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# 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 gonatropin (hCG).

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

**Results**: $E_2$, but not P4 or hCG, significantly upregulated *MFGE8* mRNA expression. hCG significantly increased *MFGE8* secretion. Microvesicels 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 $E_2$ 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

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an Arginine-Glycine-Aspartic acid (RGD) motif that serves as the ligand to its **αvβ**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 **αvβ**3/**β**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 **αvβ**3/**β**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 **αvβ**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 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% $CO_2$ 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 17β-$E_2$ (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 17β-$E_2$ $10^{-7}$ 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 17β-$E_2$ $10^{-7}$ 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 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.

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### 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 ± 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 (**αvβ**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/

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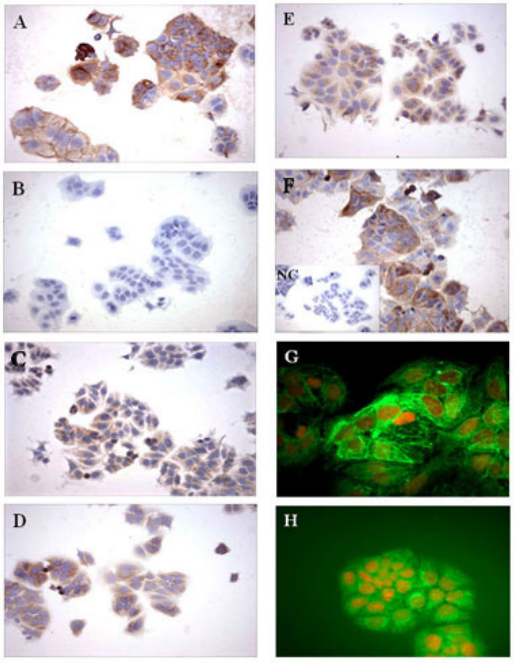


**Figure 1**. Immunocytochemistry results of untreated Ishikawa cells (magnification x200): (**A**) Cytokeratin at 1:30 dilution, marker of epithelial cells. (**B**) Vimentin at 1:50, marker of stromal cells. (**C**) ERα at 1:25. (**D**) ERβ at 1:25. (**E**) PR at 1:25. (**F**) *MFGE8* at 1:1000, note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

**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).

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 –1$^{st}$ half site–3nt spacer–2$^{nd}$ 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 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.

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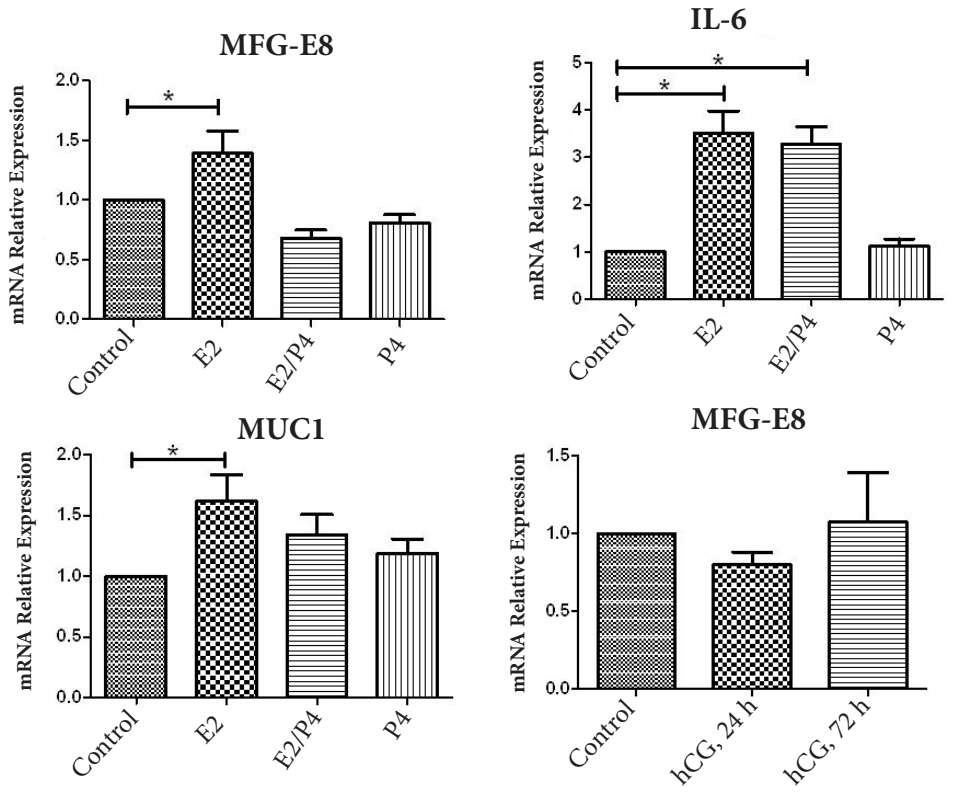


**Figure 2**. Quantitative changes in *MFGE8*, IL-6 and MUC1 mRNA expression in Ishikawa cells (relative expression of target transcripts in relation to the reference gene β-actin). (**A**) The relative gene expression of *MFGE8* was significantly increased by E2 after 24 hours (*P <0.05). On the other 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 MFG-E 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

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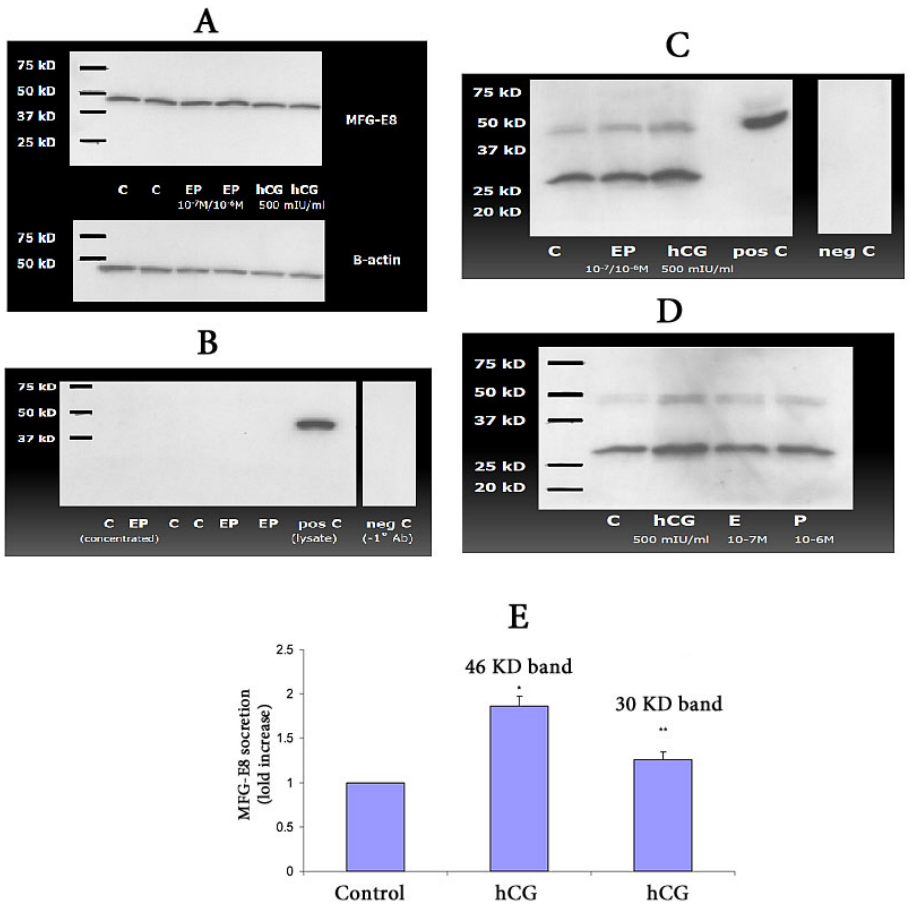


**Figure 3**. Immunoblotting results for *MFGE8*: (**A**) Representative immunoblot of intracellular *MFGE8* protein after 72 hour treatment of Ishikawa cells with E2 and P4, and hCG, with corresponding β-actin immunoblot. (**B**) Representative immunoblot of secreted *MFGE8* protein in the medium of 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: $E_2$ 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 $E_2$ 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), glyceraldehide 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

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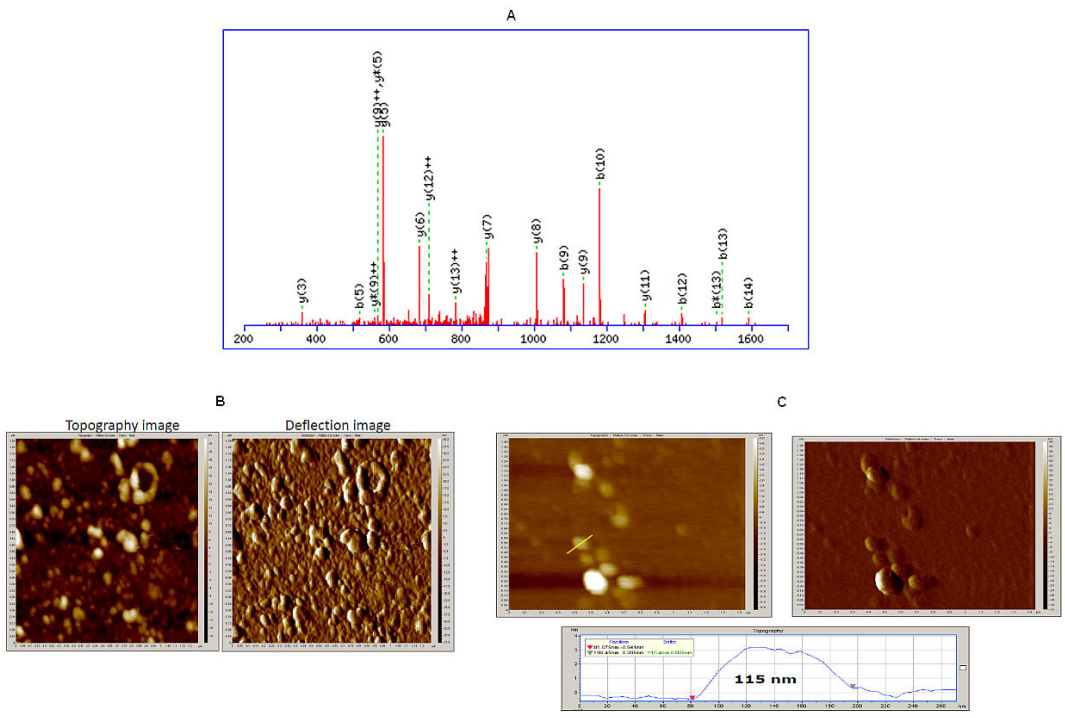


**Figure 4**. **A**. Identification of the secreted *MFGE8* 30 kD band: MS/MS spectra of *MFGE8* peptide 1765.657048 from (883.835800, 2+) with the sequence VTFLGLQHWVPELAR. The y and b ion series corresponding to the various fragments are annotated in the spectrum. The high intensity y5 ion further confirms the validity of the identification. X-axis: intensity, Y-axis: m/z. **B**. Atomic force microscopy of Ishikawa cell microvesicles (microparticles) after isolation from culture medium and immobilization on mica substrate. 1.25 µm topography (left) and deflection (right) images acquired simultaneously in air, in contact mode. Taller features appear lighter in color in the topograph images. Deflection images are included for their improved contrast. (**C**) 1.4 µm topography (left) and deflection (right) images of diluted sample (1:100). Underneath: Cross sectional showing that the measurement of the microvesicle highlighted in (**B**) is approximately 115 nm in diameter.

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 up regulated *MFGE8* expression at the transcriptional level, and P4 blunted the estrogen effect. However, neither $E_2$ 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 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 hypotesis 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 | ✓ | ✓ | -- | -- | -- | -- | -- | -- | -- | -- | -- |

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