Human endometrial milk fat globule-epidermal growth factor 8 (*MFGE8*) is up regulated by estradiol at the transcriptional level, and its secretion via microvesicles is stimulated by human chorionic gonadotropin (hCG)

Abbaa Sarhan^{1,5}, Silvina Bocca^{1,4*}, Liang Yu¹, Sandra Anderson¹, Terry Jacot¹, Tanya Burch², Julius O. Nyalwidhe², Claretta Sullivan³, Mandeep Kaur⁶, Vladimir B. Bajic⁶ and Sergio Oehninger¹

*Correspondence: boccaS@evms.edu

¹The Jones institute for Reproductive Medicine, Department of Obstetrics and Gynecology, USA.

²Department of Microbiology and Molecular Cell Biology -Center for Biomedical Proteomics, USA.

³Department of Surgery, Eastern Virginia Medical School, Norfolk, Virginia 23507, USA.

⁴The Jones institute for Reproductive Medicine, Eastern Virginia Medical School, Department of Obstetrics and Gynecology, USA.

⁵Columbia Fertility Associates, 2440 M St NW, Suite 401, Washington DC 20037, USA.

⁶Computational Bioscience Research Center, King Abdullah University of Science and Technology, Thuwal 23955-6900, Kingdom of Saudi Arabia.

Abstract

Objective: We have recently showed that *MFGE8*, a novel epithelial cell protein in the human endometrium, upregulated during the window of implantation. We hypothesized that *MFGE8* may act as a key modulator of endometrial remodeling and trophoblast invasion. The aims of this study were (i) to investigate the *in vitro* regulation of human endometrial epithelial cells *MFGE8* transcription, translation, and secretion by sex steroids and hCG; and (ii) to examine the possibility of *MFGE8* secretion via microvesicles.

Design: Experimental *in vitro* study using Ishikawa cells.

Setting: University center.

Interventions: Treatment with estradiol (E₂), progesterone (P₄), and human chorionic gonadotropin (hCG).

Main outcome measures: *MFGE8* mRNA and protein expression, and identification of secreted microvesicles by mass spectrometry (MS) and immunoblotting.

Results: E₂, but not P₄ or hCG, significantly upregulated *MFGE8* mRNA expression. hCG significantly increased *MFGE8* secretion. Microvesicles obtained after ultracentrifugation were visualized with atomic force microscopy ranging from ~100 to 200 nm. In addition to the expected 46 kD protein, the microvesicles contained a second form of secreted *MFGE8* measuring ~30 kD which was confirmed by MS.

Conclusions: We demonstrated (i) dual effects of E₂ and hCG on the regulation of *MFGE8*, and (ii) *MFGE8* protein secretion in association with microvesicles. *MFGE8* has the potential to modulate endometrial function and implantation via exocrine and/or paracrine-autocrine effects. To the best of our knowledge, this is the first demonstration of microvesicular secretion of any regulatory protein by endometrial epithelial cells, providing initial evidence suggestive of microvesicular participation in cellular trafficking information in the non-pregnant and pregnant endometrium.

Keywords: Endometrium, gene expression, *MFGE8*, microvesicles

Introduction

The nature and roles of the key molecules involved in blastocyst implantation is still under investigation [1-5]. Decidualization is the progressive remodeling of the endometrium that provides a receptive environment referred to as the "window of implantation" [6-10]. The gene expression profiles of the receptive endometrium have been characterized [11-13]. Among them, our group showed that milk fat globule EGF factor 8 (*MFGE8*), an endometrial gene not previously linked to the endometrium or implantation, was transcriptionally up-regulated 2.6- fold

during the receptive phase [14]. We postulated *MFGE8* as a novel glycoprotein with key roles in the regulation of endometrial function [15].

The secreted glycoprotein, *MFGE8* (also known as lactadherin, SED1, or breast antigen 46 [BA46]) was initially described as a component of the milk fat globule membrane, which is mainly secreted in microvesicles. It has also been suggested as a tumor marker in breast carcinomas [16,17]. Human *MFGE8* is a 46 kD glycoprotein peripherally associated with the cell membrane having two N-terminal EGF-like repeats, one of which includes

an Arginine-Glycine-Aspartic acid (RGD) motif that serves as the ligand to its $\alpha v\beta 3$ integrin receptor, facilitating cell adhesion and signal transduction. The two C-terminal discoidin C1-C2 domains are homologous to coagulation factors V and VIII, and are responsible for binding to cell membrane phospholipids or cell surface carbohydrate moieties as well as to the extracellular matrix [18,19].

MFGE8 has a variety of functions in many extra-uterine tissues related to apoptosis, cell adhesion and remodeling, neovascularization, and immunomodulation. In the breast it promotes phagocytosis of apoptotic cells by $\alpha v\beta 3/\beta 5$ integrin-expressing phagocytes [20], as well as in endothelial and epithelial cells [21,22]. Prolactin is a potent inducer of *MFGE8* expression in macrophages [23]. In systemic endothelial cells, *MFGE8* binding to $\alpha v\beta 3/\beta 5$ integrins promotes VEGF-induced survival and proliferation, leading to angiogenesis [24]. *MFGE8* also stimulates cell/cell and cell/extracellular matrix adhesion during sperm-oocyte interaction [18,25]. In intestinal, mammary gland, and epididymal epithelia, *MFGE8* regulates migration of epithelial cells [18,26-28]. *MFGE8* also protects against symptomatic rotavirus infection [29].

We recently demonstrated that *MFGE8* protein is predominantly localized to the epithelial compartment of the human endometrium, both in luminal and glandular epithelial cells, and with intense staining at both apical and basal cellular compartments [15]. The *MFGE8* receptor, integrin $\alpha v\beta 3$, was present in the epithelial and stromal compartments, with cycle-dependent and coincident peak expressions at mid-secretory phase [15]. We also showed that *MFGE8* protein was up regulated by prolactin in primary endometrial epithelial cell cultures. This finding supports a modulatory role for prolactin as a stromal-epithelial paracrine factor controlling *MFGE8* production [15]. Furthermore, we reported that *MFGE8* protein is highly expressed in human chorionic villi at all trimesters of gestation (in both cytotrophoblasts and syncytiotrophoblasts), and in murine implantation sites [30]. We also observed that *MFGE8* has pro-apoptotic activity in human endometrial stromal cells suggesting paracrine modulation [31]. Finally, we presented new evidence that recombinant *MFGE8* modulates endometrial endothelial cell proliferation and adhesion under *in vitro* conditions [32].

Here, the objective was to investigate the *in vitro* regulation of human *MFGE8* transcription, production and secretion in endometrial epithelial cells by estradiol (E_2), progesterone (P4), and human chorionic gonadotropin (hCG). To accomplish this goal, we used Ishikawa cells, an established *in vitro* cell culture model surrogate for human endometrial epithelium and known to display estrogen (ER) and progesterone (PR) receptors in culture [33-36]. Given that E_2 priming and P4 are active participants in the luteal phase endometrial transformation, and that hCG is one of the earliest embryonic products in a pregnancy cycle [37], we hypothesized that these hormones may directly regulate endometrial *MFGE8* production.

Materials and methods

Ishikawa cells culture and treatment

Ishikawa cells were cultured in DMEM/F12 containing 100 U/ml penicillin and 100 μ g/ml streptomycin (Gibco, Grand Island, NY) and supplemented with 5% fetal bovine serum (FBS, Atlanta Biological, Lawrenceville, GA) at 37°C in a 5% CO_2 atmosphere. The cells were trypsinized and seeded in DMEM/F12 containing 5% charcoal stripped FBS (Gibco) in either 6 or 12- well plates (for experiments measuring mRNA and intracellular protein) or 75 ml flasks (for experiments measuring secreted protein), and upon reaching 50-80% confluence used in experiments with hormone treatments as described below [34,36,38].

For mRNA studies, the Ishikawa cells were then treated with 0.1% ethanol (as vehicle control), with $17\beta-E_2$ (Sigma Aldrich Co., St. Louis, MO) for 24 hours (10^{-8} M), with P4 (10^{-6} M, Sigma) for 24 hours, or with a sequential treatment of E_2 for 24 hours (10^{-8} M), then followed by P4 (10^{-6} M) for another 24 hours, (n=6 experiments per treatment). The cells were collected and stored at -80°C until later use for mRNA extraction. In further experiments, Ishikawa cells were treated with recombinant hCG at 500 mIU/ml (Sigma) for 24 and 72 hours (n=6 per time) [31,39].

For intracellular protein studies, Ishikawa cells grown in the 12-well plates were treated with $17\beta-E_2$ 10^{-7} M or P4 10^{-6} M, alone and also in combination, and additionally with recombinant hCG 500 mIU/ml alone (without steroids), in duplicates, for 24, 48, and 72 hours. Finally, for secreted protein studies, the 75 ml flasks were treated with $17\beta-E_2$ 10^{-7} M or P4 10^{-6} M (alone and also in combination), and additionally with hCG at 500 mIU/ml alone (without steroids) (n=8 experiments) in 8 ml of media for 72 hours. The 8 ml of media was centrifuged at 1000 rpm for 5 minutes to remove cellular debris before storage at -80°C.

Immunohistochemistry, immunofluorescence, and RNA extraction and real time RT-PCR

Antibodies and dilutions used for immunostaining, as well as primers and PCR quantification and analysis methods are presented in Supplemental Materials.

In silico promoter analysis for EREs and PREs

We analyzed the 5'-flanking regions of *MFGE8* gene for the presence of putative estrogen and P4-response elements (EREs and PREs), as previously described by us [14,36,40]. For this *in silico* identification of EREs and PREs, we used two tools, DEREf (Dragon ERE Finder) [40] and Dragon PRE Locator (<http://apps.sanbi.ac.za/PRE/index.php>). We extracted promoter regions that correspond to 3000 bp upstream and 200 bp downstream (-3000, +200) relative to the 5' end of Exon 1. The ortholog promoter sequences of 11 mammalian species (including human) were extracted using TOUCAN 2 [41]. The ortholog species included were: human, cow, dog, hedgehog, elephant, opossum, monkey, mouse, rabbit, chimp and rat.

Protein extraction, SDS-PAGE, and immunoblotting

Methods for total lysate total protein extraction from Ishikawa cells, and *MFGE8* immuno blotting are presented in Supplemental Materials. For the secreted *MFGE8* protein quantification, the 8 ml of media previously collected was initially subjected to ultracentrifugation at 100,000 x g (50TI rotor, Beckman Coulter, Brea, CA) for 2 hours followed by PBS wash and repeat ultracentrifugation for another 2 hours following a similar protocol to other previously published studies [42-43]. The entire pellet was sonicated and then loaded onto the gel followed by the protocol mentioned above. Total protein quantification of the 75 ml flasks was performed to ensure similar cell numbers between the control and treatment samples for normalization. A sample from cell lysates was run simultaneously as a positive control.

In-gel digestion and MS

Immunoblot analysis of secreted *MFGE8* protein in the culture media demonstrated, in addition to the expected 46 kD band, a smaller sized band detected at ~30 kD. The bands were absent in negative control experiments that were run simultaneously to confirm the specificity of the antibody. While the 46 kD band was seen in the cells and in the medium, the lower molecular mass band was detected exclusively in the media and not in the cell lysate. To confirm that the ~30 kD band was, indeed, *MFGE8*, in addition to the immunoblotting, tandem MS analyses was performed after in-gel digestion with trypsin to generate peptides.

Briefly, pellets from two samples were loaded and separated on the same gel by SDS-PAGE as described above. One part of the gel was stained overnight with colloidal Coomassie to visualize the separated protein bands. The other part of the gel was electrotransferred onto a PVDF membrane and stained with Ponceau A to visualize the transferred proteins. Images of the separated proteins in the gel and membrane were taken and upon overlaying the images were identical. The PVDF membrane was processed for immunoblot detection of *MFGE8*. The immunoblot and Ponceau S images were used to precisely determine the corresponding band on the Coomassie stained gel for in-gel digestion and processing. The band was excised, destained, and the protein reduced with 10 mM dithiothreitol (DTT) in 50 mM NH_4HCO_3 before alkylation with 55 mM iodoacetamide in 50 mM NH_4HCO_3 . Digestion with sequence grade trypsin was done overnight at a concentration of 20 ng/ μl . The generated peptides were extracted with 50% acetonitrile/0.1% formic acid, and dried before reconstituting in 0.1% formic acid. The peptides were separated on a C18 column and subjected to liquid chromatography MS (LC-ESI-MS/MS) on a LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA).

Atomic force microscopy (AFM)

To determine whether microvesicles were produced by Ishikawa cells, pellets formed after ultracentrifugation of the

Ishikawa cell culture media were subjected to AFM. Images are generated using a force sensitive, scanning probe that flexes due to the interatomic forces it encounters at the surface of the sample. This point-by-point deflection is converted into high-resolution topographs of the surface. An aliquot of the pellet resuspension (in PBS) was added to uncoated muscovite mica (Electron Microscopy Sciences, Hatfield, PA) and allowed to air dry to encourage firm attachment to the substrate. Samples were subsequently rinsed with water to eliminate any crystals that might have formed from the buffer salts on the mica surface during drying. AFM images were collected with an Agilent 5500 scanning probe microscope (Agilent Technology, Chandler, AZ). Silicon nitride cantilevers (0.01-0.1 N/m) were used to scan the sample in contact mode at a rate of 0.5-2.0 lines per second at 512 points per line resolution. Images were processed as first-order flattened.

Statistical analysis

Gene expression levels (relative expression of *MFGE8* to b-actin) with different doses of E_2 and P4 treatments were compared using the Kruskal-Wallis test. The Holm-Sidak and Dunnet post hoc tests for pair-wise multiple comparisons were used as appropriate. Protein expression of *MFGE8* with different treatments was analyzed using paired t-test. Significance was defined as $P < 0.05$. Results are presented as mean $\pm$ standard error.

Results

Presence of epithelial cells markers in Ishikawa cells by immunocytochemistry

Immunocytochemical studies (Figures 1A-1H) confirmed the purity and epithelial phenotypic characteristics of the Ishikawa cells with positive staining for cytokeratin and negative staining for vimentin. Positive staining was also noted for ER α , ER β , PR, and *MFGE8*, confirming the suitability of this cell line as an *in vitro* model for testing *MFGE8* hormonal regulation in endometrial epithelial cells. Furthermore, the presence of *MFGE8* receptor ($\alpha\text{v}\beta 3$ integrin), and of hCG-R, were demonstrated in the Ishikawa cells by immunofluorescence (nuclei counterstained with DAPI).

Estradiol, but not progesterone or hCG, increases *MFGE8* mRNA expression in Ishikawa cells

Quantitative changes in *MFGE8* mRNA expression, as well as IL-6 and MUC1 (genes known to be estrogen-responsive in endometrial epithelial cells) were examined. The relative gene expression of *MFGE8* ($n = 6$) was significantly increased by E_2 after 24 hours ($P < 0.05$). On the other hand, P4 appeared to inhibit the estrogen stimulation but did not have a direct effect (Figure 2A). For IL-6: E_2 alone and E_2 plus P4 significantly increased IL-6 gene expression compared to controls ($P < 0.05$) (Figure 2B). For MUC1: E_2 significantly increased gene expression ($*P < 0.05$), but no effect was seen after E_2 plus P4 treatment, or with P4 alone (Figure 2C). The treatment with hCG at 500 mIU/

Figure 1. Immunocytochemistry results of untreated Ishikawa cells (magnification x200): (A) Cytokeratin at 1:30 dilution, marker of epithelial cells. (B) Vimentin at 1:50, marker of stromal cells. (C) ERα at 1:25. (D) ERβ at 1:25. (E) PR at 1:25. (F) *MFGE8* at 1:1000, note intense staining using low dilution. Insert: NC: negative control using a non-immune IgG control antibody. Immunofluorescence results of untreated Ishikawa cells: (G) $\alpha v \beta 3$ integrin at 1:50. (H) hCG-R (1:100). Note predominant cell membrane location for these proteins, with a filamentous distribution for $\alpha v \beta 3$ integrin, and a more diffuse punctuate form for hCG-R (x630 for both).

ml did not affect *MFGE8* mRNA expression levels by 24 or 72 hours (**Figure 2D**).

Prediction of EREs and PREs in the *MFGE8* gene

We looked for EREs of 17 bases length [42] (2 flanking nt –1st half site–3nt spacer–2nd half site–2 flanking nt) on 11 ortholog promoters of *MFGE8* gene. **Table 1A** shows the summary of ERE predictions in these promoters at the tool sensitivity of 87%.

DEREF predicted EREs on promoters of 8 orthologous species. Human and Chimp promoters have conserved 16 bp ERE sequences (shown in red) with only most 3' nucleotide mismatched. Human and Mouse have 15 bp ERE conserved sequences (shown as bold black) with one mismatch in the second half-site and one mismatch in the second flanking nucleotide on the 5' end respectively. Mouse and Rat promoters contain fully conserved 17 bp ERE (shown in blue).

PREs of length 15 bases were searched on 11 ortholog promoters of *MFGE8* gene. At DPREL's default threshold we

Table 1A. Predicted EREs for *MFGE8* gene.

Species	start	strand	Pattern
Human	2372	-	TT-GGTCA-GGC-TGGTC-TC
	1149	-	TT-GGCCA-GGC-TGGTC-TT
Dog	1575	+	GG-GGTGA-CAC-TGTCC-CA
Hedgehog	1023	-	AA-GGACT-AGC-TAACC-AC
Elephant	248	+	GA-GGGCT-GTG-TGGCC-GA
Monkey	2675	+	CT-AATCA-CTG-TGATC-CA
	136	-	TG-GGTCT-GCC-TGCCC-GC
Mouse	3031	-	TA-GGCCA-GGC-TGGCC-TT
	81	-	TG-AGTCC-CTC-TGGCC-TC
Chimp	2421	-	TT-GGTCA-GGC-TGGTC-TT
Rat	86	-	TG-AGTCC-CTC-TGGCC-TC

Human and Chimp promoters have conserved sequences of EREs (shown in red). Human and Mouse have conserved sequences of EREs (shown as bold black). Mouse and Rat promoters contain fully conserved ERE (shown in blue).

Table 1B. Predicted PREs for *MFGE8* gene.

Species	Start	Score	Strand	Pattern
Human	2830	0.701900	+	AGGACA-TGG-TGTTCT
Dog	240	0.702296	+	GGGAAA-TAA-TGTTCT
Chimp	2724	0.701900	+	AGGACA-TGG-TGTTCT

For PREs fully conserved PRE "AGGACA-TGG-TGTTCT" is present in both Human and Chimp promoters (shown in red).

were able to predict PREs on the promoters of three species (**Table 1B**). We observed that fully conserved PRE "AGGACA-TGG-TGTTCT" is present in both Human and Chimp promoters (shown in red) at the tool sensitivity of 96%.

hCG stimulates secretion of *MFGE8* in the form of microvesicles

Immunoblot analyses showed no significant difference in intracellular *MFGE8* protein after 24, 48, and 72 hour treatment with E_2 (10^{-7} M) and/or P (10^{-6} M), or with hCG (500 mIU/ml). A representative blot is shown in **Figure 3A**. Detection of secreted *MFGE8* was initially attempted using the 1 ml of media per sample from the 12 well plates, but was not possible even after concentration of the media (**Figure 3B**). This obstacle was overcome by collecting media from more cells. Specifically, 8 ml of media were available to assay when cells were grown in 75 ml flasks instead of 12 well plates. After ultracentrifugation of the media as described above, the resultant pellet was sonicated and loaded in its entirety for SDS-PAGE and immunoblotting. Protein detection was compromised if less than the entire pellet was loaded. Ultracentrifugation protocols have been used in other studies (cited above) investigating secreted *MFGE8* associated with microvesicles, and appears to be necessary for protein detection.

Figure 2. Quantitative changes in *MFGE8*, *IL-6* and *MUC1* mRNA expression in Ishikawa cells (relative expression of target transcripts in relation to the reference gene β -actin). (A) The relative gene expression of *MFGE8* was significantly increased by E2 after 24 hours (* $P < 0.05$). On the other hand, P4 inhibited the estrogen stimulation, and P4 alone did not have an effect. (B) For *IL-6*: E2 alone and E2 plus P4 significantly increased *IL-6* gene expression compared to controls (* $P < 0.05$), whereas P4 alone had no effect. (C) For *MUC1*: E2 significantly increased gene expression (* $P < 0.05$), but no effect was seen after E2 plus P4 treatment, or with P4 alone. (D) After 24 or 72 hours, 500 mIU hCG did not modify *MFGE8* mRNA expression levels ($P > 0.5$).

Using this ultracentrifugation/sonication protocol, a second *MFGE8* secreted band was consistently visualized in addition to the expected 46 kD band (Figures 3C and 3D). This second band was more intense and with a lower molecular weight at ~30 kD, suggesting that another form of *MFGE8*, not evident in any of the cell lysate samples, was also being secreted. Semi-quantification of the original 46 kD band showed a significant 1.8 fold increase in secreted *MFGE8* after 72 hour treatment with hCG at 500 mIU/ml ($P = 0.03$) as seen in Figure 3E. Unlike hCG, E_2 (10^{-7} M) and/or P4 (10^{-6} M) 72 hour treatments did not show a significant upregulation of secreted *MFGE8*. In addition, the ~30 kD *MFGE8* form also showed a significant increase after hCG treatment, with a 1.25 fold increase ($p < 0.05$).

Characterization of secreted microvesicles by MS and AFM

The LC-ESI-MS/MS unequivocally confirmed the identity of the second band as *MFGE8* by the identification of an *MFGE8* peptide with m/z of 1765.9747 and sequence VTFLGLQH-

WVPELAR. The identification was with a significant Mascot Ion score of 55 and a significant Mascot Expect score of .0013. The sequence corresponds to the amino acids 93 -107 of the protein. The MS/MS spectra for the peptide is rich in both b and y ion series with very high intensities that validate the identification (Figure 4A). The ions score for the MS/MS match is based on the calculated probability, P, that the observed match between the experimental data and the database sequence is a random event, scores greater than 46 being statistically significant identifications. The expect score indicates the probability that the observed match experimental data and the database peptide sequence is random. For confident matches the significance threshold for the Expect values is < 0.1 .

The in-gel digestion and MS experiments revealed that in addition to *MFGE8*, several other proteins were identified in the culture medium, with molecular weights in the vicinity of the ~30 kD target, including proteins previously described in human plasma microvesicles [44]. Among those proteins were

Figure 3. Immunoblotting results for *MFGE8*: (A) Representative immunoblot of intracellular *MFGE8* protein after 72 hour treatment of Ishikawa cells with E2 and P4, and hCG, with corresponding β -actin immunoblot. (B) Representative immunoblot of secreted *MFGE8* protein in the medium of Ishikawa cells (without ultracentrifugation of the media) after 24 hour treatment with E2 at 10^{-7} M and P4 at 10^{-6} M. No *MFGE8* band was detected even when the media was concentrated prior to loading onto SDS-PAGE. C; controls; EP: E₂ at 10^{-7} M and P4 at 10^{-6} M; pos C: positive control; neg C: negative control. Immunoblotting results for *MFGE8*: (C) and (D) Representative immunoblots of secreted *MFGE8* protein after ultracentrifugation of the media of cultured Ishikawa cells after 72 hour treatment with E₂ and/or P4, or hCG. (E) Summary of data from 72 hour treatment with hCG showing a statistically significant increase in secreted *MFGE8* after ultracentrifugation of media (n=8), for the 46 kD protein (1.8 fold increase, * P= 0.03) and for the ~30 kD protein (1.25 fold increase, **p<0.05) compared to control conditions.

(protein score and mass-kD): annexin (182, 38690), actin (708, 41710-41766), glyceraldehyde 3-phosphate dehydrogenase (47, 36030), heat shock cognate (88, 70854), proteasome subunit alpha (52, 29537), and tubulin alpha (110, 49863-50120).

AFM imaging confirmed the presence of secreted membranous structures, likely microvesicles from the pellets after ultracentrifugation of the Ishikawa cells media. Images from the original undiluted sample showed such an abundance of microvesicles that it made the measurement of individual dimensions difficult. However, after 1:100 dilution of the original sample, clearer images were obtained of individual microvesicles which ranged in size from ~100 to 200 nm (Figures 4B and 4C).

Discussion

Dysregulation of *MFGE8* has been linked to autoimmune and inflammatory diseases such as systemic lupus erythematosus, and severe pregnancy complications [45-47]. Given its myriad of functions, it is not surprising that *MFGE8* has now been linked to such a dynamic organ as the endometrium [15,31-32]. Here, we further investigated the regulation of endometrial epithelial *MFGE8* production by ovarian sex steroids, which regulate every ovulatory cycle regardless of pregnancy, and with hCG of trophoblastic origin, which is only present in a pregnant cycle. We used an established human uterine-derived epithelial cell line (Ishikawa), which shares many phenotypic

hCG was shown having direct paracrine effects on the endometrium that precede its endocrine role of rescuing the corpus luteum, and regulates IGFBP-1 and prolactin in decidualized human endometrial stromal cells [51–54]. Here, hCG did not affect *MFGE8* gene expression levels, consistent with previous results from our laboratory showing no change in primary human endometrial epithelial cells [31]. Contrarily, hCG significantly stimulated *MFGE8* secretion. Our data thus far demonstrate hCG regulation of *MFGE8* secretion at the protein level and not at the transcriptional level, and support the concept that *MFGE8* secretion is mainly associated with microvesicles. The fact that hCG enhanced *MFGE8* protein secretion but not mRNA expression has at least two possible explanations: (i) it could be a simple reflection of the shorter incubation times used for the RNA extraction studies [34]; (ii) on the other hand, the discrepancy between protein and mRNA expression suggests various levels of regulation dur-

ing protein synthesis, e.g., posttranscriptional, translational, or posttranslational regulation [55].

Following the realization that microvesicles (microparticles) and exosomes are bioactive structures, scientific interest in their origin and roles in diseases has increased tremendously [44,56]. AFM imaging in this study confirmed the presence of secreted microvesicles from the Ishikawa cells, which has not been previously reported in the literature. Immunoblotting of the lysed microvesicles confirmed the association of MFGE-8 with microvesicles and explained why concentrated media did not show any detectable MFGE-8. Measurements of individual microvesicles from multiple samples showed a range of 100 to 200 nm, which is consistent with other studies in mouse mammary epithelial cells and adipocytes [44,46,57-58].

Another interesting finding was the identification of a second secreted form of *MFGE8* (~30 kD), in addition to the expected 46 kD form. Mass spectrometry data confirmed the presence of a peptide matching *MFGE8* in that band along with other proteins typically found in plasma microvesicles at that molecular weight [44]. In contrast, the cell lysates consistently showed the expected single distinct 46 kD *MFGE8* band. This finding suggests a post-translational secretory modification to *MFGE8* as opposed to the presence of two distinct mRNA transcripts, which should result in the visualization of two protein products or isoforms. It is unlikely that the second band is an artifact of proteolytic cleavage during isolation since it was consistently present along with the 46 kD band. Furthermore, it was located at the same molecular size in all independently isolated samples, and mild conditions were used in the protein isolation process.

A similar 30 kD truncated variant of secreted *MFGE8* was reported from human breast milk of healthy females [59]. That study presented the possibility that this truncated C-terminal fragment is produced from the 46 kD membrane bound or intracellular form by enzymatic cleavage. The Uniprot Consortium (www.uniprot.org) describes three isoforms for the human *MFGE8* protein produced by alternative splicing with isoform 1 representing the intact 387 amino acid protein, while isoforms 2 and 3 are missing amino acid 1-75 and 291-342, respectively. In addition, there are reports suggesting that aberrant splicing of *MFGE8*, which results in a truncated protein product due to a premature stop codon, and genetic polymorphisms in *MFGE8*, may lead to the development of lupus [60].

Conclusions

In conclusion, this study showed that estrogen regulates *MFGE8* gene expression at transcriptional level, and that hCG stimulated *MFGE8* protein secretion. Moreover, hCG stimulated *MFGE8* secretion in association with microvesicles, pointing to an early dialogue between the trophoblast and the endometrium. To the best of our knowledge this is the first demonstration of microvesicular secretion of a regulatory protein by human endometrial epithelial cells. We acknowledge

that these studies were performed in an established epithelial cell line and need to be recapitulated in primary cells. Taking together our previous data in human endometrium, primary cells and now Ishikawa cells [15,30-32], we propose a unifying hypothesis that a multifactorial *MFGE8* regulation is maintained by estrogen upregulation of mRNA, prolactin upregulation of intracellular protein production, and hCG triggers the actual secretion of *MFGE8*. These data support that *MFGE8* is involved in the estrogen-, prolactin- and hCG-dependent regulation of endometrial-embryonic effects at the time of implantation, and also that *MFGE8* could be a key modulator of endometrial physiology acting as an exocrine, paracrine and/or autocrine factor, with microvesicular participation in cellular trafficking information in the non-pregnant and pregnant endometrium.

Competing interest

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	AS	SB	LY	SA	TJ	TB	JON	CS	MK	VBB	SO
Research concept and design	✓	✓	–	–	✓	✓	✓	–	–	–	✓
Collection and/or assembly of data	✓	–	✓	✓	–	–	–	✓	–	–	–
Data analysis and interpretation	✓	✓	–	–	–	–	–	–	✓	✓	–
Writing the article	✓	–	–	–	–	–	–	–	–	–	✓
Critical revision of the article	–	✓	–	–	–	–	–	–	–	–	–
Final approval of article	–	✓	–	–	–	–	–	–	–	–	✓
Statistical analysis	✓	✓	–	–	–	–	–	–	–	–	–

Acknowledgement and funding

We thank Ms. Natalie Lonergan from the Department of Surgery for assistance with atomic force microscopy experiments. This work was supported by a research grant from The Jones Institute Foundation and Eastern Virginia Medical School to SO and SB.

Publication history

Editor: Edgar Serfling, University of Würzburg, Germany.
EICs: Matthias Gaestel, Hannover Medical University, Germany.
Ulhas P. Naik, University of Delaware, USA.
Received: 20-Sep-2013 Revised: 27-Sep-2013
Re-Received: 30-Sep-2013 Accepted: 01-Oct-2013
Published: 17-Oct-2013

References

- Dey SK, Lim H, Das SK, Reese J, Paria BC, Daikoku T and Wang H. **Molecular cues to implantation.** *Endocr Rev.* 2004; **25**:341-73. | [Article](#) | [PubMed](#)
- Maruyama T and Yoshimura Y. **Molecular and cellular mechanisms for differentiation and regeneration of the uterine endometrium.** *Endocr J.* 2008; **55**:795-810. | [Article](#) | [PubMed](#)
- Yoshinaga K. **Research on Blastocyst Implantation Essential Factors (BIEFs).** *Am J Reprod Immunol.* 2010; **63**:413-24. | [Article](#) | [PubMed](#)
- Singh M, Chaudhry P and Asselin E. **Bridging endometrial receptivity and implantation: network of hormones, cytokines, and growth factors.** *J Endocrinol.* 2011; **210**:5-14. | [Article](#) | [PubMed](#)
- Haouzi D, Dechaud H, Assou S, De Vos J and Hamamah S. **Insights into**

- human endometrial receptivity from transcriptomic and proteomic data. *Reprod Biomed Online*. 2012; **24**:23-34. | [Article](#) | [PubMed](#)
6. Telgmann R and Gellersen B. **Marker genes of decidualization: activation of the decidual prolactin gene.** *Hum Reprod Update*. 1998; **4**:472-9. | [Article](#) | [PubMed](#)
7. Dunn CL, Kelly RW and Critchley HO. **Decidualization of the human endometrial stromal cell: an enigmatic transformation.** *Reprod Biomed Online*. 2003; **7**:151-61. | [Article](#) | [PubMed](#)
8. Gellersen B and Brosens J. **Cyclic AMP and progesterone receptor cross-talk in human endometrium: a decidualizing affair.** *J Endocrinol*. 2003; **178**:357-72. | [Article](#) | [PubMed](#)
9. Jabbour HN, Kelly RW, Fraser HM and Critchley HO. **Endocrine regulation of menstruation.** *Endocr Rev*. 2006; **27**:17-46. | [Article](#) | [PubMed](#)
10. Gellersen B, Brosens IA and Brosens JJ. **Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives.** *Semin Reprod Med*. 2007; **25**:445-53. | [Article](#) | [PubMed](#)
11. Horcajadas JA, Riesewijk A, Dominguez F, Cervero A, Pellicer A and Simon C. **Determinants of endometrial receptivity.** *Ann N Y Acad Sci*. 2004; **1034**:166-75. | [Article](#) | [PubMed](#)
12. Giudice LC. **Application of functional genomics to primate endometrium: insights into biological processes.** *Reprod Biol Endocrinol*. 2006; **4 Suppl 1**:S4. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
13. Aghajanova L, Hamilton AE and Giudice LC. **Uterine receptivity to human embryonic implantation: histology, biomarkers, and transcriptomics.** *Semin Cell Dev Biol*. 2008; **19**:204-11. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
14. Mirkin S, Arslan M, Churikov D, Corica A, Diaz JI, Williams S, Bocca S and Oehninger S. **In search of candidate genes critically expressed in the human endometrium during the window of implantation.** *Hum Reprod*. 2005; **20**:2104-17. | [Article](#) | [PubMed](#)
15. Franchi A, Bocca S, Anderson S, Riggs R and Oehninger S. **Expression of milk fat globule EGF-factor 8 (MFG-E8) mRNA and protein in the human endometrium and its regulation by prolactin.** *Mol Hum Reprod*. 2011; **17**:360-71. | [Article](#) | [PubMed](#)
16. Peterson JA, Zava DT, Duwe AK, Blank EW, Battifora H and Ceriani RL. **Biochemical and histological characterization of antigens preferentially expressed on the surface and cytoplasm of breast carcinoma cells identified by monoclonal antibodies against the human milk fat globule.** *Hybridoma*. 1990; **9**:221-35. | [Article](#) | [PubMed](#)
17. Taylor MR, Couto JR, Scallan CD, Ceriani RL and Peterson JA. **Lactadherin (formerly BA46), a membrane-associated glycoprotein expressed in human milk and breast carcinomas, promotes Arg-Gly-Asp (RGD)-dependent cell adhesion.** *DNA Cell Biol*. 1997; **16**:861-9. | [Article](#) | [PubMed](#)
18. Ensslin MA and Shur BD. **The EGF repeat and discoidin domain protein, SED1/MFG-E8, is required for mammary gland branching morphogenesis.** *Proc Natl Acad Sci U S A*. 2007; **104**:2715-20. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
19. Raymond A, Ensslin MA and Shur BD. **SED1/MFG-E8: a bi-motif protein that orchestrates diverse cellular interactions.** *J Cell Biochem*. 2009; **106**:957-66. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
20. Hanayama R, Tanaka M, Miwa K, Shinohara A, Iwamatsu A and Nagata S. **Identification of a factor that links apoptotic cells to phagocytes.** *Nature*. 2002; **417**:182-7. | [Article](#) | [PubMed](#)
21. Nandrot EF, Anand M, Almeida D, Atabai K, Sheppard D and Finnemann SC. **Essential role for MFG-E8 as ligand for alpha5beta1 integrin in diurnal retinal phagocytosis.** *Proc Natl Acad Sci U S A*. 2007; **104**:12005-10. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
22. Fens MH, Mastrobattista E, de Graaff AM, Flesch FM, Ultee A, Rasmussen JT, Molema G, Storm G and Schiffelers RM. **Angiogenic endothelium shows lactadherin-dependent phagocytosis of aged erythrocytes and apoptotic cells.** *Blood*. 2008; **111**:4542-50. | [Article](#) | [PubMed](#)
23. Aziz MM, Ishihara S, Rumi MA, Mishima Y, Oshima N, Kadota C, Moriyama I, Li YY, Rahman FB, Otani A, Oka A, Ishimura N, Kadowaki Y, Amano Y and Kinoshita Y. **Prolactin induces MFG-E8 production in macrophages via transcription factor C/EBPbeta-dependent pathway.** *Apoptosis*. 2008; **13**:609-20. | [Article](#) | [PubMed](#)
24. Silvestre JS, Thery C, Hamard G, Boddaert J, Aguilar B, Delcayre A, Houbbron C, Tamarat R, Blanc-Brude O, Heeneman S, Clergue M, Duriez M, Merval R, Levy B, Tedgui A, Amigorena S and Mallat Z. **Lactadherin promotes VEGF-dependent neovascularization.** *Nat Med*. 2005; **11**:499-506. | [Article](#) | [PubMed](#)
25. Ensslin MA and Shur BD. **Identification of mouse sperm SED1, a bimotif EGF repeat and discoidin-domain protein involved in sperm-egg binding.** *Cell*. 2003; **114**:405-17. | [Article](#) | [PubMed](#)
26. Aoki N. **Regulation and functional relevance of milk fat globules and their components in the mammary gland.** *Biosci Biotechnol Biochem*. 2006; **70**:2019-27. | [Article](#) | [PubMed](#)
27. Bu HF, Zuo XL, Wang X, Ensslin MA, Koti V, Hsueh W, Raymond AS, Shur BD and Tan XD. **Milk fat globule-EGF factor 8/lactadherin plays a crucial role in maintenance and repair of murine intestinal epithelium.** *J Clin Invest*. 2007; **117**:3673-83. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
28. Raymond AS, Elder B, Ensslin M and Shur BD. **Loss of SED1/MFG-E8 results in altered luminal physiology in the epididymis.** *Mol Reprod Dev*. 2010; **77**:550-63. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
29. Newburg DS, Peterson JA, Ruiz-Palacios GM, Matson DO, Morrow AL, Shults J, Guerrero ML, Chaturvedi P, Newburg SO, Scallan CD, Taylor MR, Ceriani RL and Pickering LK. **Role of human-milk lactadherin in protection against symptomatic rotavirus infection.** *Lancet*. 1998; **351**:1160-4. | [Article](#) | [PubMed](#)
30. Bocca SM, Anderson S, Amaker B, Swanson RJ, Franchi A, Lattanzio F and Oehninger S. **Milk fat globule epidermal growth factor 8 (MFG-E8): a novel protein in the mammalian endometrium with putative roles in implantation and placentation.** *Placenta*. 2012; **33**:795-802. | [Article](#) | [PubMed](#)
31. Riggs RM, Bocca S, Anderson S, Franchi A, Rhavi BS and Oehninger S. **Epithelial cell protein milk fat globule-epidermal growth factor 8 and human chorionic gonadotropin regulate stromal cell apoptosis in the human endometrium.** *Fertil Steril*. 2012; **98**:1549-56 e3. | [Article](#) | [PubMed](#)
32. Bocca S, Anderson S and Oehninger S. **Milk fat globule epithelial growth factor 8 (MFG-E8) regulates human endometrial endothelial cell adhesion and proliferation.** *Fertil Steril*. 2010; **94**:S215-S216. | [Article](#)
33. Nishida M. **The Ishikawa cells from birth to the present.** *Hum Cell*. 2002; **15**:104-17. | [Article](#) | [PubMed](#)
34. Bocca SM and Archer DF. **Raloxifene (Rlx) has antiestrogenic effect on Vascular Endothelial Growth Factor (VEGF) mRNA and protein while down-regulating progesterone receptors in a human endometrial cell line in vitro.** *Fertil Steril*. 2005; **84**:S119-120. | [Article](#)
35. Heneweer C, Schmidt M, Denker HW and Thie M. **Molecular mechanisms in uterine epithelium during trophoblast binding: the role of small GTPase RhoA in human uterine Ishikawa cells.** *J Exp Clin Assist Reprod*. 2005; **2**:4. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
36. Zhang X, Bocca S, Franchi A, Anderson S, Kaur M, Bajic VB and Oehninger S. **Do GnRH analogues directly affect human endometrial epithelial cell gene expression?** *Mol Hum Reprod*. 2010; **16**:347-60. | [Article](#) | [PubMed](#)
37. Sherwin JR, Sharkey AM, Cameo P, Mavrogianis PM, Catalano RD, Edassery S and Fazleabas AT. **Identification of novel genes regulated by chorionic gonadotropin in baboon endometrium during the window of implantation.** *Endocrinology*. 2007; **148**:618-26. | [Article](#) | [PubMed](#)
38. Krikun G, Sakkas D, Schatz F, Buchwalder L, Hylton D, Tang C and Lockwood CJ. **Endometrial angiopoietin expression and modulation by thrombin and steroid hormones: a mechanism for abnormal angiogenesis following long-term progestin-only contraception.** *Am J Pathol*. 2004; **164**:2101-7. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
39. Yang H, Lei CX and Zhang W. **Human chorionic gonadotropin (hCG) regulation of galectin-3 expression in endometrial epithelial cells and endometrial stromal cells.** *Acta Histochem*. 2013; **115**:3-7. | [Article](#) | [PubMed](#)
40. Bajic VB, Tan SL, Chong A, Tang S, Strom A, Gustafsson JA, Lin CY and Liu ET. **Dragon ERE Finder version 2: A tool for accurate detection and**

- analysis of estrogen response elements in vertebrate genomes. *Nucleic Acids Res.* 2003; **31**:3605-7. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
41. Aerts S, Van Loo P, Thijs G, Mayer H, de Martin R, Moreau Y and De Moor B. **TOUCAN 2: the all-inclusive open source workbench for regulatory sequence analysis.** *Nucleic Acids Res.* 2005; **33**:W393-6. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
 42. Veron P, Segura E, Sugano G, Amigorena S and Thery C. **Accumulation of MFG-E8/lactadherin on exosomes from immature dendritic cells.** *Blood Cells Mol Dis.* 2005; **35**:81-8. | [Article](#) | [PubMed](#)
 43. Aoki N, Jin-no S, Nakagawa Y, Asai N, Arakawa E, Tamura N, Tamura T and Matsuda T. **Identification and characterization of microvesicles secreted by 3T3-L1 adipocytes: redox- and hormone-dependent induction of milk fat globule-epidermal growth factor 8-associated microvesicles.** *Endocrinology.* 2007; **148**:3850-62. | [Article](#) | [PubMed](#)
 44. Jin M, Drwal G, Bourgeois T, Saltz J and Wu HM. **Distinct proteome features of plasma microparticles.** *Proteomics.* 2005; **5**:1940-52. | [Article](#) | [PubMed](#)
 45. Hanayama R, Tanaka M, Miyasaka K, Aozasa K, Koike M, Uchiyama Y and Nagata S. **Autoimmune disease and impaired uptake of apoptotic cells in MFG-E8-deficient mice.** *Science.* 2004; **304**:1147-50. | [Article](#) | [PubMed](#)
 46. Toth B, Lok CA, Boing A, Diamant M, van der Post JA, Friese K and Nieuwland R. **Microparticles and exosomes: impact on normal and complicated pregnancy.** *Am J Reprod Immunol.* 2007; **58**:389-402. | [Article](#) | [PubMed](#)
 47. Yamaguchi H, Takagi J, Miyamae T, Yokota S, Fujimoto T, Nakamura S, Ohshima S, Naka T and Nagata S. **Milk fat globule EGF factor 8 in the serum of human patients of systemic lupus erythematosus.** *J Leukoc Biol.* 2008; **83**:1300-7. | [Article](#) | [PubMed](#)
 48. Lessey BA, Ilesanmi AO, Castelbaum AJ, Yuan L, Somkuti SG, Chwalisz K and Satyaswaroop PG. **Characterization of the functional progesterone receptor in an endometrial adenocarcinoma cell line (Ishikawa): progesterone-induced expression of the alpha1 integrin.** *J Steroid Biochem Mol Biol.* 1996; **59**:31-9. | [Article](#) | [PubMed](#)
 49. Castelbaum AJ, Ying L, Somkuti SG, Sun J, Ilesanmi AO and Lessey BA. **Characterization of integrin expression in a well differentiated endometrial adenocarcinoma cell line (Ishikawa).** *J Clin Endocrinol Metab.* 1997; **82**:136-42. | [Article](#) | [PubMed](#)
 50. Hannan NJ, Paiva P, Dimitriadis E and Salamonsen LA. **Models for study of human embryo implantation: choice of cell lines?** *Biol Reprod.* 2010; **82**:235-45. | [Article](#) | [PubMed](#)
 51. Bonduelle ML, Dodd R, Liebaers I, Van Steirteghem A, Williamson R and Akhurst R. **Chorionic gonadotrophin-beta mRNA, a trophoblast marker, is expressed in human 8-cell embryos derived from trippronucleate zygotes.** *Hum Reprod.* 1988; **3**:909-14. | [PubMed](#)
 52. Licht P, Fluhr H, Neuwinger J, Wallwiener D and Wildt L. **Is human chorionic gonadotropin directly involved in the regulation of human implantation?** *Mol Cell Endocrinol.* 2007; **269**:85-92. | [Article](#) | [PubMed](#)
 53. Fluhr H, Krenzer S, Deperschmidt M, Zwirner M, Wallwiener D and Licht P. **Human chorionic gonadotropin inhibits insulin-like growth factor-binding protein-1 and prolactin in decidualized human endometrial stromal cells.** *Fertil Steril.* 2006; **86**:236-8. | [Article](#) | [PubMed](#)
 54. Mansour R, Tawab N, Kamal O, El-Faissal Y, Serour A, Aboulghar M and Serour G. **Intrauterine injection of human chorionic gonadotropin before embryo transfer significantly improves the implantation and pregnancy rates in in vitro fertilization/intracytoplasmic sperm injection: a prospective randomized study.** *Fertil Steril.* 2011; **96**:1370-1374 e1. | [Article](#) | [PubMed](#)
 55. Tian Q, Stepaniants SB, Mao M, Weng L, Feetham MC, Doyle MJ, Yi EC, Dai H, Thorsson V, Eng J, Goodlett D, Berger JP, Gunter B, Linseley PS, Stoughton RB, Aebersold R, Collins SJ, Hanlon WA and Hood LE. **Integrated genomic and proteomic analyses of gene expression in Mammalian cells.** *Mol Cell Proteomics.* 2004; **3**:960-9. | [Article](#) | [PubMed](#)
 56. Diamant M, Tushuizen ME, Sturk A and Nieuwland R. **Cellular microparticles: new players in the field of vascular disease?** *Eur J Clin Invest.* 2004; **34**:392-401. | [Article](#) | [PubMed](#)
 57. Oshima K, Aoki N, Kato T, Kitajima K and Matsuda T. **Secretion of a peripheral membrane protein, MFG-E8, as a complex with membrane vesicles.** *Eur J Biochem.* 2002; **269**:1209-18. | [Article](#) | [PubMed](#)
 58. Gyorgy B, Szabo TG, Pasztoi M, Pal Z, Misjak P, Aradi B, Laszlo V, Pallinger E, Pap E, Kittel A, Nagy G, Falus A and Buzas EI. **Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles.** *Cell Mol Life Sci.* 2011; **68**:2667-88. | [Article](#) | [PubMed Abstract](#) | [PubMed Full Text](#)
 59. Giuffrida MG, Cavaletto M, Giunta C, Conti A and Godovac-Zimmermann J. **Isolation and characterization of full and truncated forms of human breast carcinoma protein BA46 from human milk fat globule membranes.** *J Protein Chem.* 1998; **17**:143-8. | [Article](#) | [PubMed](#)
 60. Yamaguchi H, Fujimoto T, Nakamura S, Ohmura K, Mimori T, Matsuda F and Nagata S. **Aberrant splicing of the milk fat globule-EGF factor 8 (MFG-E8) gene in human systemic lupus erythematosus.** *Eur J Immunol.* 2010; **40**:1778-85. | [Article](#) | [PubMed](#)

Citation:

Sarhan A, Bocca S, Yu L, Anderson S, Jacot T, Burch T, Nyalwidhe JO, Sullivan C, Kaur M, Bajic VB and Oehninger S. **Human endometrial milk fat globule-epidermal growth factor 8 (MFG-E8) is up regulated by estradiol at the transcriptional level, and its secretion via microvesicles is stimulated by human chorionic gonadotropin (hCG).** *Cell Signal Traffic.* 2013; **1**:1.
http://dx.doi.org/10.7243/2054-1481-1-1