

# **Hormonal Regulation of Endometrial Olfactomedin Expression and Its Suppressive Effect on Spheroids Attachment onto Endometrial Epithelial Cells**

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**Abbreviations:** hCG, human chorionic gonadotrophin; IVF, in vitro fertilization; LH, luteinizing hormone; Olfm-1, olfactomedin 1.

*Key words:* Adhesions/ Endometrial Receptivity/ Olfactomedin/ spheroids/ Attachment

## Abstract

25 **Background:** Olfactomedin is a member of a diverse group of extracellular matrix proteins important for neuronal growth. Recent microarray studies identified olfactomedin (Olfm) as one of the down-regulated transcripts in receptive endometrium for embryo attachment and implantation. However, the underlying molecular mechanisms that govern Olfm expression and its effect on embryo  
30 attachment and implantation remain unknown.

**Objectives:** To study the expression of Olfm in the human endometrium by real-time PCR and immunohistochemistry, and the function of Olfm in trophoblast-endometrial cell attachment.

**Setting:** University reproduction unit and laboratory.

35 **Intervention(s):** Real-time PCR, Western blotting and immunohistochemistry on human endometrial biopsies from natural and ovarian stimulated cycles. In vitro spheroids-endometrial cells co-culture study.

**Results:** Human endometrial Olfm-1 and Olfm-2 transcripts decreased significantly from the proliferative to the secretory phases of the menstrual cycle. Olfm protein was  
40 strongly expressed in the luminal and glandular epithelium and moderately in the stromal cells of human endometria. Ovarian stimulation significantly decreased ( $p<0.05$ ) the expression of endometrial Olfm-1 and -2 transcripts in patients receiving IVF treatment when compared with those in the natural cycle. Importantly, recombinant Olfm-1 suppressed JAr spheroids attachment onto Ishikawa cells and this  
45 was not associated with changes of  $\beta$ -catenin and E-cadherin expression in trophoblast and endometrial cells.

**Conclusion(s):** Decreased expression of Olfm during the receptive phase of the endometrium may allow successful trophoblast attachment for implantation.

## 50 **Introduction**

The attachment of a preimplantation embryo onto the uteri endometrium initiates the establishment of pregnancy. The endometrium is receptive to the implanting embryo only within a short period, termed the “Window of Implantation” (WOI), which lies between days 19–24 of the menstrual cycle in humans. Recent  
55 microarray studies have identified a number of genes that are differentially regulated during the WOI. In mice, the gene expression profiles of whole uterine tissue before and after implantation (Yoshioka et al., 2000), and between implantation and inter-implantation sites (Reese et al., 2001) has been studied. Due to ethical reasons, similar studies in humans with embryos at the implantation site are not possible.  
60 However, four microarray studies (Carson et al., 2002; Kao et al., 2002; Borthwick et al., 2003; Riesewijk et al., 2003) compared the gene expression of the endometrium at the WOI with that at the proliferative phase or early secretory phase, and found differential expression of a large number of genes. While these studies demonstrated the developmental complexity of the endometrium, there were only a few similarities  
65 between their results, in which only three genes (Dickkopf homolog 1, osteopontin and apolipoprotein D) were up-regulated and one gene (Olfactomedin, Olfm) was down-regulated (Horcajadas et al., 2004). Previously, we reported that down-regulation of endometrial olfactomedin-1 (Olfm-1) expression was found in patients receiving ovarian stimulation for IVF treatment (Liu et al., 2008).

70 Olfm was first cloned from the olfactory mucus of *Rana catesbeiana* (Yokoe et al., 1993), and is a member of a diverse group of extracellular matrix proteins including the neural cell adhesion molecule, laminin, fibronectin and proteoglycans (Reichardt et al., 1991). More than 100 known Olfm members have been discovered in various species (Zeng et al., 2005). Molecular analysis has shown that Olfms are  
75 glycoproteins that can form stable polymers through interaction with the glycans of

the neighboring molecules (Yokoe et al., 1993). An increasing number of proteins are found to contain an Olfm domain, including amassin in sea urchins, tiarin in amphibians, and noelin and myocilin/TIGR in vertebrates. Although the biological functions of members in the Olfm family are not well understood, they are likely to be  
80 involved in the formation of the extracellular matrix (Karavanich et al., 1998). Clinically, an increased expression of Olfm-1 is associated with unexplained recurrent spontaneous abortion (Lee et al., 2007). Forced expression of Olfm-1 in a human endometrial cell line inhibited cell growth and induced cell cycle arrest at the S and G<sub>2</sub>-M phases (Lee et al., 2007). Yet, Olfm-1 transcript was down-regulated in  
85 endometrial cancer with uncontrolled cell proliferation (Wong et al., 2007). A recently identified novel human Olfm-like protein-1 (hOLFML1), which contains an Olfm domain, enhances proliferation of the HeLa cells in vitro (Wan et al., 2008). Zebrafish Olfm-1 interacts with WIF-1, a secreted inhibitor of Wnt-signaling, and regulates retinal axon elongation in vivo (Nakaya et al., 2008). Moreover, an  
90 Olfm-like glycoprotein, hOLF44, has been isolated and suggested to be involved in human placental and embryonic development (Zeng et al., 2004). The widespread occurrence of Olfm-related proteins in vertebrates and invertebrates suggests that this family has functions of universal importance.

In the present study, we hypothesized that Olfm is differentially expressed in the  
95 endometrium of a normal menstrual cycle and that it modulates implantation. We aimed to determine the expression of Olfm transcripts in human endometria taken in natural and stimulated cycles, to correlate their expression with the hormonal status of the women, and to study the role of Olfm in regulating trophoblast attachment onto endometrial epithelial cells in vitro.

## 100 **Methods and Materials**

### **Patients**

Infertile women who attended the Assisted Reproduction Unit, The University of Hong Kong–Queen Mary Hospital for IVF treatment were recruited for this study. They had regular menstrual cycles, normal uterus and no significant intrauterine or  
105 ovarian abnormalities as determined by transvaginal ultrasonography. Only women with regular menstrual cycles and male factor infertility who had not received any steroidal hormones for 2 months or more prior to the study and also agreed to the use of condoms for contraception during the study cycle were recruited for endometrial biopsies in the natural cycle (Liu et al., 2010). The study protocol was approved by  
110 the Institutional Review Board of The University of Hong Kong/Hospital Authority Hong Kong West Cluster. Written informed consent was obtained from all patients prior to participation in the study.

Endometrial biopsies were taken from patients undergoing diagnostic laparoscopy for assessment of tubal patency from 2004 to 2005. These samples  
115 were taken in different phases of the menstrual cycle including early-/mid-proliferate phase (Day 1-9, EPMP, n=8), late proliferate phase (Day 10-14, LP, n=8), early secretory phase (Day 15-18, ES, n=8), middle secretory phase (Day 19-23, MS, n=15), and late secretory phase (Day 24-28, LS, n=6). The donors of these samples in different groups did not differ significantly ( $p>0.05$ ) in terms of age and duration of  
120 infertility.

Human endometrial samples from the stimulated cycle were collected from early 2002 to December 2008. The ovarian stimulation was carried out as described previously using the long protocol (Ng et al., 2000). Endometrial biopsies in the stimulated cycles (n=32) were obtained seven days after the hCG injection (hCG+7),  
125 and endometrial biopsies in natural cycles (n=15) were taken on day LH+7 (7 days

after luteinizing hormone surge) as described previously (Liu et al., 2008). Those patients were recruited when embryo transfer was not performed due to the failure of fertilization caused by male factors, absence of spermatozoa in testicular sperm extraction on the day of oocyte retrieval or when the serum E<sub>2</sub> level was >20,000pmol/L on the day of hCG injection.

### **Tissue Collection**

Biopsies were performed as an outpatient procedure from the fundal and upper part of the body of the uterus using the Pipelle device (CCD Laboratories, France). The biopsy specimen was snap-frozen in liquid nitrogen and was stored at -80° C until total RNA isolation was achieved. Serum E<sub>2</sub> and progesterone (P<sub>4</sub>) levels were also measured on the day of the biopsy by commercially available chemiluminescent-based immunoassay kits as reported previously (Ng et al., 2000).

### **RNA extraction, reverse transcription and real-time PCR**

Total RNAs from human endometrial biopsies were extracted using the Absolutely RNA Microprep Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. RNA samples (300 ng) were reverse-transcribed into cDNA using TaqMan® Reverse Transcription Reagents (PE Applied Biosystems, Foster City, CA). Multiplex real-time polymerase chain reaction using 18S as an internal control for the normalization of RNA loading was performed in a 20 µl reaction mixture with Assays-on-Demand Gene Expression Assay for human Olfm-1, -2, -3 and -4 (PE Applied Biosystems). Samples from the mid-secretory phase were used as calibrators for different PCR experiments. The human Olfm-1 (Hs00255159\_m1), Olfm-2 (Hs00608880\_m1), Olfm-3 (Hs00379238\_m1), Olfm-4 (Hs00197437\_m1), estrogen receptor-α (Hs00174860\_m1), estrogen receptor-β

(Hs00230957\_m1) and ribosomal 18S (Hs99999901\_s1) TaqMan probes were used (PE Applied Biosystems).

## 155 **Protein extraction and Western blotting**

Total proteins from endometrial biopsies and cell lysates [Ishikawa, JAr and fallopian tube epithelial OE-E6/E7 (Lee et al., 2001) cells] were dissolved in RIPA solution (1X PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors. The Olfm-1,  $\beta$ -catenin, E-cadherin protein expression was analyzed by Western blotting. The membranes were probed with affinity purified rabbit anti-human Olfm polyclonal antibody (1:1000 dilution, Zymed Lab, Burlingame, CA) raised against Olfm-1 peptide (aa 246-259, C-YMDGYHNNRFVREY-NH<sub>2</sub>), mouse anti-E-cadherin (1:1000, Abcam, Cambridge, MA), or mouse anti- $\beta$ -catenin (1:2500, BD Bioscience, San Jose, CA) in blocking solution overnight at 4°C. The anti-rabbit or anti-mouse secondary antibody conjugated with horseradish peroxidase (1:5000, GE Healthcare, Pittsburgh, PA) was used. Olfm-2 and Olfm-3 peptides (C-YMDGYKGRRVLEF-NH<sub>2</sub> and C-YMDSYTNNKIVREY-NH<sub>2</sub>, respectively) were synthesized (GenScript, Piscataway, NJ) and used to neutralize Olfm-1 antibody (5-fold excess) at 4°C for 4 hours. After thorough washing, the membrane was visualized by enhanced chemiluminescence reagent (Santa Cruz). To normalize the protein loading, the membranes were stripped and detected for  $\beta$ -actin using anti- $\beta$ -actin antibody (Sigma, St Louis, MO).

## 175 **Production, purification and labeling of human recombinant Olfm (rhOlfm)**

The full length Olfm-1 coding sequence was amplified from cDNA reverse transcribed from total RNA isolated from human endometrial epithelial cells by PCR

using the Expand Long Template PCR system with proof reading Taq DNA polymerase (Roche, Mannheim, Germany) and gene-specific primers (Forward: 180 5'-CGGATCATATGCCAGGTCGTTGGAGGTGG-3'; Reverse: 5'-CATGCTCAGC CTACAACTCGTCGGAGCGGAT-3, restriction sites underlined) containing *Nde*I and *Bln*I restriction sites, and cloned into a pET15b expression vector (Novagen, Gibbstown, NJ). One positive clone was transformed into *E. coli* BL21 (DE3) RIL cells (Stratagene, La Jolla, CA), and human recombinant Olfm-1 (rhOlfm-1) protein 185 was induced with 1 mM isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) for 3 hrs at 37°C. rhOlfm-1 was purified using a protein refolding kit (Novagen, Gibbstown, NJ) as described previously (Lee et al., 2006). The identity of the purified protein was confirmed by Western blotting and mass spectrometry analysis. To determine the binding of rhOlfm-1 to the endometrial and trophoblast cells, 50  $\mu$ g of the 190 recombinant protein was labeled with the AlexaFluor® 488 Microscale Protein Labeling kit (Molecular Probes, Invitrogen, Carlsbad, CA) using the protocol described by the manufacturer. Then, the cells were incubated with the medium containing the labeled recombinant protein (5  $\mu$ g/ml) for 3 hrs and washed twice with PBS, and the signal was detected under a fluorescence microscope.

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### **Immunohistochemistry**

Human endometrial biopsies were fixed in buffered formalin, embedded in paraffin, sectioned at 5  $\mu$ m in thickness, and mounted on poly-lysine coated-slides. Tissue sections were de-paraffinized, which was followed by antigen retrieval using 200 the Target Retrieval Solution (DakoCytomation, Carpinteria, CA) as previously described (Lee et al., 2006). A rabbit anti-human Olfm polyclonal antibody (1:200), no primary antibody or an antibody pre-absorbed with the antigen/blocking peptide (Zymed Lab) was included. Finally, positive signal was detected using



3,3'-diaminobenzidine (DakoCytomation). Nuclei were counter-stained with  
205 hematoxylin. The sections were observed with a Zeiss Axioskop microscope  
(Photometrics Sensys, Roper Scientific, Tucson, AZ) with bright-field optics.

### **Spheroids-endometrial cell attachment assay**

Human choriocarcinoma cells (JAR, ATCC HTB-144) and endometrial  
210 adenocarcinoma cells (Ishikawa, ECACC 99040201) were cultured at 37°C in a  
humid atmosphere with 5% CO<sub>2</sub>. Adhesion of choriocarcinoma JAR cells to  
endometrial Ishikawa cells was quantified using an adhesion assay as described (Hohn  
et al., 2000; Uchida et al., 2007; Liu et al., 2010) with modifications. For the  
co-culture study, JAR cells were treated with 0.1-10 µg/ml Olfm-1, 5 µM methotrexate  
215 (MTX) as a positive control and 1 µg/ml BSA as a negative control for 48 hrs.  
Multi-cellular spheroids were generated by rotating the trypsinized cells at 4g force  
for 24 hrs. The spheroids of sizes 60–200 µm were transferred onto the surface of a  
confluent monolayer of the Ishikawa cells pre-treated with Olfm-1 for 24 hrs. The  
cultures were maintained in MEM medium with rhOlfm-1 for 1 hr at 37°C in a  
220 humidified atmosphere with 5% CO<sub>2</sub>. Non-adherent spheroids were removed by  
centrifugation at 10g for 10 min. To study the effect of Olfm-1 on JAR cells only, the  
JAR cells were treated with rhOlfm-1 for 48 hrs before spheroid generation. The  
spheroids were then co-cultured with the untreated Ishikawa cells for an hour. The  
effect of Olfm-1 on Ishikawa cells was studied by treating the cells with rhOlfm-1 for  
225 24 hrs before co-culture with untreated JAR spheroids for an hour. Attached spheroids  
were counted under a dissecting microscope, and expressed as the percentage of the  
total number of spheroids used (% adhesion). Photographs of the cultures were taken  
with a Nikon Eclipse TE300 inverted microscope (Nikon, Tokyo, Japan).

230 **Statistical analysis**

All the data were analyzed by statistical software (SigmaPlot 10.0 and SigmaStat 2.03; Jandel Scientific, San Rafael, CA). The non-parametric analysis of variance on rank test for multiple comparisons followed by the Mann-Whitney U test was used when the data were not normally distributed. A probability value  $<0.05$

235 was considered to be statistically significant.

## Results

### Expression of Olfms in human endometrium in the menstrual cycle

Olfm has four known transcripts (Olfm-1, -2, -3 and -4) and their expressions in  
240 different phases of the menstrual cycle were studied by real-time PCR. Olfm-1 and  
-2 transcripts were highly expressed in the proliferative phase, but not at the secretory  
phase of the menstrual cycle. Significant differences ( $p < 0.05$ ,  $> 3$ -fold) in the  
expression levels of these transcripts were found between the proliferative phases and  
the mid-/late-secretory phases (Fig. 1A). Yet, no significant difference ( $p > 0.05$ ) was  
245 found in the expression of Olfm-3 or -4 transcripts in samples from different phases of  
the cycle (Fig. 1A).

The polyclonal antibody against Olfm-1 peptide was designed and raised from  
the rabbit. Western blotting confirmed a specific band of the expected size (50-kDa)  
in the human endometrial lysate (Fig. 1B). Olfm-1 antibody could not be neutralized  
250 by Olfm-2 and -3 peptides. The expression of Olfm-1 protein was higher in the  
proliferative phase of the menstrual cycle (Fig. 1C). Immunohistochemistry on a  
paraffin-embedded human endometrial section identified strong Olfm-1  
immunoreactivity in the luminal and glandular epithelium, and a weaker signal in the  
stromal cells (Fig. 1D). The signal could be nullified when the antibody was  
255 pre-absorbed with Olfm-1 peptide.

### Effect of ovarian stimulation on endometrial Olfm transcripts expression

The expression of Olfm transcripts in human endometria isolated from patients  
who underwent ovarian stimulation was compared with that from the patients from  
260 natural cycles. Both Olfm-1 and -2 transcripts were found to be significantly  
( $p < 0.05$ ) down-regulated in the stimulated group (Fig. 2). Although there was a

slight increase in the expression of Olfm-4 transcript in the stimulated group, the difference was not statistically significant.

## 265 **Association of Olfm-1 and -2 expressions with serum estradiol, progesterone concentrations and estrogen receptor levels**

Linear regression and Pearson correlation analysis were used to analyze the relationship of Olfm-1 and Olfm-2 transcripts with serum E<sub>2</sub> and P<sub>4</sub> concentrations and estrogen receptor  $\alpha$  and  $\beta$  transcript levels (Fig. 3). Serum E<sub>2</sub> levels on hCG/LH  
270 day and P<sub>4</sub> levels on hCG/LH+7 day were negatively correlated ( $p < 0.05$ ) with Olfm-2, but not Olfm-1 transcript levels. Interestingly, estrogen receptor  $\alpha$  was positively correlated ( $p < 0.001$ ) with both Olfm-1 and Olfm-2 levels. Yet, estrogen receptor  $\beta$  was negatively correlated ( $p < 0.01$ ) with Olfm-2 but not Olfm-1 levels in the human endometrial samples.

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## **Effect of Olfm-1 on Spheroids-endometrial cell attachment assay**

rhOlfm-1 protein was expressed in *E. coli* as inclusion bodies. Soluble rhOlfm-1 was obtained from the inclusion bodies after dialysis. The binding of AlaxaFlour-labeled Olfm-1 was tested on JAr and Ishikawa cells. It was found that  
280 both JAr and Ishikawa bound AlaxaFlour-labeled rhOlfm-1 (Fig. 4A). To study how rhOlfm-1 modulates the attachment process in vitro, we used the spheroids-endometrial cells co-culture assay to study the attachment of spheroids onto Ishikawa cells. Treatment of JAr cells for 48 hrs with rhOlfm-1 (0.1-10  $\mu\text{g/ml}$ ) did not affect the attachment rate onto Ishikawa cells (Fig. 4B). However, there was a  
285 significant decrease ( $p < 0.05$ ) in the attachment rate when 10  $\mu\text{g/ml}$  rhOlfm-1 was used to treat the Ishikawa cells (Fig. 4C). When both JAr and Ishikawa cells were treated with rhOlfm-1 (0.1-10  $\mu\text{g/ml}$ ), there was a dose-dependent suppression of JAr

spheroids attachment onto the Ishikawa cells (Figure 4D). The percentage of attachment decreased from 87% to 57% from 0.1 to 10  $\mu\text{g/ml}$  Olfm-1 used. 290 Similarly, the positive control MTX (5  $\mu\text{M}$ ) strongly suppressed the attachment of JAr onto the Ishikawa cells in all the experiments performed. No significant decrease in attachment was observed when 1 $\mu\text{g/ml}$  BSA was used (97% vs 95%) when compared with the untreated control. The average viability of the JAr and Ishikawa cells in all the groups after treatment was 90–94% as determined by Trypan Blue staining (data 295 not shown).

#### **Effect of Olfm on E-cadherin and $\beta$ -catenin expression in JAr and Ishikawa cells**

To study whether Olfm regulates Wnt-signaling and extracellular matrix molecule expression in JAr and Ishikawa cells during the attachment process, the 300 expression levels of  $\beta$ -catenin and E-cadherin were determined. Treatment of the JAr or Ishikawa cells with Olfm-1 at 0-10 $\mu\text{g/ml}$  for 24 hrs and 28 hrs, respectively, did not affect E-cadherin and  $\beta$ -catenin expression (Fig. 5). No observable change in  $\beta$ -actin expression was detected.

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## Discussion

Previous microarray analysis suggested that down-regulation of Olfm-1 was found in the receptive endometrium (Horcajadas et al., 2004). Our findings further suggested that both endometrial Olfm-1 and -2 transcripts, as well as Olfm-1 protein  
310 were down-regulated in the mid-secretory phase of the natural cycle and in patients who received ovarian stimulation for IVF treatment. Moreover, recombinant human Olfm-1 protein binds to JAr and Ishikawa cells, and suppresses spheroid-endometrial cell attachment in vitro. The suppressive effect was not associated with down-regulation of  $\beta$ -catenin or E-cadherin expression in neither Ishikawa nor JAr  
315 cells.

Both Olfm-1 and -2 transcripts were down-regulated in the secretory phase of the menstrual cycle when the serum estrogen and progesterone levels were high. Estrogen promotes, while progesterone suppresses the expression of ER and PR in the endometrial cells during the late proliferative phase and secretory phase of the  
320 menstrual cycle (Nisolle et al., 1994). Since the expression of Olfm-1 and -2 transcripts were positively correlated with the endometrial ER $\alpha$  level and high serum progesterone level suppresses ER $\alpha$  expression, it is possible that the steroid hormones may play both direct and indirect roles in regulating Olfm expression in vitro and in vivo. Yet, no consensus estrogen responsive element (ERE) or the progesterone  
325 responsive element (PRE) were found in the Olfm proximal promoter regions, suggesting that the steroid regulation of Olfm-1 and -2 expression may be indirect.

Accumulating evidence suggested that high serum estradiol level advances endometrial development (Basir et al., 2001) not conducive to embryo implantation. Results from our laboratory demonstrated an advancement of gene expression profiles  
330 in patient with ovarian hyperstimulation (Liu et al., 2008). However, Olfm transcripts remained at similar levels at the secretory phase of the natural cycle,

suggesting that down-regulation of Olfm expression by ovarian stimulation may not be associated with an advancement of endometrial development.

Fluorescent-labeled Olfm-1 strongly binds to JAr and Ishikawa cells. It is possible that Olfm-1 may form a protective layer on the apical surface of the luminal epithelium, and its down-regulation in the peri-implantation period allows apposition and adhesion of the blastocysts onto the receptive endometrium while allowing the other molecules also to expose and interact with the embryo. Yet, further investigations are needed to study if Olfm-1 binds to a specific receptor onto these cell lines and whether these bindings are specific to cells in reproductive tract.

A similar function has been proposed for MUC-1, a potent anti-adhesive extracellular matrix molecule in the endometrium (Meseguer et al., 1998). MUC-1 is down-regulated at the implantation site during the receptive period (Carson et al., 1998). Results from the JAr spheroids-Ishikawa cells attachment assay support our hypothesis that rhOlfm-1 may play a similar role to MUC-1 in embryo attachment. Interestingly, treatment of the JAr trophoblast cells with Olfm-1 had no effect on attachment, but treatment of the Ishikawa cells with Olfm-1 at a high concentration (10 µg/ml) significantly reduced the attachment rate, albeit that the Ishikawa cells express a detectable amount of Olfm protein (data not shown). Importantly, when both JAr and Ishikawa cells were treated with Olfm-1, the change in the attachment rate became more prominent, showing a dose-dependent inhibition on attachment (from 97% to 57%).

Yet, results from this study suggested that further decrease in Olfm-1 expression in the hyperstimulated endometrium is not associated with an increase in endometrial receptivity in vivo. In fact, the expression of Olfm-1 transcript in the secretory phase of natural cycle is low. It is likely that down-regulation of other implantation-related (e.g. Wnt-signaling) signaling molecules may affect embryo

attachment in vivo. For example, high level of DKK-1, a Wnt-signaling inhibitor which was commonly found in the endometria of ovarian stimulated patients, inhibits spheroids attachment in vitro (Liu et al., 2010). Similarly, inactivation of nuclear Wnt- $\beta$ -catenin signaling limits blastocyst competency for implantation (Xie et al., 2008; Chen et al., 2009).

A differential expression of cadherins and integrins may be involved in the successful attachment of an embryo onto the human endometrium (van der Linden et al., 1995). A recent study demonstrated that an increased E-cadherin expression is associated with an increase in receptivity in non-receptive endometrial (AN3-CA) cells to BeWo spheroids attachment (Rahnama et al., 2009). However, we did not observe detectable changes in  $\beta$ -catenin and E-cadherin expression in Ishikawa or JAr cells, although rhOlfm-1 binds to both cell types. Therefore, it is likely that either Olfm-1 functions as a physical barrier for implantation or another unidentified signaling pathway may play an important role in the implantation process.

In summary, we first demonstrated the down-regulation of endometrial Olfm transcripts and protein at the secretory phase of the menstrual cycle. Human recombinant Olfm-1 suppresses the attachment of spheroids onto endometrial Ishikawa cells and down-regulation of Olfm-1 during the receptive period may favor embryo attachment for successful implantation.

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## Figure Legends

### Figure 1

485 **The expression of olfactomedin (Olfm) transcripts in human endometrium.** The expression levels of (A) Olfm-1, -2, -3 and -4 transcripts were determined by real-time PCR. The values were the mean of  $\log_2$  (RQ)  $\pm$  standard deviation of fold-induction of Olfm mRNA in multiple comparison calculated by the  $2^{\Delta\Delta Ct}$  method. a-b denotes significant difference ( $p < 0.05$ ) between groups. EP+MP: Early-Mid Proliferative, LP: Late Proliferative, ESMS: Early-Mid Secretory, LS: Late Secretory.

490 (B) Sequence alignment of the Olfm peptides revealed higher homology between Olfm-1, -2 and -3 (blue). Western blotting using Ishikawa, JAr and OE-E6/E7 cells showed a single band of about 50-kDa in size. The band could be neutralized with Olfm-1, but not Olfm-2 and -3 peptides.  $\beta$ -actin protein was used as loading control.

495 (C) Western blotting was performed for Olfm-1 expression in human endometrial samples (proliferative vs secretory phases). The protein loading was normalized with  $\beta$ -actin expression. (D) Immunohistochemical staining of Olfm-1 (brown staining) in human endometrium at the proliferative phase of the menstrual cycle. Strong immunostaining was localized at the glandular and luminal epithelial cells. No signal

500 was found when the antibody was pre-absorbed with blocking peptide. Scale bar = 10 $\mu$ m.

### Figure 2

**The expression of olfactomedin in the endometrium of IVF patients.** Human endometrial samples were taken from patients receiving IVF treatment. The patients were divided into two groups: natural (n=15) and stimulated (n=32) cycles. The expression levels of Olfm-1, -2, -3 and -4 transcripts in these groups were determined

by real-time PCR analysis. \* denotes significant difference ( $p < 0.05$ ) between groups.

510 **Figure 3**

**Linear regression and Pearson correlation analysis on the expression of Olfm-1 and Olfm-2 transcripts with serum  $E_2$  and  $P_4$  levels, and  $ER\alpha$  and  $ER\beta$  mRNA expression in endometrial tissues.** Pearson correlation of 47 endometrial samples

was performed for Olfm-1 and Olfm-2 mRNA expression with the serum  $E_2$  level  
515 (pmol/L) at LH/hCG day, LH/hCG+7 day, serum  $P_4$  level (nmol/L) at LH/hCG+7 day, and tissue  $ER\alpha$  and  $ER\beta$  mRNA expression (fold-change normalized with 18S RNA). A p-value  $< 0.05$  denotes significant difference and the R value denotes the correlation coefficient.

520 **Figure 4**

**Effect of Olfm-1 on the attachment of JAr spheroids to Ishikawa cells.** (A)

Immunofluorescence staining of labeled rhOlfm-1. Both JAr and Ishikawa bind to the labeled rhOlfm-1. (B) The effect of rhOlfm-1 treatment on JAr cells and the changes in attachment rate. JAr cells treated with rhOlfm-1 protein were trypsinized

525 and shaken for 24 hours to obtain spheroids of 60–200 $\mu$ m in size. The spheroids were put onto Ishikawa monolayer for an hour and the number of spheroids attached was determined as a percentage of the number of spheroids added. The effect of MTX

and/or BSA and Olfm-1 recombinant protein on the attachment of JAr spheroids was determined. (C) The effect of rhOlfm-1 treatment on Ishikawa cells on the

530 attachment rate in a co-culture study. (D) The effect of rhOlfm-1 treatment on both

JAr and Ishikawa cells. Olfm-1 dose-dependently suppressed JAr attachment, while BSA at 1 $\mu$ g/ml did not affect spheroids attachment under the same culturing condition.

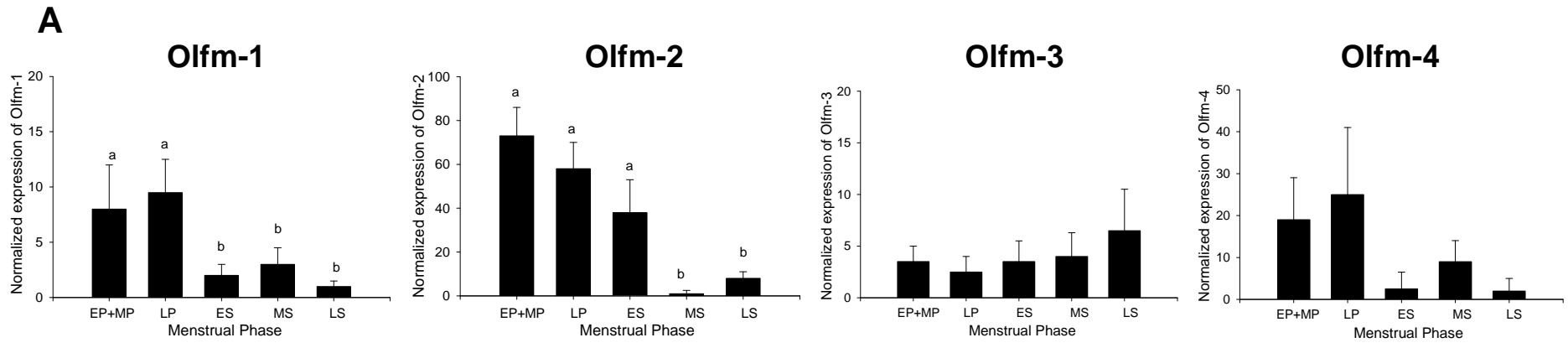
The results were pooled from at least 4 independent experiments using more than

2000 spheroids. a-b, b-c and a-c denotes significant difference ( $p < 0.01$ ) from the  
535 control by One way ANOVA followed by Scheffe's mean separation test.

### **Figure 5**

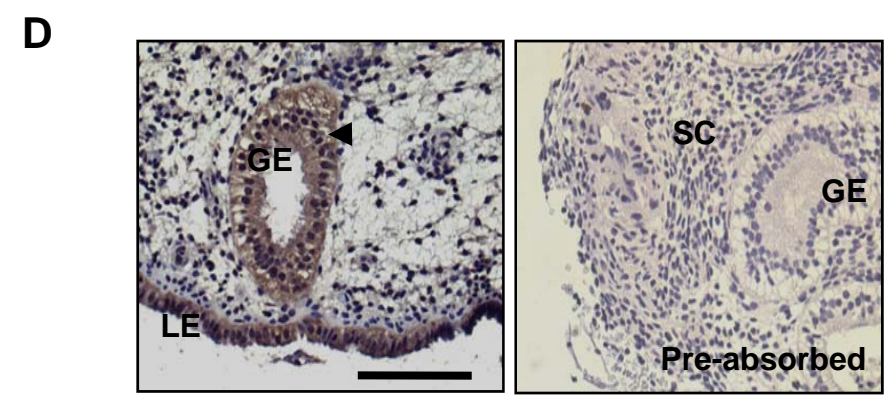
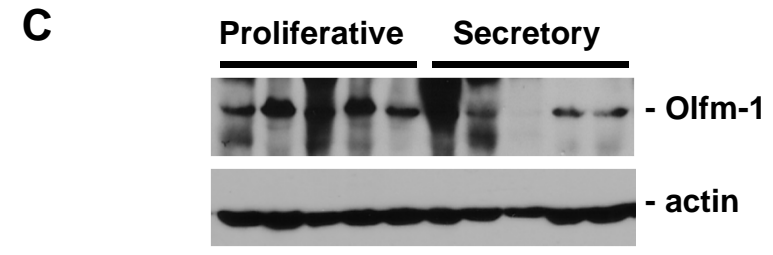
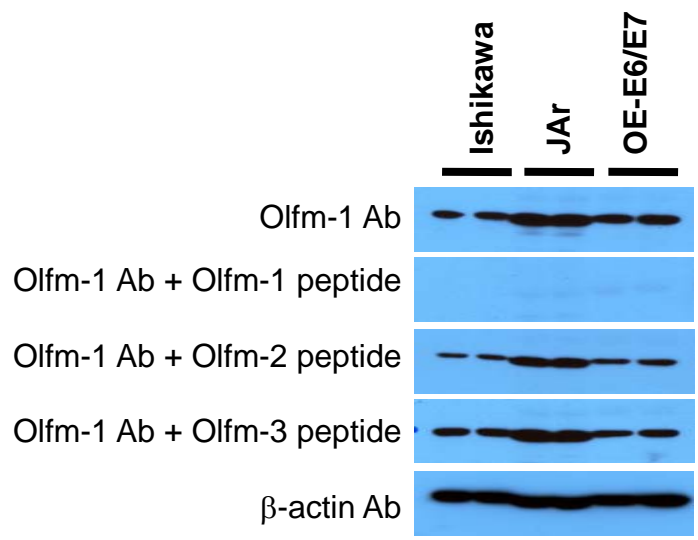
**Effect of Olfm-1 on the expression of E-cadherin and  $\beta$ -catenin in Ishikawa and JAr cells.** Ishikawa and JAr cells were treated with 0.1-10 $\mu$ g/ml rhOlfm-1 protein  
540 for 24 hrs and 48 hrs, respectively. The expression levels of E-cadherin and  $\beta$ -catenin proteins were determined by Western blotting. The protein loading was normalized by  $\beta$ -actin expression.

**Figure 1**



**B**

HuOlfm-1	238	PEGDNRVWYMDGYHNNRFVREYKSMVD
HuOlfm-2	224	PSADSRVWYMDGYKGRVLEFRTLGD
HuOlfm-3	228	SEKNNRVWYMDSYTNNKIVREYKSIAD
HuOlfm-4	281	QHPNKGLYWVAPLNTDGRLLLEYRLYN





**Figure 2**

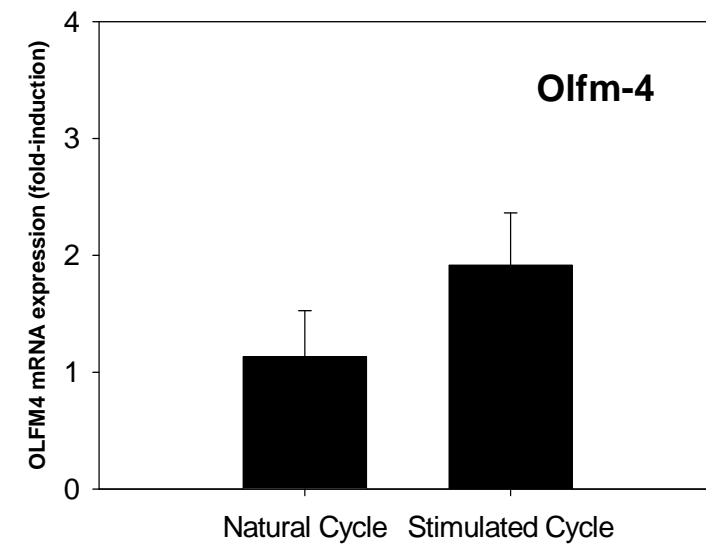
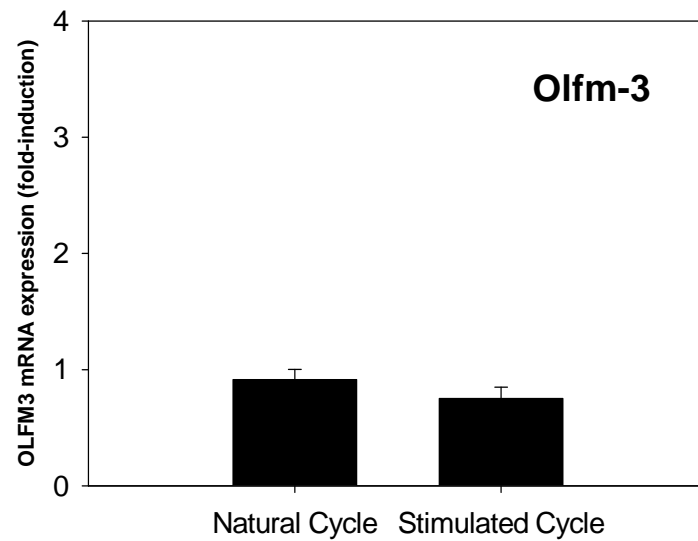
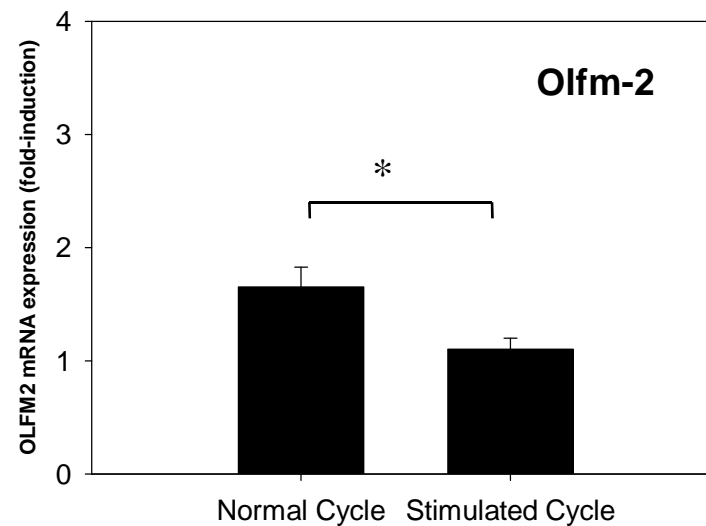
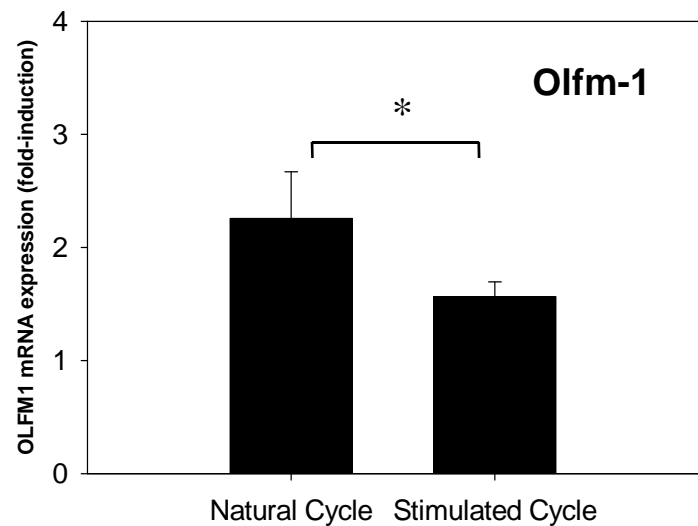


Figure 3

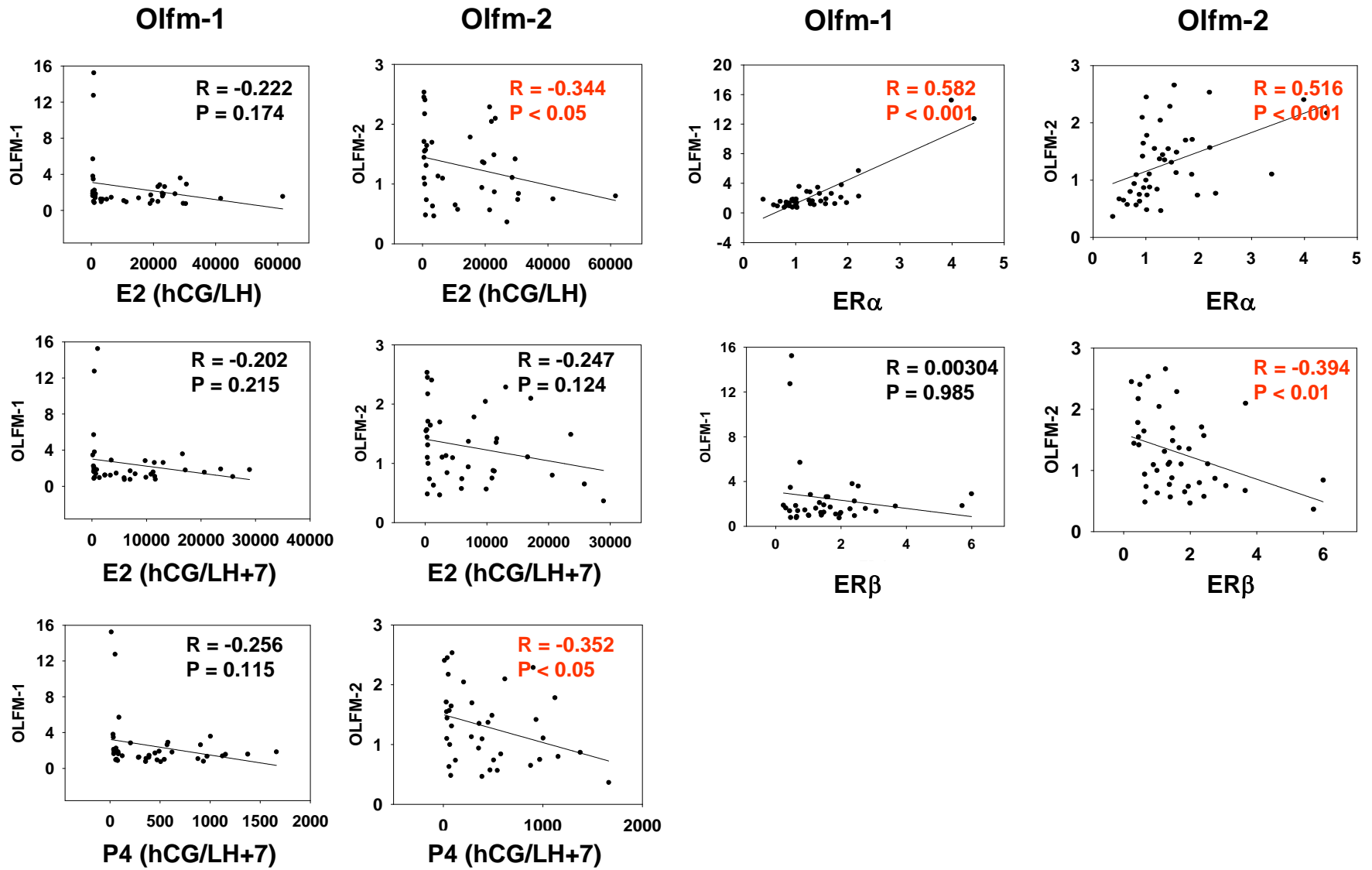
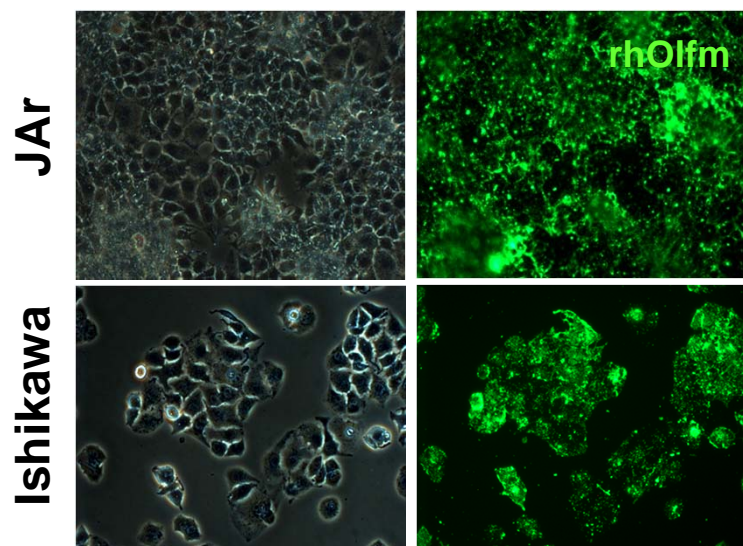
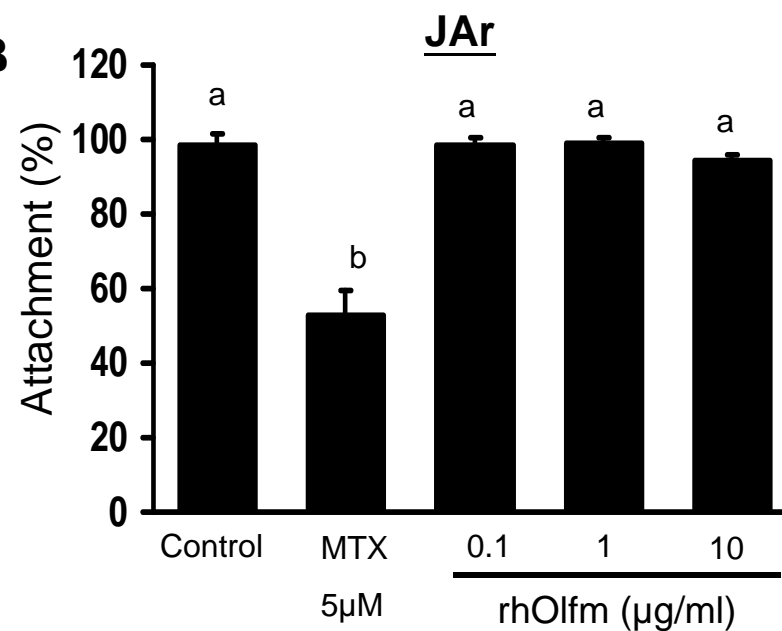


Figure 4

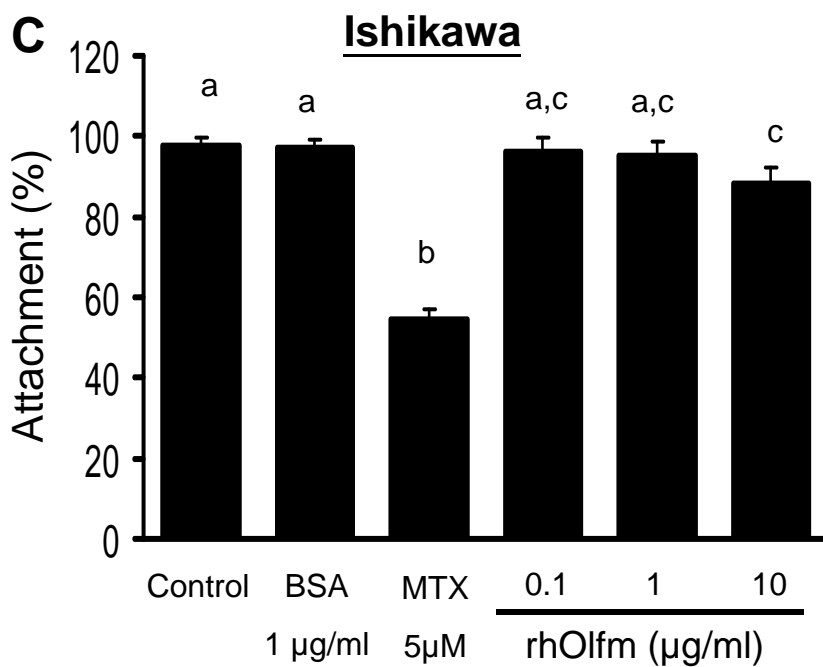
A



B



C



D

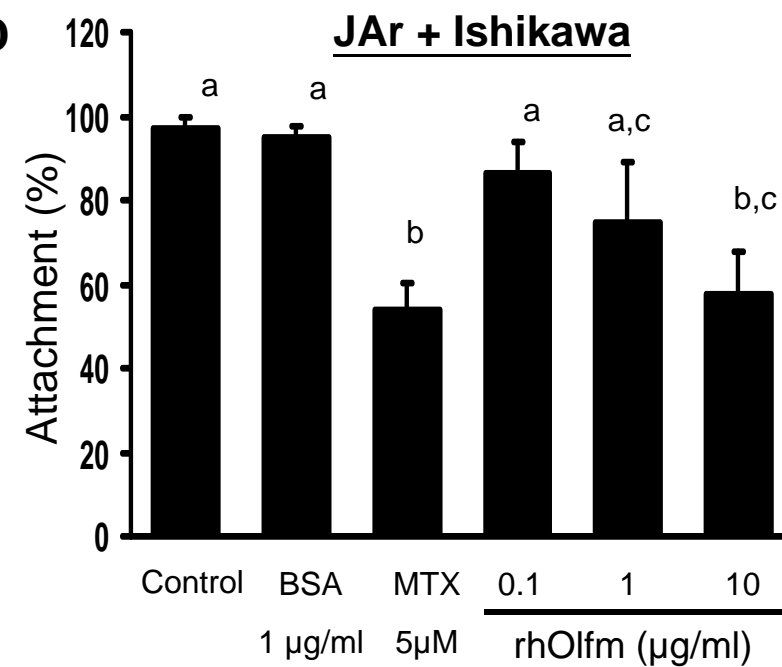


Figure 5

