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)

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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 αvβ3 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 αvβ3 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 αvβ3/β5 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 αvβ3, 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 choriocarcinoma 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 (E2), 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 E2 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 μg/ml streptomycin (Gibco, Grand Island, NY) and supplemented with 5% fetal bovine serum (FBS, Atlanta Biological, Lawrenceville, GA) at 37°C in a 5% CO2 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 17β-E2 (Sigma Aldrich Co., St. Louis, MO) for 24 hours (10⁶ M), with P4 (10⁶ M, Sigma) for 24 hours, or with a sequential treatment of E2 for 24 hours (10⁶ M), then followed by P4 (10⁶ 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 17β-E2, 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 17β-E2, 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 Exon1. 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 55 mM iodoacetamide in 50 mM NH₄HCO₃.

The band was excised, destained, and the protein reduced with 55 mM iodoacetamide in 50 mM NH₄HCO₃. 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 E₂ 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 ± 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 ERα, ERβ, 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 (αvβ3 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 E₂ 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: E₂ alone and E₂ plus P₄ significantly increased IL-6 gene expression compared to controls (P<0.05) (Figure 2B). For MUC1: E₂ significantly increased gene expression (*P <0.05), but no effect was seen after E₂ plus P₄ treatment, or with P₄ alone (Figure 2C). The treatment with hCG at 500 mIU/
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 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 1A. Predicted EREs for MFGE8 gene.

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<td>TT-GGCCA-GGC-TGGTC-TC</td>
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<td>Dog</td>
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<td>+</td>
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<td>1023</td>
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<td>Rat</td>
<td>86</td>
<td>-</td>
<td>TG-AGTCC-CTC-TGGCC-TC</td>
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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).

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

Table 1B. Predicted PREs for MFGE8 gene.

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<td>Chimp</td>
<td>2724</td>
<td>0.7019</td>
<td>+</td>
<td>AGGACA-TGG-TGTTCT</td>
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For PREs 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 E2 (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.
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, E2 (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 VTFGLQLQH-WVPHELAR. The identification was with a significant Mascot Ion score of 55 and a significant Mascot Expect score of 0.0013. 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
(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
features with normal human endometrial epithelial cells [33-36,48]. Ishikawa cells have been shown to represent an excellent in vitro model to study the regulation of hormonally mediated events surrounding implantation [49-50].

Estradiol significantly upregulated MFGE8 expression at the transcriptional level, and P4 blunted the estrogen effect. However, neither E2 nor P4 appeared to modulate MFGE8 intracellular production or secretion. The identification of the palindromic sequence that defines EREs and PREs allows for the in silico discovery of putative hormone receptor targets in the genome. We demonstrated the presence of putative EREs and PREs in the promoters of several mammalian species suggesting a potential for control of MFGE8 by these hormones [36,40]. While on its own, the presence of hormone response elements in the promoter region of a gene is not evidence of the hormonal control of expression of that gene, their presence suggests that such sites could be used by activated hormone receptors and consequently could affect gene expression. The good conservation of these motifs between Human and Chimp, associated with the positional conservation of these motifs (less than 100 nt) and strand preservation, suggest conservation in closely related species.

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 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

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