phenol red-free RPMI 1640 culture medium supplemented with 5% fetal bovine serum, 5% bovine calf serum, 120 U/ml penicillin/streptomycin (Cambrex, Walkersville, MD), and 3.2 mM L-glutamine (Cambrex). Once cells reached confluence, they were transferred to serum-free medium containing only L-glutamine and antibiotics for 36–40 h. After this washout period, the cells were treated with 10 ng/ml IL-1β (catalog no. 201-LB, R&D Systems, Inc., Minneapolis, MN) or 0.5 and 1 μg/ml purified EMMPRIN for 24 h in serum-free conditioned medium. Conditioned medium samples were collected, and cells were harvested in TRIzol reagent (Invitrogen Life Technologies, Inc., Carlsbad, CA) for RNA isolation. Cell lines were established from three different patients and were used at passage 5 in these studies.

RNA isolation and quantitative RT-PCR

Total RNA was extracted from cells or tissue using TRIzol (Invitrogen Life Technologies, Inc.) according to the manufacturer's instructions. One microgram of total RNA was used in a 20-μl volume of RT reactions using RETROscript First Strand Synthesis Kit for RT-PCR following the manufacturer's instructions (Ambion, Inc., Austin, TX). Synthesized cDNA was used for real-time PCR analysis.

Real-time PCR analyses were performed in a 25-μl volume containing 1× TaqMan Universal PCR Master Mix No AmpErase UNG (catalog no. 4324018, Applied Biosystems, Atlanta, GA), diluted cDNA, and ribonuclease-free water. Genes were amplified using either 1× Assays-on-Demand Gene Expression Assay Mix (Applied Biosystems) or 500 nM primers and 150 nM TaqMan minor groove binding probe sets. MMP-1 and -2 primer probe sets were designed using Primer Express software (PerkinElmer, Norwalk, CT). The 20× Assay-on-Demand Gene Expression Assays were purchased for MMP-3, EMMPRIN, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; catalog no. Hs00233962m1, Hs00174305m1, and Hs99999905m1, respectively) sequences or reference sequences as listed in Table 1. Minor groove binding probes were labeled with the fluorescent reporter dye 6-carboxyfluorescein to directly detect PCR products. Real-time PCR amplification and detection were performed in MicroAmp optical 96-well reaction plates using the ABI PRISM 7700 sequence detection system (Applied Biosystems). Amplification conditions included holding for 10 min at 95 C, 40 thermal cycles of denaturing for 15 sec at 95 C, and annealing/extending for 1 min at 60 C. Relative fold induction levels were calculated using the comparative threshold cycle method for separate tube amplification. GAPDH gene expression served as an endogenous control.

Immunoblotting

Conditioned media were concentrated 75-fold with Amicon Ultra-15 10-kDa centrifugal filters (Millipore Corp., Billercia, MA). Equal volumes of concentrated media were denatured in Laemmli sample buffer at 95 C for 5 min. Proteins were separated by SDS-PAGE (10% acrylamide) and transferred onto 0.45-μm pore size Protran nitrocellulose membranes (Schleicher & Schuell, Keene, NH) overnight at 4 C. The membranes were incubated in Tris-buffered saline containing 0.1% Tween 20 with 5% nonfat dry milk for 2 h at 25 C to block nonspecific binding. Antibodies were purchased from R&D Systems, Inc. (MMP-1 MAB901, MMP-3 MAB905, and EMMPRIN AF972) or Calbiochem (MMP-2 IM33L). Membranes were incubated with 0.1–1 μg/ml specific antibody in Tris-buffered saline containing 0.1% Tween 20 containing

TABLE 1. Sequences of primer sets and probes used for TaqMan PCR.

Gene	Reference sequence	Forward primer	Reverse primer	Probe
MMP-1		CCTCGCTGGGAGCAAACA	TTGGCAAATCTGGCGTGTAAT	ATCTGACCTACAGGATTG
MMP-2		CCCTCGCTGGGAGCAAA	CTTGGCAAATCTGGCGTGTAAT	ATCTGACCTACAGGATTGA
MMP-3	TGAACCTTGTTCAGAAATATCTAGA			
EMMPRIN	GGGGCTGCCGGCACAGTCTTCACT			
GAPDH	GGGCGCCTGGTCACCAGGGCTGCTT			

2.5% nonfat dry milk for 90 min at 25 C and then washed five times to remove unbound antibody. Membranes were incubated with a 1:15,000 dilution of antimouse IgG-HRP in Tris-buffered saline containing 0.1% Tween 20 with 2.5% nonfat dry milk for 45 min at 25 C. Antibodies were detected using the Super Signal chemiluminescence system (Pierce Chemical Co., Rockford, IL). Unstained protein molecular weight markers were used as standards (Bio-Rad Laboratories, Hercules, CA) and detected with Ponceau staining. Densitometry was performed on Kodak ID 3.5 software (Eastman Kodak Co., Rochester, NY), and net intensity was recorded as the number of pixels of band minus background.

Immunohistochemistry

Human uterine tissues were fixed in either 10% neutral buffered formalin or 1X Histochoice (Amresco, Solon, OH) and embedded into paraffin blocks. Tissue blocks were sectioned at 5 μm and mounted on poly-L-lysine-coated slides. Antigen retrieval was performed by boiling sections in 10 mM citrate buffer (pH 6.0) for 10 min, and endogenous peroxidase activity was quenched with methanol containing 0.3% hydrogen peroxide for 15 min. Nonspecific binding was blocked with 5% normal rabbit serum in PBS containing 1% BSA for 20 min. Slides were then incubated with 2 μg/ml goat polyclonal antibody against recombinant human EMMPRIN (R&D Systems, Inc.) in 1% BSA in PBS at 4 C overnight. Nonspecific goat IgG (2 μg/ml) in 1% BSA in PBS served as a negative control. Slides were washed, then incubated with biotinylated rabbit antigoat IgG (Vector Laboratories, Inc., Burlingame, CA) diluted 1:100 with 1% BSA in PBS for 60 min at room temperature. Detection of EMMPRIN was performed by incubation of sections for 45 min with avidin-biotinylated peroxidase complex, reacted with 0.2 mg/ml metal-3,3′-diaminobenzidine (Sigma-Aldrich Corp., St. Louis, MO) in Tris-HCl buffer (pH 7.6), then counterstained with hematoxylin.

Statistical analysis

An ANOVA model to evaluate experimental variability was used to determine differences in treatments. The difference between the threshold cycle of target gene and GAPDH was used to determine statistical significance. Threshold cycle was defined as the cycle number at which all transcripts are in the linear phase of amplification. The difference between target and GAPDH was then normalized for control treatment expression and expressed as the relative fold difference. The statistical significance between treatments is indicated by different letters above treatments. Tables 2 and 3 show the average change in threshold cycles and SE values of each treatment for all genes.

Results

EMMPRIN protein localization and expression in eutopic human endometrium

EMMPRIN protein localization followed a cyclical pattern throughout the menstrual cycle and was quite different for epithelial cells compared with the fibroblast cells. During the menstrual phase, EMMPRIN protein expression was quite strong in fibroblasts, but little immunoreactivity was evident in glandular epithelial cells (Fig. 1B). During the proliferative phase, EMMPRIN protein was strongly expressed in epithelial cells (Fig. 1, C and D), whereas expression in uterine fibroblast cells was more stratified, with only the fibroblast cells near the lumen showing positive immunoreactivity. During the secretory phase of the cycle, EMMPRIN expression became progressively stronger and more widespread in fibroblasts, but became weaker and spotted in glandular and luminal epithelial cells (Fig. 1, E–G). Thus, EMMPRIN protein expression in epithelial cells was highest during the proliferative phase when estradiol levels were elevated, but was downregulated throughout the secretory phase when progesterone levels were high, and epithelial cells lose their expression of both progesterone (PR) and estrogen (ER) receptors. In contrast,

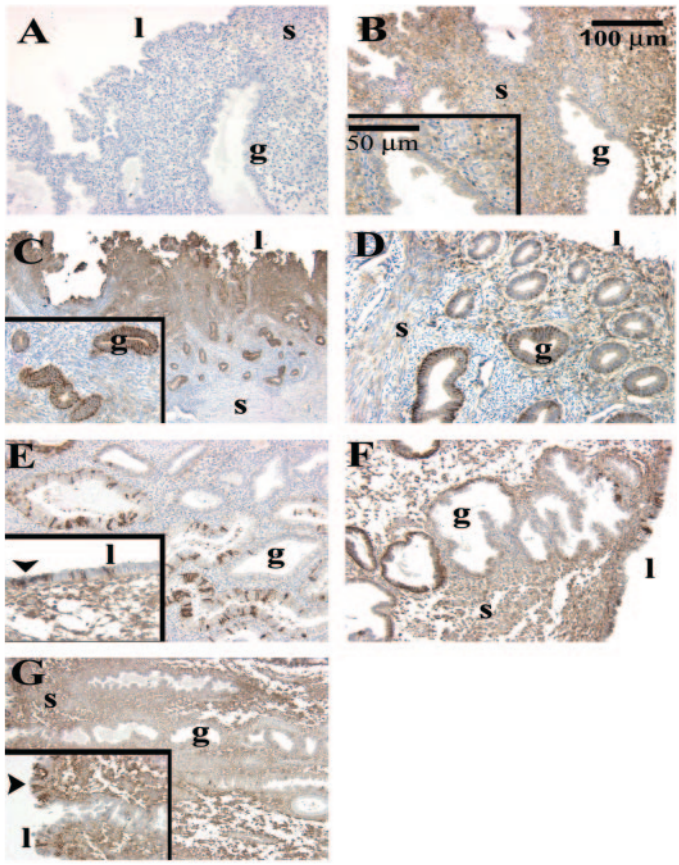

FIG. 1. EMMPRIN protein localization in cycling human eutopic uterine endometrium. Tissue sections were probed with human anti-EMMPRIN antibody or nonspecific IgG. The stages of the menstrual cycle shown are: A, early menses; control, nonspecific IgG; B–G, probed with anti-EMMPRIN antibody; B, early menses (*inset*, ×40); C, early proliferative (*inset*, ×40); D, midproliferative (×20); E, early secretory (*inset* luminal epithelium, ×40); F, midsecretory; and G, late secretory (*inset* luminal epithelium; ×40). Unless noted, all pictures were taken at ×20 magnification. *Arrowheads* denote EMMPRIN localization to luminal epithelial cells. s, Stroma; l, uterine lumen; g, gland.

the fibroblasts located closer to the uterine lumen showed increased EMMPRIN immunoreactivity in the early and midsecretory phases of the cycle at the time of high progesterone levels and high PR expression. During the late secretory and menstrual phases, when progesterone levels declined, the entire stroma showed immunostaining for EMMPRIN.

Quantitative expression of EMMPRIN mRNA in cycling endometrium

Endometrial mRNA samples were collected from four stages of the menstrual cycle, proliferative (cycle d 7–14), early secretory (cycle d 14–19), midsecretory (cycle d 20–23), and late secretory (cycle d 24–27). EMMPRIN expression was normalized to GAPDH and calibrated to late secretory stage mRNA levels. EMMPRIN expression was significantly higher in the proliferative phase (1.9-fold; $P < 0.05$) than in any of the secretory phases of the cycle (Fig. 2 and Table 2). The midsecretory phase group had significantly higher expression of EMMPRIN mRNA ($P < 0.05$) than either the early or late secretory stage (Fig. 2 and Table 2). The profile of

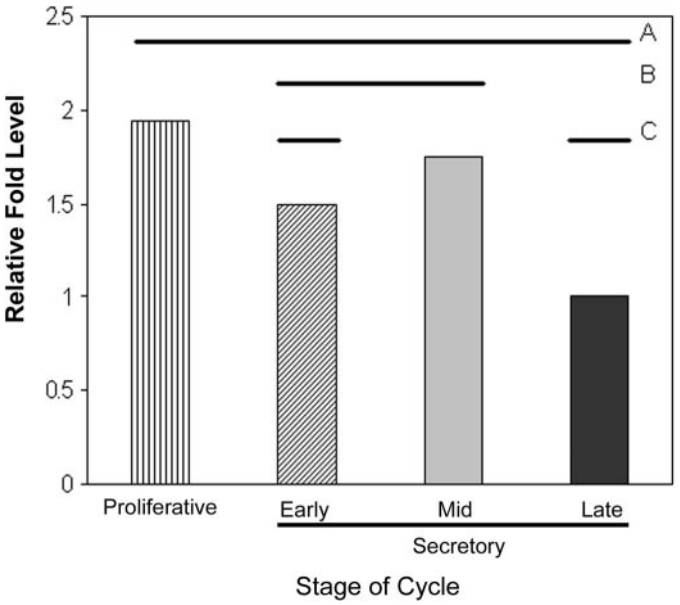

FIG. 2. Changes in EMMPRIN mRNA levels in uterine endometrium throughout the menstrual cycle. Biopsies of eutopic endometrium from throughout the menstrual cycle were analyzed by real-time PCR for EMMPRIN mRNA expression. EMMPRIN mRNA levels were normalized to GAPDH and calibrated to the late secretory stage to determine relative expression levels. Bar A indicates significant differences between proliferative and secretory stages, bar B indicates significant differences between mid- and late secretory stages, and bar C indicates significant differences between early and late secretory stages (n = 4 for each cycle stage; $P < 0.05$).

EMMPRIN gene expression indicates that EMMPRIN mRNA levels were positively correlated with estrogen and ER expression throughout the menstrual cycle.

EMMPRIN stimulates MMP-1, 2, and -3 and EMMPRIN gene expression and protein secretion in HUF cells

To determine whether EMMPRIN regulates the expression of MMPs in the uterine endometrium, we treated HUF cells with purified native EMMPRIN. IL-1β, a well-known stimulator of MMP production by fibroblast cells, was used as a positive control. We found that EMMPRIN stimulation of MMP-1 and -2 mRNA levels in HUF cells was comparable to that observed for IL-1β treatment after 24 h. EMMPRIN stimulated MMP-1 mRNA levels 5.2-fold (0.5 μg/ml) and 2.46-fold (1 μg/ml), whereas IL-1β stimulated MMP-1 mRNA levels 5.6-fold over control levels. EMMPRIN also stimulated MMP-2 mRNA levels by 8.55-fold (0.5 μg/ml) and 4.68-fold (1 μg/ml), whereas IL-1β stimulated MMP-2 mRNA 8.23-fold over control levels (Fig. 3A and Table 3). Treatment with EMMPRIN did not increase MMP-3 mRNA

TABLE 2. Average change in cycle threshold (Avg ΔCt) and SE values of EMMPRIN mRNA levels for normal cycling endometrium

Stage of cycle	EMMPRIN	
	Avg ΔCt	SE
Proliferative	−0.271	0.196
Early secretory	0.106	0.108
Midsecretory	−0.121	0.171
Late secretory	0.685	0.202

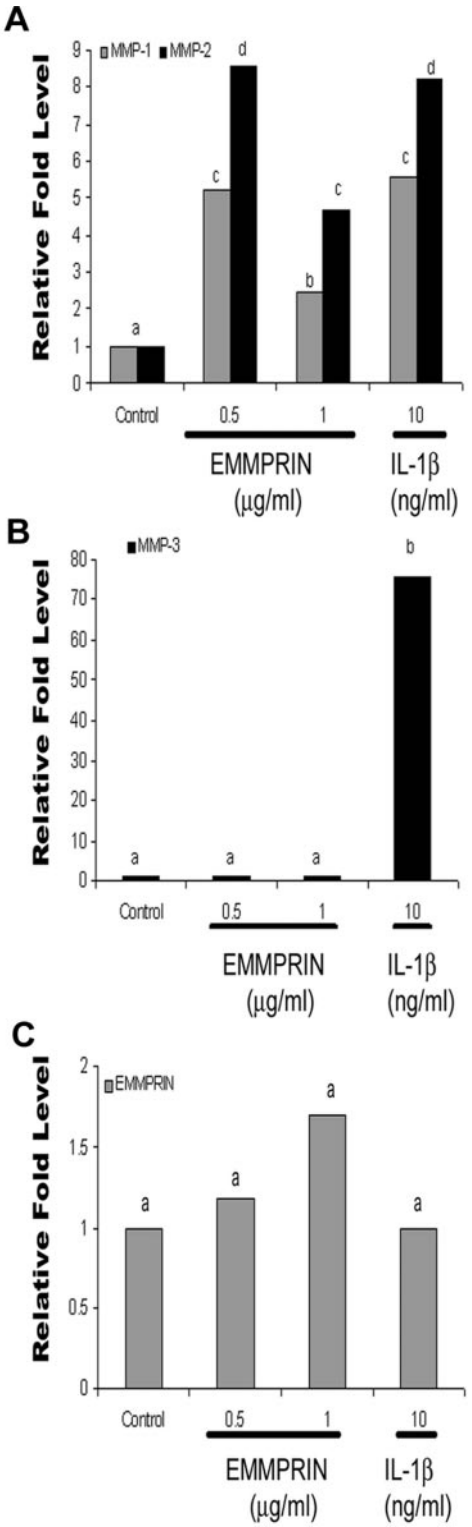

FIG. 3. Changes in MMP-1, -2, and -3 and EMMPRIN mRNA levels in response to treatment with EMMPRIN or IL-1β. RNA was collected after treatment of HUF cells with either IL-1β or EMMPRIN at the indicated concentrations for 24 h. Fold changes in mRNA levels were quantitated by TaqMan PCR normalized to GAPDH and calibrated to control levels. A, Changes in MMP-1 and -2 mRNA levels; B, changes in MMP-3 mRNA levels; C, changes in EMMPRIN mRNA levels (n = 3; $P < 0.05$). Statistical significance was calculated from normalized data and is indicated by *different letters* above treatments.

TABLE 3. Average change in cycle threshold (Avg ΔCt) and SE values for MMP-1, MMP-2, MMP-3, and EMMPRIN mRNA levels for each treatment

Treatment	MMP-1		MMP-2		MMP-3		EMMPRIN	
	Avg ΔCt	SE	Avg ΔCt	SE	Avg ΔCt	SE	Avg ΔCt	SE
Control EMMPRIN	7.533	0.064	7.571	0.135	6.710	0.087	1.057	0.103
0.5 μg/ml EMMPRIN	6.657	0.318	7.273	0.206	6.623	0.163	0.817	0.083
1 μg/ml EMMPRIN	7.063	0.369	7.737	0.223	6.727	0.099	0.293	0.076
Control IL-1β	8.524	0.816	9.294	1.074	9.16	0.877	0.698	1.054
10 ng/ml IL-1β	6.045	0.889	6.420	1.172	2.918	0.763	0.704	0.968

levels, whereas IL-1β caused a 75-fold increase in MMP-3 mRNA (Fig. 3B and Table 3). Treatment with IL-1β had no effect on EMMPRIN mRNA levels, whereas treatment with EMMPRIN (1 μg/ml) caused a 1.6-fold increase in endogenous EMMPRIN mRNA levels (Fig. 3C and Table 3).

Immunoblot analysis of HUF cell-conditioned medium showed that both EMMPRIN and IL-1β increased MMP-1, -2, and -3 protein secretion after 24 h of treatment (Fig. 4). Neither EMMPRIN nor IL-1β stimulated EMMPRIN protein secretion (data not shown). Expanded time-course studies

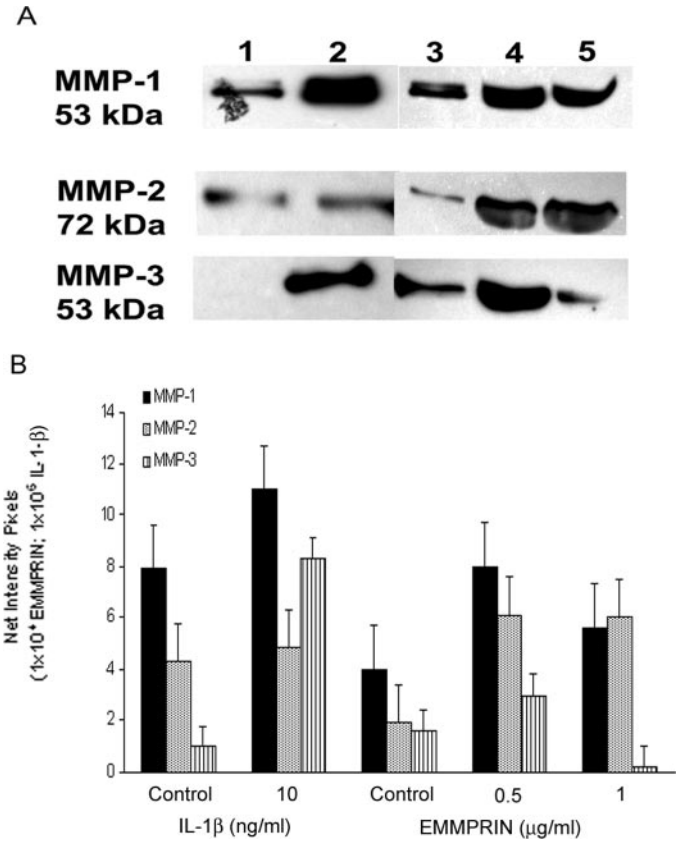

FIG. 4. Changes in MMP-1, -2, and-3 protein secretion in response to EMMPRIN or IL-1β. A, Immunoblots of conditioned medium samples collected from treated HUF cells. Membranes were probed with specific antibodies and detected with appropriate secondary antibodies. Lane 1, Control IL-1β; lane 2, 10 ng/ml IL-1β; lane 3, control EMMPRIN; lane 4, 0.5 μg/ml EMMPRIN; lane 5, 1 μg/ml EMMPRIN (n = 3). Treatments of cells with IL-1β and EMMPRIN were conducted in separate experiments; therefore, two sets of untreated control cells are shown. B, Densitometric analysis of immunoblots was normalized to the values for untreated control medium samples. *Bars* indicate the SE of treatments.

were not performed due to the limited amount of purified native EMMPRIN available.

EMMPRIN protein localization and mRNA expression in endometriotic lesions

Endometriotic lesions were analyzed for the expression of EMMPRIN mRNA and protein. Examination of histological sections from several lesions showed that EMMPRIN protein expression was highly localized to the basal side of uterine glandular epithelial cells and also to uterine fibroblast cells within the endometriotic lesions (Fig. 5, A, c–e). Immunostaining of lesions that contained peritoneal mesothelium revealed that the mesothelial cells also showed strong EMMPRIN protein expression (Fig. 5 A, f, note *arrowhead*).

Real-time PCR analysis of matched eutopic and endometriotic lesions confirmed that EMMPRIN mRNA levels were elevated in ectopic lesions collected during the follicular, early, mid-, and late secretory phases of the cycle in four of five patients compared with eutopic EMMPRIN mRNA levels (Fig. 5B). All samples were normalized to control patients at the same stage of the cycle.

Discussion

Menstruation is a process unique to women and some primates. The cyclic breakdown and subsequent regrowth of the endometrial layer of the uterus throughout each menstrual cycle are critical for maintenance of a functional endometrium. This extensive remodeling of the uterine lining requires precise regulation and activation of MMPs. A number of MMPs are expressed by fibroblasts of the endometrium, including MMP-1, -2, -3, -9, -10, and -11 (2, 27, 28). The results of our present studies confirm that EMMPRIN, a glycosylated transmembrane protein, is expressed by both uterine epithelial and fibroblast cells. Our results also show that EMMPRIN regulates the production of MMP-1, -2, and -3 by uterine fibroblast cells. In addition, we are the first to show that EMMPRIN is expressed not only by uterine cells of eutopic endometrium, but also by uterine cells and peritoneal mesothelial cells in endometriotic lesions.

Immunohistochemical analysis of EMMPRIN protein expression showed that uterine epithelial cells and fibroblasts display significantly different patterns of expression throughout the menstrual cycle. EMMPRIN protein expression in uterine epithelial cells was strongest during the proliferative phase, when ER and PR are maximally expressed in these cells (29, 30). EMMPRIN expression then decreased in epithelial cells during the secretory and menstrual phases in parallel with a loss of ER and PR expression. The expres-

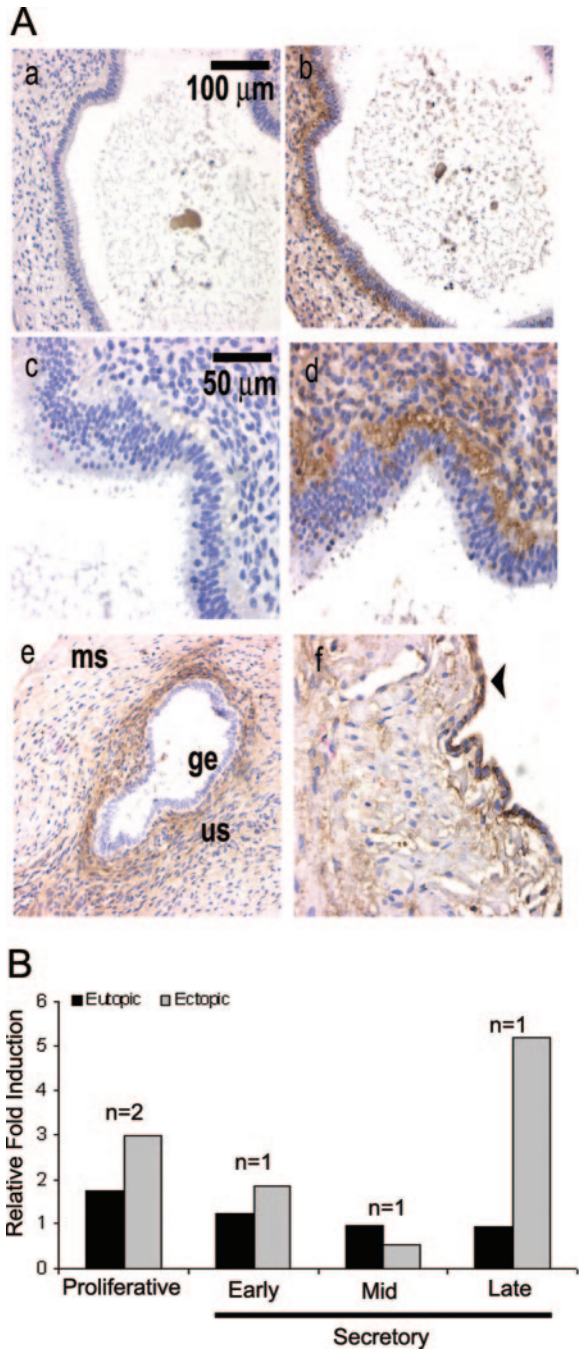

FIG. 5. EMMPRIN protein and mRNA expression in ectopic human endometrium (endometriotic lesions). A, Representative immunohistochemical analysis of EMMPRIN protein localization in lesions from three patients. Patient 1, a, Control, nonspecific IgG; c, anti-EMMPRIN antibody; patient 2, b, control, nonspecific IgG; d, anti-EMMPRIN antibody; e and f, patient 3, anti-EMMPRIN antibody. The *arrowhead* denotes EMMPRIN expression in peritoneal mesothelial cells. Magnification of photos: a and c, ×20; b and d, ×40; e, ×20; and f, ×40. ms, Mesothelial stroma; ge, uterine glandular epithelium; us, uterine stroma. B, Real-time PCR analysis of matched eutopic and ectopic endometria for EMMPRIN mRNA levels. Samples were normalized to endogenous control GAPDH mRNA levels and calibrated to eutopic endometrium of disease-free controls at the same stage of the menstrual cycle. The experimental unit is indicated above each sample.

sion pattern in uterine fibroblast cells was more complicated, showing a slow spread of EMMPRIN expression from the luminal toward the basal layers of the endometrium during the secretory phase. At menstruation, there was a high level of immunoreactivity for EMMPRIN throughout the entire stroma. Thus, in contrast to uterine epithelial cells, fibroblasts show increased expression of EMMPRIN during the secretory phase when progesterone and PR are high. Noguchi *et al.* (21) recently reported that progesterone increases the production and glycosylation of EMMPRIN in cultured uterine fibroblast cells. We hypothesize that during menstruation, in response to the withdrawal of steroid hormone support, uterine fibroblast cells secrete/release EMMPRIN protein into the surrounding extracellular areas, and this contributes to the increase in MMP proteins in the endometrium that is seen at this time of the cycle.

The results of our immunostaining studies differ from those reported by Noguchi *et al.* (21). These investigators reported that there was no immunoreactivity for EMMPRIN in luminal epithelial cells at any time throughout the menstrual cycle. In contrast, we observed expression of EMMPRIN in some, but not all, luminal epithelial cells at various stages of the menstrual cycle, including the secretory phase. EMMPRIN was localized primarily to the lateral cell membranes of luminal epithelial cells, whereas it was usually more basolateral in glandular epithelial cells. Noguchi *et al.* (21) also reported weaker immunoreactivity in fibroblast cells during the secretory phase than what we observed in our own studies. They did not examine the expression of EMMPRIN protein during the menstrual phase of the cycle. The differences in our results may be due to the use of antibodies of differing sensitivities and would also be dependent on the accuracy of dating of the endometrial samples used in our respective analyses. EMMPRIN gene expression followed a pattern similar to that of protein expression during the menstrual cycle. The levels of EMMPRIN mRNA were highest during the proliferative phase of the cycle when the immunoreactivity in glandular epithelium was strongest, and some areas of the stroma also showed protein expression.

Immunohistochemical analysis of endometriotic lesions showed that both uterine cells and peritoneal mesothelial cells in these lesions express EMMPRIN protein and mRNA. The expression of EMMPRIN by ectopic endometrium could be involved in stimulating MMP production at the site of invasion into the peritoneal wall or on the surface of the ovary. Moreover, EMMPRIN may serve an additional important function during the initial establishment of these lesions. EMMPRIN is known to bind to itself through homophilic interactions and is thought to serve as an attachment molecule (18). The fact that both ectopic endometrium and peritoneal mesothelial cells express EMMPRIN suggests that EMMPRIN may aid in the initial attachment of endometrial cells to the peritoneal mesothelium via homophilic interactions. Both of these functions of EMMPRIN allow for the establishment of endometriotic lesions.

Primary cultures of human uterine fibroblast cells were used to examine the effects of purified native EMMPRIN on MMP-1, -2, and-3 production. IL-1β treatment was used as a positive control to compare the effectiveness of EMMPRIN

stimulation of MMPs to a known MMP stimulator. Our results showed that EMMPRIN increased MMP-1 and -2 mRNA levels and protein secretion by 5- to 8-fold. A similar level of stimulation was seen with IL-1β. The effects of EMMPRIN and IL-1β on MMP-3 production were somewhat different. EMMPRIN had no effect on MMP-3 mRNA levels and increased MMP-3 protein secretion by approximately 3-fold at the 0.5 μg/ml concentration. In contrast, IL-1β treatment resulted in a 70-fold increase in MMP-3 mRNA levels and a 12-fold increase in MMP-3 protein levels in conditioned medium. Throughout these experiments, we observed that the 0.5 μg/ml concentration of EMMPRIN was somewhat more effective than the 1.0 μg/ml concentration. However, a broader dose-response curve was not performed due to the very limited amount of protein available. The concentrations of native, purified EMMPRIN we used are similar to those used in other studies to assess its effects on MMP production by cancer cells and fibroblasts (31). IL-1β had no effect on EMMPRIN mRNA levels in uterine fibroblast cells and did not stimulate the secretion of EMMPRIN into the culture medium. EMMPRIN caused a modest 1.6-fold increase in EMMPRIN mRNA levels in fibroblasts, but did not increase the secretion of EMMPRIN protein into the medium. This result is consistent with the finding that lung fibroblasts had increased EMMPRIN gene and protein expression after treatment with a soluble recombinant form of EMMPRIN (32). In this study, cells were treated with a higher concentration (5 μg/ml) of EMMPRIN and showed a 4.5-fold increase compared with the 1.6-fold increase we observed with 0.5 μg/ml native purified EMMPRIN.

The signaling pathway used by EMMPRIN to stimulate MMPs is poorly understood. Previous studies reported that EMMPRIN stimulates MMP-1 or -2 production by fibroblasts using the p38 MAPK pathway (31), but stimulates MMP production by breast cancer cells using the phospholipase A_2 and 5-lipoxgenase pathways (19). Another potential signaling pathway may involve ErbB2. Recent studies by Toole and colleagues (33, 34) reported that EMMPRIN stimulates hyaluronan production by colon carcinoma and mammary carcinoma cells. Hyaluronan binds to CD44 on the surface of these cells, and this interaction leads to elevated ErbB2 phosphorylation. A receptor for EMMPRIN has not yet been identified, although it is known that EMMPRIN can activate MMP production through homophilic binding (18). The results of our studies confirm a role for EMMPRIN in the uterine endometrial remodeling that occurs during the menstrual cycle and suggest that EMMPRIN may be involved in the development of endometriosis. Additional studies to identify the specific signaling pathway of EMMPRIN will be critical to understanding how EMMPRIN regulates MMP production and secretion in both eutopic and ectopic human uterine endometria.

Acknowledgments

We thank Dr. Masaaki Nakai and Meagan Grudzien for their help with the immunostaining. We also thank Drs. Li Chen and Robert Belton, Jr., for helpful discussion throughout the course of this work.

Received March 18, 2005. Accepted February 28, 2006.

Address all correspondence and requests for reprints to: Dr. Romana Nowak, University of Illinois, 1207 West Gregory Drive, Urbana, Illinois 61801. E-mail: ranowak@uiuc.edu.

This work was supported by National Institutes of Health Grants NIH U54 HD 40093 (to R.A.N.) and NIH T32 HD 007028.

References

1. **Marbaix E, Donnez J, Courtoy PJ, Eeckhout Y** 1992 Progesterone regulates the activity of collagenase and related gelatinases A and B in human endometrial explants. Proc Natl Acad Sci USA 89:11789–11793
2. **Rodgers WH, Matrisian LM, Giudice LC, Dsupin B, Cannon P, Svitek C, Gorstein F, Osteen KG** 1994 Patterns of matrix metalloproteinase expression in cycling endometrium imply differential functions and regulation by steroid hormones. J Clin Invest 94:946–953
3. **Rodgers WH, Osteen KG, Matrisian LM, Navre M, Giudice LC, Gorstein F** 1993 Expression and localization of matrilysin, a matrix metalloproteinase, in human endometrium during the reproductive cycle. Am J Obstet Gynecol 168:253–260
4. **Salamonsen LA, Butt AR, Hammond FR, Garcia S, Zhang J** 1997 Production of endometrial matrix metalloproteinases, but not their tissue inhibitors, is modulated by progesterone withdrawal in an *in vitro* model for menstruation. J Clin Endocrinol Metab 82:1409–1415
5. **Cramer DW, Wilson E, Stillman RJ, Berger MJ, Belisle S, Schiff I, Albrecht B, Gibson M, Stadel BV, Schoenbaum SC** 1986 The relation of endometriosis to menstrual characteristics, smoking, and exercise. JAMA 255:1904–1908
6. **Halme J, Strovall D** 1995 Endometriosis and its medical management. St. Louis, MO: Mosby
7. **Curry Jr TE, Osteen KG** 2001 Cyclic changes in the matrix metalloproteinase system in the ovary and uterus. Biol Reprod 64:1285–1296
8. **Birkedal-Hansen H, Moore WG, Bodden MK, Windsor LJ, Birkedal-Hansen B, DeCarlo A, Engler JA** 1993 Matrix metalloproteinases: a review. Crit Rev Oral Biol Med 4:197–250
9. **Leppert D, Waubant E, Galardy R, Bunnett NW, Hauser SL** 1995 T cell gelatinases mediate basement membrane transmigration in vitro. J Immunol 154:4379–4389
10. **Matrisian LM** 1992 The matrix-degrading metalloproteinases. Bioessays 14: 455–463
11. **Okada S, Kita H, George TJ, Gleich GJ, Leiferman KM** 1997 Migration of eosinophils through basement membrane components in vitro: role of matrix metalloproteinase-9. Am J Respir Cell Mol Biol 17:519–528
12. **Biswas C, Zhang Y, DeCastro R, Guo H, Nakamura T, Kataoka H, Nabeshima K** 1995 The human tumor cell-derived collagenase stimulatory factor (renamed EMMPRIN) is a member of the immunoglobulin superfamily. Cancer Res 55:434–439
13. **Ellis SM, Nabeshima K, Biswas C** 1989 Monoclonal antibody preparation and purification of a tumor cell collagenase-stimulatory factor. Cancer Res 49: 3385–3391
14. **Guo H, Zucker S, Gordon MK, Toole BP, Biswas C** 1997 Stimulation of matrix metalloproteinase production by recombinant extracellular matrix metalloproteinase inducer from transfected Chinese hamster ovary cells. J Biol Chem 272:24–27
15. **Kataoka H, DeCastro R, Zucker S, Biswas C** 1993 Tumor cell-derived collagenase-stimulatory factor increases expression of interstitial collagenase, stromelysin, and 72-kDa gelatinase. Cancer Res 53:3154–3158
16. **Kirk P, Wilson MC, Heddle C, Brown MH, Barclay AN, Halestrap AP** 2000 CD147 is tightly associated with lactate transporters MCT1 and MCT4 and facilitates their cell surface expression. EMBO J 19:3896–3904
17. **Igakura T, Kadomatsu K, Kaname T, Muramatsu H, Fan QW, Miyauchi T, Toyama Y, Kuno N, Yuasa S, Takahashi M, Senda T, Taguchi O, Yamamura K, Arimura K, Muramatsu T** 1998 A null mutation in basigin, an immunoglobulin superfamily member, indicates its important roles in peri-implantation development and spermatogenesis. Dev Biol 194:152–165
18. **Sun J, Helmer M** 2001 Regulation of MMP-1 and MMP-2 production through CD147/extracellular matrix metalloproteinase inducer interactions. Cancer Res 61:2276–2281
19. **Taylor PM, Woodfield RJ, Hodgkin MN, Pettitt TR, Martin A, Kerr DJ, Wakelam MJ** 2002 Breast cancer cell-derived EMMPRIN stimulates fibroblast MMP2 release through a phospholipase A_2 and 5-lipoxygenase catalyzed pathway. Oncogene 21:5765–5772
20. **Sidhu SS, Mengistab AT, Tauscher AN, LaVail J, Basbaum C** 2004 The microvesicle as a vehicle for EMMPRIN in tumor-stromal interactions. Oncogene 23:956–963
21. **Noguchi Y, Sato T, Hirata M, Hara T, Ohama K, Ito A** 2003 Identification and characterization of extracellular matrix metalloproteinase inducer in human endometrium during the menstrual cycle *in vivo* and *in vitro*. J Clin Endocrinol Metab 88:6063–6072
22. **Noyes RW, Hertig AT, Rock J** 1975 Dating the endometrial biopsy. Am J Obstet Gynecol 122:262–263
23. **Strakova Z, Srisuparp S, Fazleabas AT** 2000 Interleukin-1β induces the ex-

pression of insulin-like growth factor binding protein-1 during decidualization in the primate. Endocrinology 141:4664–4670
24. **Frank GR, Brar AK, Jikihara H, Cedars MI, Handwerger S** 1995 Interleukin 1β and the endometrium: an inhibitor of stromal cell differentiation and possible autoregulator of decidualization in humans. Biol Reprod 52:184–191
25. **Kessler CA, Moghadam KK, Schroeder JK, Buckley AR, Brar AK, Handwerger S** 2005 Cannaboid receptor I activation markedly inhibits human decidualization. Mol Cell Endocrinol 229:65–74
26. **Richards RG, Brar AK, Grank GR, Hartman SM, Jikihara H** 1995 Fibroblast cells from term deciduas closely resemble stromal cells: induction of prolactin and insulin-like growth factor binding protein-1 expression. Biol Reprod 52: 609–615
27. **Kokorine I, Marbaix E, Henriet P, Okada Y, Donnez J, Eeckhout Y, Courtoy PJ** 1996 Focal cellular origin and regulation of interstitial collagenase (matrix metalloproteinase-1) are related to menstrual breakdown in the human endometrium. J Cell Sci 109:2151–2160
28. **Rawdanowicz TJ, Hampton AL, Nagase H, Woolley DE, Salamonsen LA** 1994 Matrix metalloproteinase production by cultured human endometrial stromal cells: identification of interstitial collagenase, gelatinase-A, gelatinase-B, and stromelysin-1 and their differential regulation by interleukin-1α and tumor necrosis factor-α. J Clin Endocrinol Metab 79:530–536
29. **Lessey BA, Killam AP, Metzger DA, Haney AF, Greene GL, McCarty KSJ** 1988 Immunohistochemical analysis of human uterine estrogen and progesterone receptors throughout the menstrual cycle. J Clin Endocrinol Metab 67:334–340
30. **Lessey BA, Metzger DA, Haney AF, McCarty Jr KS** 1989 Immunohistochemical analysis of estrogen and progesterone receptors in endometriosis: comparison with normal endometrium during the menstrual cycle and the effect of medical therapy. Fertil Steril 51:409–415
31. **Lim M, Martinez T, Jablons D, Cameron R, Guo H, Toole B, Li JD, Basbaum C** 1998 Tumor-derived EMMPRIN (extracellular matrix metalloproteinase inducer) stimulates collagenase transcription through MAPK p38. FEBS Lett 441:88–92
32. **Tang Y, Kesavan P, Nakada MT, Yan L** 2004 Tumor-stroma interaction: positive feedback regulation of extracellular matrix metalloproteinase inducer (EMMPRIN) expression and matrix metalloproteinase-dependent generation of soluble EMMPRIN. Mol Cancer Res 2:73–80
33. **Ghatak S, Misra S, Toole BP** 2005 Hyaluronan constitutively regulates ErbB2 phosphorylation and signaling complex formation in carcinoma cells. J Biol Chem 280:8875–8883
34. **Marieb EA, Zoltan-Jones A, Li R, Misra S, Ghatak S, Cao J, Zucker S, Toole BP** 2004 Emmprin promotes anchorage-independent growth in human mammary carcinoma cells by stimulating hyaluronan production. Cancer Res 64:1229–1232

JCEM is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.