Title	Human chorionic gonadotropin stimulates spheroid attachment on fallopian tube epithelial cells through the mitogen-activated protein kinase pathway and down-regulation of olfactomedin-1
Author(s)	So, KH; Kodithuwakku, SPK; Kottawatta, KSA; Li, RHW; Chiu, PCN; Cheung, ANY; Ng, EHY; Yeung, WSB; Lee, CKF
Citation	Fertility and Sterility, 2015, v. 104 n. 2, p. 474-482
Issued Date	2015
URL	http://hdl.handle.net/10722/214727
Rights	Creative Commons: Attribution 3.0 Hong Kong License

hCG promote implantation in oviduct **F & S 19650 style revision**

Human chorionic gonadotropin stimulates spheroid attachment on Fallopian tube epithelial cells through the mitogen-activated protein kinase pathway and down-regulation of Olfactomedin-1

Kam-Hei So, M.Phil.,[1] Suranga P. Kodithuwakku, Ph.D.,[1,2] Kottawattage S. A. Kottawatta, M.Phil.,[1,3] Raymond H.W. Li, M.B.B.S., F.R.C.O.G.,[1,4,5] Philip C.N. Chiu, Ph.D.,[1,4,5] Annie N.Y. Cheung, M.D., F.R.C.Path.,[6] Ernest H.Y. Ng, M.D., F.R.C.O.G.,[1,4,5] William S.B. Yeung, Ph.D.,[1,4,5] and Kai-Fai Lee, Ph.D.,[1,4,5,*]

[1]Department of Obstetrics and Gynaecology, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong SAR, China
[2]Department of Animal Science, Faculty of Agriculture, The University of Peradeniya, Peradeniya, Sri Lanka
[3]Department of Veterinary Public Health and Pharmacology, Faculty of Veterinary Medicine and Animal Science, The University of Peradeniya, Peradeniya, Sri Lanka
[4]Centre for Reproduction, Development and Growth, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong SAR, China
[5]ShenZhen Key Laboratory of Fertility Regulation. The University of Hong Kong-Shenzhen Hospital, Haiyuan 1st Road, Futian District, Shenzhen, 518053, China
[6]Department of Pathology, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong SAR, China

*Corresponding author: Kai-Fai Lee (PhD), Department of Obstetrics and Gynaecology, The University of Hong Kong, Pokfulam, Hong Kong. Fax: 852-28161947 and E-mail: ckflee@hku.hk

K.H.S. has nothing to disclose. S.P.K. has nothing to disclose. K.S.A.K. has nothing to disclose. R.H.W.L. has nothing to disclose. P.C.N.C. has nothing to disclose. A.N.Y.C. has nothing to disclose. E.H.Y.N. has nothing to disclose. W.S.B.Y. has nothing to disclose. K.F.L. has nothing to disclose.

Capsule

hCG increases the attachment of trophoblastic spheroids on human Fallopian tube epithelial cells through activation of Erk and Wnt/β-catenin signaling pathways leading to down-regulation of Olfm1 expression.

Objective: To study the effect of human chorionic gonadotropin (hCG) on Olfactomedin-1 (Olfm1) expression and spheroid attachment in human Fallopian tube epithelial cells in vitro.

Design: Experimental study

Setting: Reproductive biology laboratory

Patient(s): Healthy non-pregnant women

Intervention(s): No patient interventions

Main Outcome Measure(s): Luteinizing hormone/chorionic gonadotropin receptor (LHCGR) and Olfm1 expression in Fallopian tube epithelium cell line (OE-E6/E7 cells). OE-E6/E7 cells treated with hCG, U0126 Erk inhibitor or XAV939 Wnt/β-catenin inhibitor were analyzed by Western blotting, RT-PCR, and *in vitro* spheroid attachment assay.

Result(s): hCG increased spheroid attachment on OE-E6/E7 cells through down-regulation of Olfm1 and activation of Wnt and MAPK signaling pathways. U0126 down-regulated both MAPK and Wnt/β-catenin signaling pathways and up-regulated Olfm1 expression. XAV939 down-regulated only the Wnt/β-catenin-signaling pathway but up-regulated Olfm1 expression.

Conclusion(s): hCG activated both Erk and Wnt/β-catenin signaling pathways and enhanced spheroid attachment on Fallopian tube epithelial cells through down-regulation of Olfm1 expression.

Key Words: Olfactomedin-1; Fallopian tube; tubal ectopic pregnancy; Wnt-signaling; MAPK signaling

Introduction

The Fallopian tube, also known as oviduct, consists of epithelial and stromal cells forming the mucosal layer, which is surrounded by a layer of smooth muscle cells. The Fallopian tube has several important functions, including transport of gametes for fertilization and transport of the developing embryo to the uterus for implantation. Ectopic pregnancies occur in approximately 2% of all pregnancies (1), of which about 70% occur in the ampullary region of the tube (2, 3). Tubal ectopic pregnancies (TEP) may lead to complications such as tubal rupture, hemorrhage and maternal mortality. The predisposing factors for TEP were thought to be the impaired transport of the embryo in the Fallopian tube and modifications in the tubal environment favoring tubal implantation (4). Modification of the Fallopian tube environment could be induced by inflammation or modulated by signals from the embryo itself.

Human chorionic gonadotropin (hCG) is a peptide hormone secreted by the pre-implantation embryo starting from the 8-cell stage (5, 6). This heterodimeric glycoprotein is structurally similar to luteinizing hormone (LH) and can act as a luteal phase support in assisted reproduction (7). Both LH and hCG interact with the cell surface luteinizing hormone/chorionic gonadotropin receptor (LHCGR). Recent studies have suggested that hCG may play important roles during embryo implantation by modulating the maternal immune system, down-regulating anti-adhesion molecules, and controlling trophoblast invasion (8-10). However, the mechanisms by which hCG regulates embryo implantation remain largely unknown.

Functionally and biologically active LHCGRs are expressed in several tissues, including the female reproductive tract from the Fallopian tubes to the uterus (11-13). However, some studies have reported that hCG can carry out its action independent of LHCGR (14, 15). LHCGR is a G-protein coupled receptor that is capable of acting through multiple signal transduction pathways, including phospholipid-specific phospholipase C and adenylyl cyclase, which activate the mitogen-activated protein kinase (MAPK) pathway (16, 17). The MAPK pathway contains many components including extracellular signal-regulated kinase (Erk), c-Jun N-terminal kinase (JNKs), and p38. It has been reported that hCG can stimulate production of prostaglandin in endometrial epithelial cells through the phosphatidylinositol 3-kinase-extracellular regulatory kinase pathway (18). Recent studies have suggested that Erk can crosstalk and regulate the Wnt/β-catenin signaling pathway, which is essential for implantation (19-21).

In the Wnt/β-catenin signaling pathway, the Wnt ligand binds to the cell surface Frizzled (Fz) receptor, which is a seven-transmembrane domain receptor (22). Interaction between the Wnt ligand and Fz receptor activates Dishevelled (Dvl), which leads to inactivation of glycogen synthase kinase-3β (GSK-3β) (23). GSK-3β is one of the core components of the β-catenin destruction complex, which consists of GSK-3β, phosphorylated adenomatous polyposis coli (APC), and Axin (24). Inactivation of any of the molecules in the β-catenin destruction complex allows β-catenin to escape proteasomal degradation, resulting in cytoplasmic accumulation of β-catenin that can enter the nucleus for gene activation (25).

The Wnt/β-catenin signaling pathway has been widely studied and found to be important in normal pregnancy (26, 27). Activation of the Wnt/β-catenin signaling pathway is essential for embryo adhesion onto the endometrium, trophoblast migration, vascularization, and angiogenesis of the placenta (28). The Wnt/β-catenin signaling pathway has been found to be involved in Fallopian tube inflammation and tubal ectopic pregnancy (29). Wnt activation down-regulated olfactomedin-1 (Olfm1) in oviductal epithelial cells, resulting in a microenvironment which may predispose to TEP (30). Therefore, it is important to understand the regulation and effects of Wnt/β-catenin signaling in implantation to understand tubal ectopic pregnancy.

Olfm1 expression in the endometrium is mediated by progesterone and is down-regulated during the receptive period of the cycle (31-34), which suggests the presence of Olfm1 may hinder embryo attachment. In zebrafish, Olfm1 regulated the Wnt-signaling pathway and modulated retinal axon elongation (35). Previously, we used a trophoblastic spheroid (JAr)-endometrial epithelial cell (Ishikawa) co-culture model to demonstrate the suppressive effect of Olfm1 on JAr spheroid attachment on Ishikawa cells (36). However, how Olfm1 is regulated at the feto-maternal interface remains largely unknown.

We hypothesized that hCG secreted from human pre-implantation embryos can enhance embryo attachment onto Fallopian tube epithelial cells through down-regulation of Olfm1 expression in the tube. It is likely that hCG secreted from the embryo accompanied with embryo retention in the Fallopian tube may predispose to TEP. In the present study, we investigated the effect of

142 hCG on the attachment rate of trophoblastic spheroids on Fallopian tube
143 epithelial cells (OE-E6/E7 cells) using a co-culture model.
144
145

Materials and Methods

Study Participants

Normal Fallopian tubes were collected from 15 non-pregnant women (age range, 37-51 years; mean ± SD, 42.8±4.9 years) who had undergone hysterectomy for benign gynaecological conditions at Department of Obstetrics and Gynaecology, Queen Mary Hospital, Hong Kong. All patients had regular menstrual cycles (21-30 days; mean ± SD, 26.5±3 days) and did not have history of tubal pathology. The phase of menstrual cycle of each patient sample was determined by the date of the last menstrual period. Written consent was obtained from all participants. The study was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (UW10-109)

Immunohistochemistry and Histological Scoring

Fallopian tube biopsies were fixed in 4% paraformaldehyde followed by 70% ethanol. The biopsies were embedded in paraffin wax and sectioned at a thickness of 5 μm and mounted on polylysine-coated slides. Tissue sections were deparaffinized and rehydrated, and then subjected to antigen retrieval using Target Retrieval Solution (Dako Cytomation, Carpinteria, CA, USA). Ampullary region of Fallopian tube tissues sections were incubated with anti-β-catenin (1:200, BD610153, BD Biosciences, San Jose, California, USA), anti-LHCGR (1:200; ab125214, Abcam, Cambridge, MA, USA) and anti-Olfm1 (1:50; ab71540, Abcam) antibodies for 18 h. Fallopian tube sections were incubated in 3,3'-diaminobenzidine (DAB substrate chromogen, Dako Cytomation) and the nucleus was counter-stained with Hematoxylin. Images were captured under a light microscope with a digital camera (Axioscop, Zeiss,

Göttingen, Germany). The intensity of staining of the epithelial cells in the Fallopian tube sections was quantitated by a single observer using Histological scoring (H-score) in a total of 500 cells in each section (from 4 fields with more than 100 cells in each field) as described previously (30). Results were presented as mean ± SD.

Western Blotting

For Western blot analysis, total protein extraction was performed by dissolving cell lysates from Fallopian tube epithelial cell line (OE-E6/E7 cells) or isolated primary Fallopian epithelial cells (37) in radioimmunoprecipitation assay (RIPA) buffer solution [1x phosphate-buffered saline (PBS), 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS] containing protease inhibitors (Calbiochem, Darmstadt, Germany). The membrane was probed with anti-Olfm1 (1:400, ab71540, Abcam), anti-active-β-catenin (1:1000, 05-665, Millipore, Billerica, MA, USA), anti-β-catenin (1:1000, BD610153, BD Biosciences), anti-Axin2 (1:1000, ab32197, Abcam), anti-phospho-Erk (1:1000, 9910, Cell Signaling Technology Inc., Danvers, MA, USA), anti-Erk (1;1000, 9926, Cell Signaling Technology Inc.), anti-phospho-JNK (1:1000, 9910, Cell Signaling Technology Inc.), anti-JNK (1:1000, 9926, Cell Signaling Technology Inc.), anti-phospho-p38 (1:1000, 9910, Cell Signaling Technology Inc.), and anti-p38 (1:1000, 9926, Cell Signaling Technology Inc.) antibodies in blocking solution overnight at 4^{o}C. The membrane was washed five times in PBST for 5 min and incubated with anti-rabbit or anti-mouse secondary antibody conjugated with horseradish peroxidase (1:5000, GE Healthcare, Pittsburgh, PA, USA) for 1 h. After washing five times in PBST for 5 min, the signal was visualized using an enhanced chemiluminescence reagent (Santa Cruz, Santa

198 Cruz). Protein levels were normalized by probing the membrane with β-actin
199 antibody (Sigma, St Louis, MO, USA).
200
201 **Spheroid Co-culture Assay**
202 The spheroid attachment assay was performed as previously described
203 (37, 38). Human trophoblastic JAr cells (blastocyst surrogate) and OE-E6/E7
204 cells were used as the co-culture model. Trophoblastic spheroids of about 100
205 µm in size were prepared from trypsinized JAr cells and incubated on an
206 orbital shaker rotating at 106 rpm for 24 h at 37^{o}C. The trophoblastic spheroids
207 were transferred onto a confluent monolayer of hCG-treated (0.25, 2.5 or 25
208 IU/ml) OE-E6/E7 cells (in DMEM/F12 with 10% fetal bovine serum and 1%
209 L-glutamine) in a 12-well culture plate and incubated for 1 h at 37^{o}C.
210 Unattached spheroids were removed by shaking the culture plates at 140 rpm
211 for 10 min. The attached spheroids remaining on the OE-E6/E7 monolayer
212 were counted. The attachment rate was expressed as a percentage of the
213 number of attached spheroids divided by the total number of spheroids added
214 onto the OE-E6/E7 monolayer.
215
216 **Luciferase Reporter Assay**
217 OE-E6/E7 cells were seeded at a density of 20,000 cells per well in a
218 24-well plate 24 h prior to transfection with 1 µg of TOPFLASH or FOPFLASH
219 and 0.2 µg of the internal control plasmid (pRK-TK, Promega, Madison, WI,
220 USA). TOPFLASH and FOPFLASH contain a native or mutated binding
221 sequence for TCF/LEF, respectively, which are transcription factors in the
222 Wnt/β-catenin signaling pathway. Transfection was carried out using
223 Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Total cell extracts were

prepared for the luciferase activity assay 24 h post-transfection according to the manufacturer's instructions (Promega).

Treatment with hCG, MAPK Inhibitor or Wnt Inhibitor

Both OE-E6/E7 and JAr cells were treated with hCG (0, 0.25, 2.5 and 25 IU/mL) for 24 h. For the MAPK inhibitor study, OE-E6/E7 cells were treated with 0.07 μM U0126 Erk inhibitor for 15 min. For the Wnt inhibitor study, OE-E6/E7 cells were treated with 0.1 μM XAV939 Wnt inhibitor for 24 h.

Statistical Analysis

Statistical analysis was determined by non-parametric ANOVA on Rank test. Non-parametric Mann Whitney U test or parametric Student's t-test were used where appropriate as the post-test. All data were presented as mean ± standard error mean (S.E.M.). Each experiment was repeated in triplicate. A p-value < 0.05 was considered statistically significant.

Results

OLFM1 and LHCGR Expression in the Fallopian tube at different phases of the menstrual cycle

Olfm1 and LHCGR proteins were strongly expressed in the cytoplasm of the human Fallopian epithelial cells as detected by immunohistochemistry (Fig. 1A). Olfm1 expression was significantly lower during the luteal phase of the cycle compared to the follicular and peri-ovulatory phases (Fig. 1B). In contrast, LHCGR expression in Fallopian tube epithelial cells was significantly higher during the luteal phase compared to the follicular and peri-ovulatory phases (Fig. 1B). Olfm1 and LHCGR were weakly expressed in the stromal cells of the Fallopian tube throughout all menstrual phases.

Effect of hCG treatment on the attachment of JAr spheroids on OE-E6/E7 cells

Spheroids of about 100 µm in size were prepared for the spheroid attachment assay (Fig. 1C). OE-E6/E7 or JAr cells were treated with different concentrations of hCG (0, 0.25, 2.5 and 25 IU/mL). OE-E6/E7 cells treated with a concentration of 25 IU/mL hCG significantly increased the attachment rate of non-treated JAr spheroid ($P<0.05$, Fig. 1Dtop panel). On the other hand, JAr spheroids treated with different concentrations of hCG before seeding onto the OE-E6/E7 cells untreated with hCG did not show any significant difference in attachment rate (Fig. 1D, middle panel). OE-E6/E7 cells treated with 2.5 and 25 IU/ml hCG had significantly higher attachment rate to JAr cells treated with hCG a fixed concentration of 25 IU/ml ($P<0.05$, Fig. 1D, bottom panel).

Effect of hCG on the expressions of Olfm1 and Wnt/β-catenin molecules

in OE-E6/E7 and primary Fallopian epithelial cells from tissue

OE-E6/E7 cells express hCG receptor, when OE-E6/E7 was treated with hCG (2.5 and 25 IU/mL), there was no effect on hCG receptor, but significantly decreased the Olfm1 expression (Fig. 2A). Similarly, hCG (25 IU/mL) down-regulated Olfm1 expression in primary Fallopian epithelial cells isolated from Fallopian tissue (Fig. 2B). β-catenin was localized at the epithelium of human Fallopian tube and hCG up-regulated active β-catenin expression in primary Fallopian epithelial cells isolated from Fallopian tissue (Fig. 2C). hCG down-regulated Axin2 expression, but increased the ratio of active-β-catenin/total β-catenin expressions in the OE-E6/E7 cells (Fig. 2D). The effect of hCG on Wnt-signaling activation was confirmed using the TOP/FOP Flash luciferase reporter assay. Treatment with 25 IU/mL hCG significantly increased luciferase activity in the transfected OE-E6/E7 cells (Fig. 2E).

Effect of Erk or Wnt inhibitors on MAPK and Wnt/β-catenin signaling pathways

Treatment with hCG at 2.5 and 25 IU/mL activated Erk (phosphor-Erk/Total-Erk), but not JNK (phosphor-JNK/total-JNK) or p38 (phosphor-p38/Total p38) (Fig. 3A). Treatment with U0126 inactivated the Erk pathway by significantly down-regulating phospho-Erk expression, while it significantly suppressed spheroid attachment (Fig. 3B). Interestingly, U0126 also down-regulated active-β-catenin expression in OE-E6/E7 cells. Treatment with XAV939 inactivated the Wnt/β-catenin signaling pathway, while it also significantly suppressed spheroid attachment. However, there was no change in the expression of Erk signaling molecules with the XAV939 treatment.

Treatment of OE-E6/E7 cells with either U0126 or XAV939 increased Olfm1 expression (Fig. 3B), but hCG could not suppress the stimulating effects of U0126 or XAV939, even though it could suppress Olfm1 expression in OE-E6/E7 cells.

Discussion

In this study, we demonstrated that human Fallopian tube epithelium and the OE-E6/E7 cell line both expressed LHCGRs. Our results suggest that OE-E6/E7 or human primary Fallopian epithelial cells treated with hCG down-regulated Olfm1 expression. hCG enhanced JAr spheroid attachment to OE-E6/E7 cells in vitro through the activation of Wnt/β-catenin and Erk pathways, but not through the JNK or p38 pathways.

Accumulating evidence has suggested that retention of pre-implantation embryo, tubal inflammation, and cigarette smoking are predisposing factors leading to TEP (39). Results from the present study suggest that hCG could promote attachment of the embryo on Fallopian tube epithelial cells possibly leading to TEP. hCG can stimulate trophoblast invasion through activation of Erk1/Erk2, up-regulation of leptin expression (40), and secretion of TIMP1 in human endometrial stromal cells (41). hCG can also trigger angiogenesis through the modulation of stromal cell responsiveness to interleukin 1 (42) and can up-regulate trophinin, a cell adhesion molecule in endometrial cells (43).

We found that Olfm1 expression was down-regulated at the ampullary region of the human Fallopian tube at the luteal phase of the cycle. The decreased expression of tubal Olfm1 was in agreement with our previous finding that endometrial Olfm1 was down-regulated in the secretory phase of the cycle (36), when serum progesterone level is high (44). hCG is known to be secreted by trophoblastic cells in human pre-implantation embryo. It has been reported that the trophoblastic JAr cells can secrete 0.1 IU/mL hCG in 24

h (45) and the trophoblastic spheroid can secrete <0.01 IU/mL hCG in 6 days of culture (46). We found that a high hCG level in the local microenvironment favored embryo attachment on epithelial cells in the Fallopian tubes. The upper dosage of hCG (25 IU/mL) used in this experiment was comparable to various studies, which range from 1 to 50 IU/ml (8-10). Assuming that the volume of Fallopian tube to be 1-10 μl, the concentration of hCG encountered by pre-implantation embryo in the blocked tube which could reach 617.5 ~ 61.8 IU/ml (9). Results from this study may shed light on the underlying mechanism on the role of hCG in regulating Olfm1 in ectopic pregnancy.

LHCGR is a G-protein coupled receptor that activates many downstream signaling pathways such as MAPK pathway (16, 17). Accumulating evidence has suggested that the MAPK pathway cross-talks with the Wnt/β-catenin signaling pathway through regulation of the β-catenin destruction complex, which consists of Axin2, APC and GSK3β (47). We previously reported that Wnt-activation down-regulated Olfm1 in Fallopian tube epithelial cells and promoted spheroid attachment (30). To elucidate the molecular mechanism involved in the hCG-mediated attachment process, we examined whether hCG regulated the Wnt/β-catenin signaling pathway. We found that β-catenin was localized in the epithelial cells of the Fallopian tube and similar expression pattern was reported in mouse oviduct (48). hCG treatment up-regulated active-β-catenin and down-regulated Axin2 expressions. This suggests that Axin2 down-regulation could allow β-catenin to enter the nucleus for the activation of gene transcription (49). Similarly, microarray profiling has also confirmed that Wnt-related genes could be activated by hCG in pre-ovulatory ovarian follicles (50).

Our result showed both Erk and Wnt/β-catenin signaling pathways were activated by hCG. However, it was still unknown if the MAPK pathway was upstream or downstream of Wnt/β-catenin signaling pathway. Accordingly, we treated OE-E6/E7 cells with either the U0126 Erk inhibitor or the XAV939 Wnt inhibitor to suppress phospho-Erk or Wnt/β-catenin signaling pathways, respectively. Interestingly, XAV939 could only inactivate the Wnt/β-catenin signaling pathway but not the Erk signaling pathway, which indicated the Erk signaling pathway was likely to be an upstream regulator of Wnt/β-catenin signaling pathway in response to hCG treatment (Fig. 4).

Estradiol and progesterone together promote the synthesis of LH receptor in the epithelium of pig oviduct (51, 52). High progesterone level is associated with a lower Olfm1 expression level during the implantation window in human endometrium, and suppression of Olfm1 expression enhances spheroid attachment in OE-E6/E7 cells (36). Therefore, it is likely that steroid hormones may modulate the effect of embryo-derived hCG in activating hCG/LH receptor through Erk/Wnt-signaling pathway to suppress Olfm1 expression in the human Fallopian tube.

In summary, this study demonstrated the role of hCG in regulating the attachment of trophoblastic spheroids (blastocyst surrogate) on human Fallopian tube epithelial cells through activation of Erk and Wnt/β-catenin signaling pathways leading to down-regulation of Olfm1 expression. Our results suggest that changes in the embryo microenvironment in the Fallopian tube induced by hCG could predispose to TEP. Further studies will be needed

to focus on the role of hCG in regulating Erk and Wnt/β-catenin expressions in patients with TEP.

Acknowledgements

This study was supported in part by grants from the Committee on Research and Conference grant, The University of Hong Kong and General Research Fund (HKU770813M), Hong Kong Research Grant Council to KFL.

References

1. Marion LL and Meeks GR. Ectopic pregnancy: History, incidence, epidemiology, and risk factors. Clin Obstet Gynecol 2012;55(2):376-86.
2. Roy A and Matzuk MM. Reproductive tract function and dysfunction in women. Nat Rev Endocrinol 2011;7(9):517-25.
3. Bouyer J, Coste J, Fernandez H, Pouly JL, and Job-Spira N. Sites of ectopic pregnancy: a 10 year population-based study of 1800 cases. Hum Reprod 2002;17(12):3224-30.
4. Horne AW and Critchley HO. Mechanisms of disease: the endocrinology of ectopic pregnancy. Expert Rev Mol Med 2012;14:e7.
5. Bonduelle ML, Dodd R, Liebaers I, Van Steirteghem A, Williamson R, and Akhurst R. Chorionic gonadotrophin-β mRNA, a trophoblast marker, is expressed in human 8-cell embryos derived from tripronucleate zygotes. Hum Reprod 1988;3(7):909-914.
6. Lopata A, Oliva K, Stanton PG, and Robertson DM. Analysis of chorionic gonadotrophin secreted by cultured human blastocysts. Mol Hum Reprod 1997;3(6):517-21.
7. Huirne JF, Lambalk C, Loenen AD, Schats R, Hompes PA, Fauser BJM, et al. Contemporary Pharmacological Manipulation in Assisted Reproduction. Drugs 2004;64(3):297-322.
8. Perrier d'Hauterive S, Charlet-Renard C, Berndt S, Dubois M, Munaut C, Goffin F, et al. Human chorionic gonadotropin and growth factors at the embryonic ‐ endometrial interface control leukemia inhibitory factor (LIF) and interleukin 6 (IL-6) secretion by human endometrial epithelium. Hum Reprod 2004;19(11):2633-2643.
9. Nakayama J, Aoki D, Suga T, Akama TO, Ishizone S, Yamaguchi H, et al. Implantation-dependent expression of trophinin by maternal fallopian tube epithelia during tubal pregnancies: possible role of human chorionic gonadotrophin on ectopic pregnancy. Am J Pathol 2003;163(6):2211-9.
10. Tapia Pizarro A, Argandona F, Palomino WA, and Devoto L. Human chorionic gonadotropin (hCG) modulation of TIMP1 secretion by human endometrial stromal cells facilitates extravillous trophoblast invasion in vitro. Hum Reprod 2013;28(8):2215-27.
11. Lei ZM, Toth P, Rao CV, and Pridham D. Novel coexpression of human chorionic gonadotropin (hCG)/human luteinizing hormone receptors and their ligand hCG in human fallopian tubes. J Clin Endocrinol Metab 1993;77(3):863-72.

12. Ziecik AJ, Kaczmarek MM, Blitek A, Kowalczyk AE, Li X, and Rahman NA. Novel biological and possible applicable roles of LH/hCG receptor. Mol Cell Endocrinol 2007;269(1-2):51-60.
13. Berndt S, d'Hauterive SP, Blacher S, Péqueux C, Lorquet S, Munaut C, et al. Angiogenic activity of human chorionic gonadotropin through LH receptor activation on endothelial and epithelial cells of the endometrium. FASEB J 2006;20(14):2630-2632.
14. Lee CL, Chiu PCN, Hautala L, Salo T, Yeung WSB, Stenman UH, et al. Human chorionic gonadotropin and its free β-subunit stimulate trophoblast invasion independent of LH/hCG receptor. Mol Cell Endocrinol 2013;375(1–2):43-52.
15. Viswanath G, Chatterjee S, and Roy P. Assessment of luteinizing hormone receptor function in an endometrial cancer cell line, Ishikawa cells in response to human chorionic gonadotrophin (hCG). Mol Cell Endocrinol 2007;272(1–2):14-21.
16. Ryu KS, Gilchrist RL, Koo YB, Ji I, and Ji TH. Gene, interaction, signal generation, signal divergence and signal transduction of the LH/CG receptor. Int J Gynaecol Obstet 1998;60 Suppl 1:S9-20.
17. Leung PC and Steele GL. Intracellular signaling in the gonads. Endocr Rev 1992;13(3):476-98.
18. Banerjee P, Sapru K, Strakova Z, and Fazleabas AT. Chorionic Gonadotropin Regulates Prostaglandin E Synthase via a Phosphatidylinositol 3-Kinase-Extracellular Regulatory Kinase Pathway in a Human Endometrial Epithelial Cell Line: Implications for Endometrial Responses for Embryo Implantation. Endocrinology 2009;150(9):4326-4337.
19. Krejci P, Aklian A, Kaucka M, Sevcikova E, Prochazkova J, Masek JK, et al. Receptor Tyrosine Kinases Activate Canonical WNT/β-Catenin Signaling via MAP Kinase/LRP6 Pathway and Direct β-Catenin Phosphorylation. PLoS ONE 2012;7(4):e35826.
20. Bikkavilli RK and Malbon CC. Mitogen-activated protein kinases and Wnt/beta-catenin signaling: Molecular conversations among signaling pathways. Commun Integr Biol 2009;2(1):46-9.
21. Cervenka I, Wolf J, Masek J, Krejci P, Wilcox WR, Kozubik A, et al. Mitogen-activated protein kinases promote WNT/beta-catenin signaling via phosphorylation of LRP6. Mol Cell Biol 2011;31(1):179-89.
22. Bhanot P, Brink M, Samos CH, Hsieh JC, Wang Y, Macke JP, et al. A new member of the frizzled family from Drosophila functions as a Wingless receptor. Nature 1996;382(6588):225-30.
23. Willert K and Nusse R. β-catenin: a key mediator of Wnt signaling. Curr Opin

Genet Dev 1998;8(1):95-102.
24. Nakamura T, Hamada F, Ishidate T, Anai K-i, Kawahara K, Toyoshima K, et al. Axin, an inhibitor of the Wnt signalling pathway, interacts with β-catenin, GSK-3β and APC and reduces the β-catenin level. Genes Cells 1998;3(6):395-403.
25. van Noort M, Meeldijk J, van der Zee R, Destree O, and Clevers H. Wnt Signaling Controls the Phosphorylation Status of β-Catenin. J Biol Chem 2002;277(20):17901-17905.
26. Mohamed OA, Jonnaert M, Labelle-Dumais C, Kuroda K, Clarke HJ, and Dufort D. Uterine Wnt/beta-catenin signaling is required for implantation. Proc Natl Acad Sci U S A 2005;102(24):8579-84.
27. Xie H, Tranguch S, Jia X, Zhang H, Das SK, Dey SK, et al. Inactivation of nuclear Wnt-beta-catenin signaling limits blastocyst competency for implantation. Development 2008;135(4):717-27.
28. van der Horst PH, Wang Y, van der Zee M, Burger CW, and Blok LJ. Interaction between sex hormones and WNT/β-catenin signal transduction in endometrial physiology and disease. Mol. Cell. Endocrinol. 2012;358(2):176-184.
29. Li P, Zhu Wj, Ma Zl, Wang G, Peng H, Chen Y, et al. Enhanced beta-catenin expression and inflammation are associated with human ectopic tubal pregnancy. Hum Reprod 2013;28(9):2363-71.
30. Kodithuwakku SP, Pang RT, Ng EH, Cheung AN, Horne AW, Ho PC, et al. Wnt activation downregulates olfactomedin-1 in Fallopian tubal epithelial cells: a microenvironment predisposed to tubal ectopic pregnancy. Lab Invest 2012;92(2):256-64.
31. Borthwick JM, Charnock Jones DS, Tom BD, Hull ML, Teirney R, Phillips SC, et al. Determination of the transcript profile of human endometrium. Mol Hum Reprod 2003;9(1):19-33.
32. Horcajadas JA, Riesewijk A, Martín J, Cervero A, Mosselman S, Pellicer A, et al. Global gene expression profiling of human endometrial receptivity. J Reprod Immunol 2004;63(1):41-49.
33. Liu Y, Lee KF, Ng EHY, Yeung WSB, and Ho PC. Gene expression profiling of human peri-implantation endometria between natural and stimulated cycles. Fertil Steril 2008;90(6):2152-2164.
34. Riesewijk A, Martín J, van Os R, Horcajadas JA, Polman J, Pellicer A, et al. Gene expression profiling of human endometrial receptivity on days LH+2 versus LH+7 by microarray technology. Mol Hum Reprod 2003;9(5):253-264.
35. Nakaya N, Lee HS, Takada Y, Tzchori I, and Tomarev SI. Zebrafish Olfactomedin

1 Regulates Retinal Axon Elongation In Vivo and Is a Modulator of Wnt Signaling Pathway. J Neurosci 2008;28(31):7900-7910.

36. Kodithuwakku SP, Ng PY, Liu Y, Ng EHY, Yeung WSB, Ho PC, et al. Hormonal regulation of endometrial olfactomedin expression and its suppressive effect on spheroid attachment onto endometrial epithelial cells. Hum Reprod 2011;26(1):167-175.

37. Lee YL, Lee KF, Xu JS, Wang YL, Tsao SW, and Yeung WS. Establishment and characterization of an immortalized human oviductal cell line. Mol Reprod Dev 2001;59(4):400-9.

38. So KH, Lee CL, Yeung WSB, and Lee KF. Glycodelin suppresses endometrial cell migration and invasion but stimulates spheroid attachment. Reprod Biomed Online 2012;24(6):639-645.

39. Shaw JLV, Dey SK, Critchley HOD, and Horne AW. Current knowledge of the aetiology of human tubal ectopic pregnancy. Hum Reprod Update 2010;16(4):432-444.

40. Maymó JL, Pérez Pérez A, Sánchez-Margalet V, Dueñas JL, Calvo JC, and Varone CL. Up-Regulation of Placental Leptin by Human Chorionic Gonadotropin. Endocrinology 2009;150(1):304-313.

41. Tapia-Pizarro A, Argandona F, Palomino WA, and Devoto L. Human chorionic gonadotropin (hCG) modulation of TIMP1 secretion by human endometrial stromal cells facilitates extravillous trophoblast invasion in vitro. Hum Reprod 2013;28(8):2215-27.

42. Bourdiec A, Shao R, Rao CV, and Akoum A. Human chorionic gonadotropin triggers angiogenesis via the modulation of endometrial stromal cell responsiveness to interleukin 1: a new possible mechanism underlying embryo implantation. Biol Reprod 2012;87(3):66.

43. Sugihara K, Kabir-Salmani M, Byrne J, Wolf DP, Lessey B, Iwashita M, et al. Induction of trophinin in human endometrial surface epithelia by CGβ and IL-1β. FEBS Letters 2008;582(2):197-202.

44. Kao LC, Germeyer A, Tulac S, Lobo S, Yang JP, Taylor RN, et al. Expression profiling of endometrium from women with endometriosis reveals candidate genes for disease-based implantation failure and infertility. Endocrinology 2003;144(7):2870-81.

45. Jiang K, Chen Y, and Jarvis JN. hCG Secretion in Human Choriocarcinoma JAR Cells is MAPK but not Stat3 Dependent: Contributions of TNFα and IL-1β to Inflammation-induced hCG Secretion. Placenta 2006;27(8):853-860.

46. Wang H, Bocca S, Anderson S, Yu L, Rhavi BS, Horcajadas J, et al. Sex Steroids Regulate Epithelial-Stromal Cell Cross Talk and Trophoblast Attachment

Invasion in a Three-Dimensional Human Endometrial Culture System. Tissue Eng Part C Methods 2013;19(9):676-87.

47. Nichols AS, Floyd DH, Bruinsma SP, Narzinski K, and Baranski TJ. Frizzled receptors signal through G proteins. Cell Signal 2013;25(6):1468-1475.

48. Shao R, Feng Y, Zou S, Li X, and Billig H. The inflammatory regulation of tubal beta-catenin expression in human ectopic pregnancy: is it too early to propose a cause-and-effect relationship? Hum Reprod 2013;28(12):3378-80.

49. Koval A, Purvanov V, Egger-Adam D, and Katanaev VL. Yellow submarine of the Wnt/Frizzled signaling: submerging from the G protein harbor to the targets. Biochem Pharmacol 2011;82(10):1311-9.

50. Sun X, Mei S, Tao H, Wang G, Su L, Jiang S, et al. Microarray profiling for differential gene expression in PMSG-hCG stimulated preovulatory ovarian follicles of Chinese Taihu and Large White sows. BMC Genomics 2011;12:111.

51. Gawronska B, Paukku T, Huhtaniemi I, Wasowicz G, and Ziecik AJ. Oestrogen-dependent expression of LH/hCG receptors in pig Fallopian tube and their role in relaxation of the oviduct. J Reprod Fertil 1999;115(2):293-301.

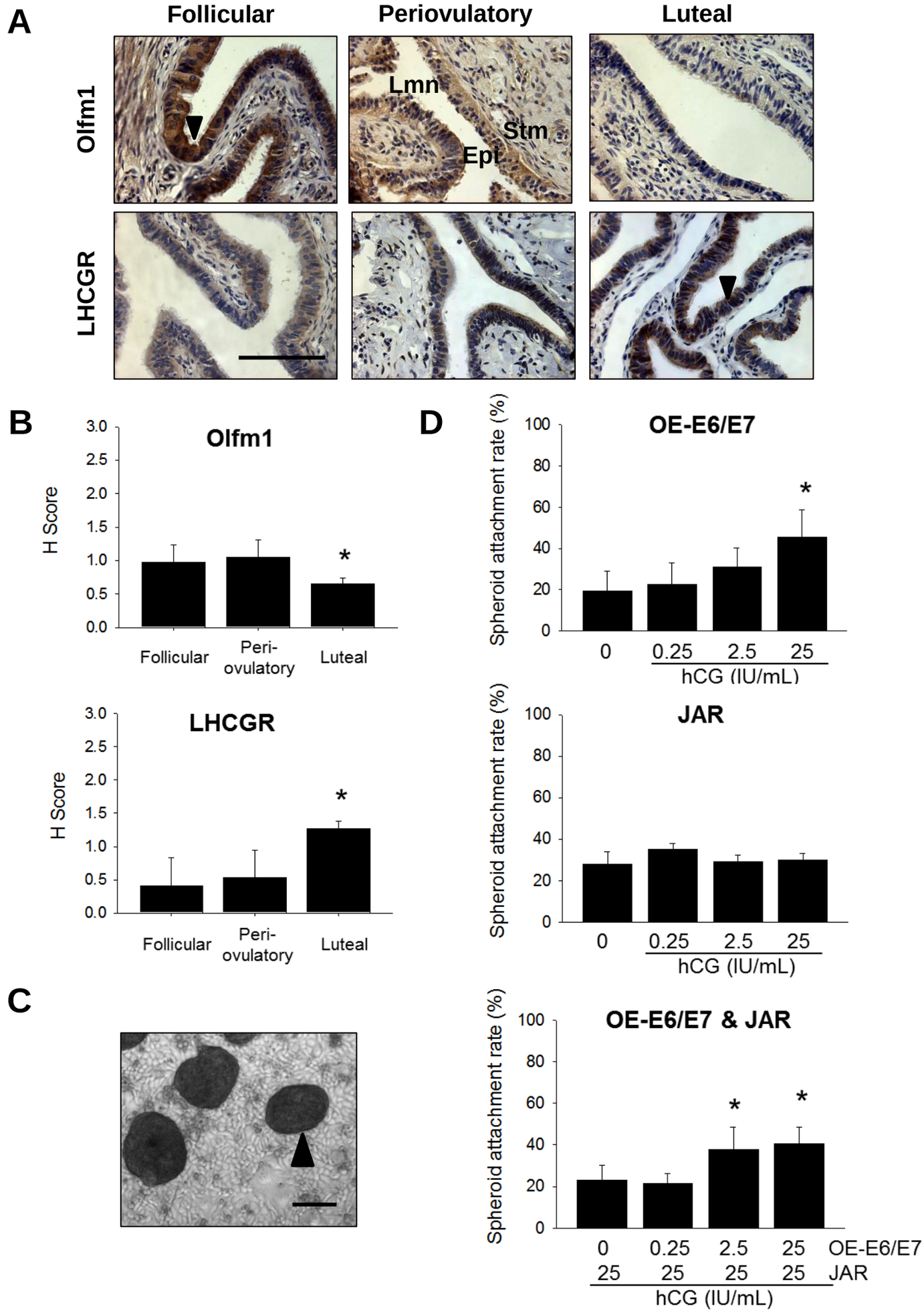
52. Gawronska B, Stepien A, and Ziecik AJ. Effect of estradiol and progesterone on oviductal LH-receptors and LH-dependent relaxation of the porcine oviduct. Theriogenology 2000;53(3):659-72.

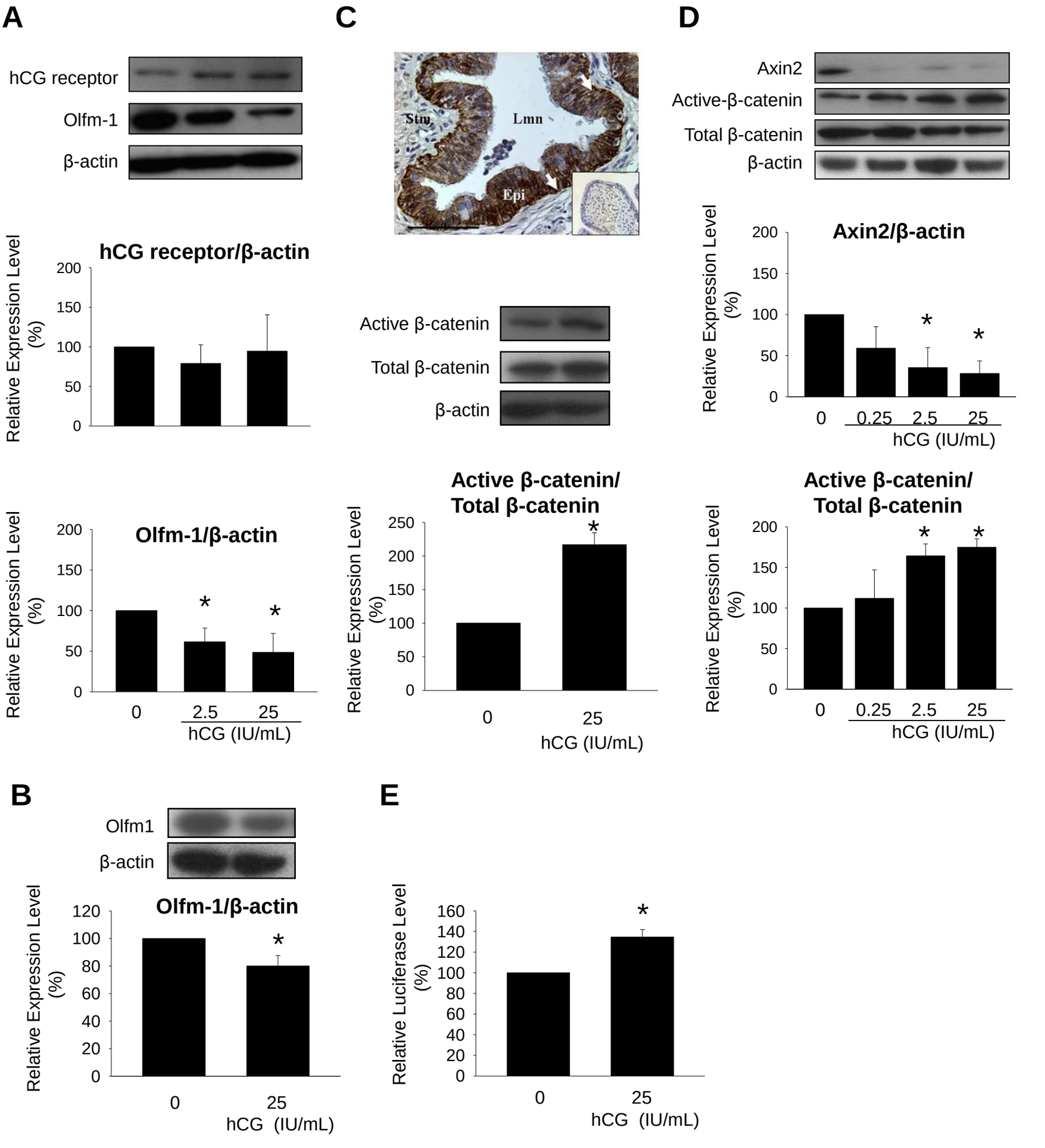
Figure Legend

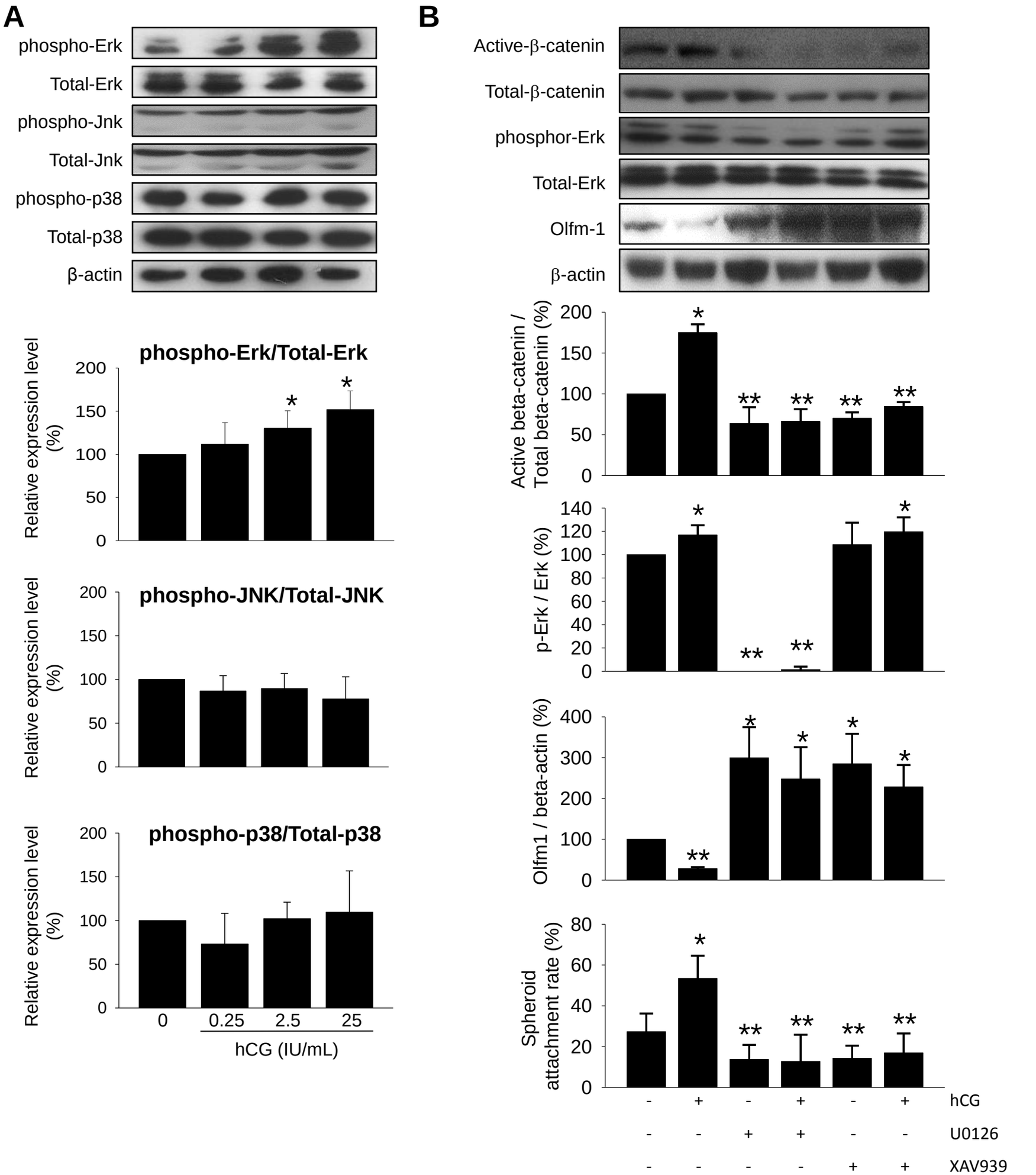
Figure 1. Olfm1 and LHCGR expression in Fallopian tubes at different phases of the menstrual cycle, and effect of hCG treatment on attachment of JAr spheroids on OE-E6/E7 cells. (A) Expression of Olfm1 and LHCGR in the ampullary region of the Fallopian tube in the follicular (n=5), periovulatory (n=5), and luteal (n=5) phases of the menstrual cycle. (B) H-score of Olfm1 and LHCGR in the epithelial cells in ampullary region of the Fallopian tube. (*$p<0.05$; Scale bar = 100 μm; Epi: epithelial cells, Stm: stromal cells, Lmn: lumen). (C) JAr spheroids of about 100 μm in size were co-cultured with OE-E6/E7 cells. (D) Effect of hCG (range, 0 - 25 IU/mL) on spheroid attachment on OE-E6/E7 cells at 1 h co-culture. Top panel represents treatment on OE-E6/E7 cells only, middle panel represents treatment on trophoblastic JAr cells only, and bottom panel represents treatment on both trophoblastic JAr and OE-E6/E7 cells. (* $p<0.05$ compared to control)

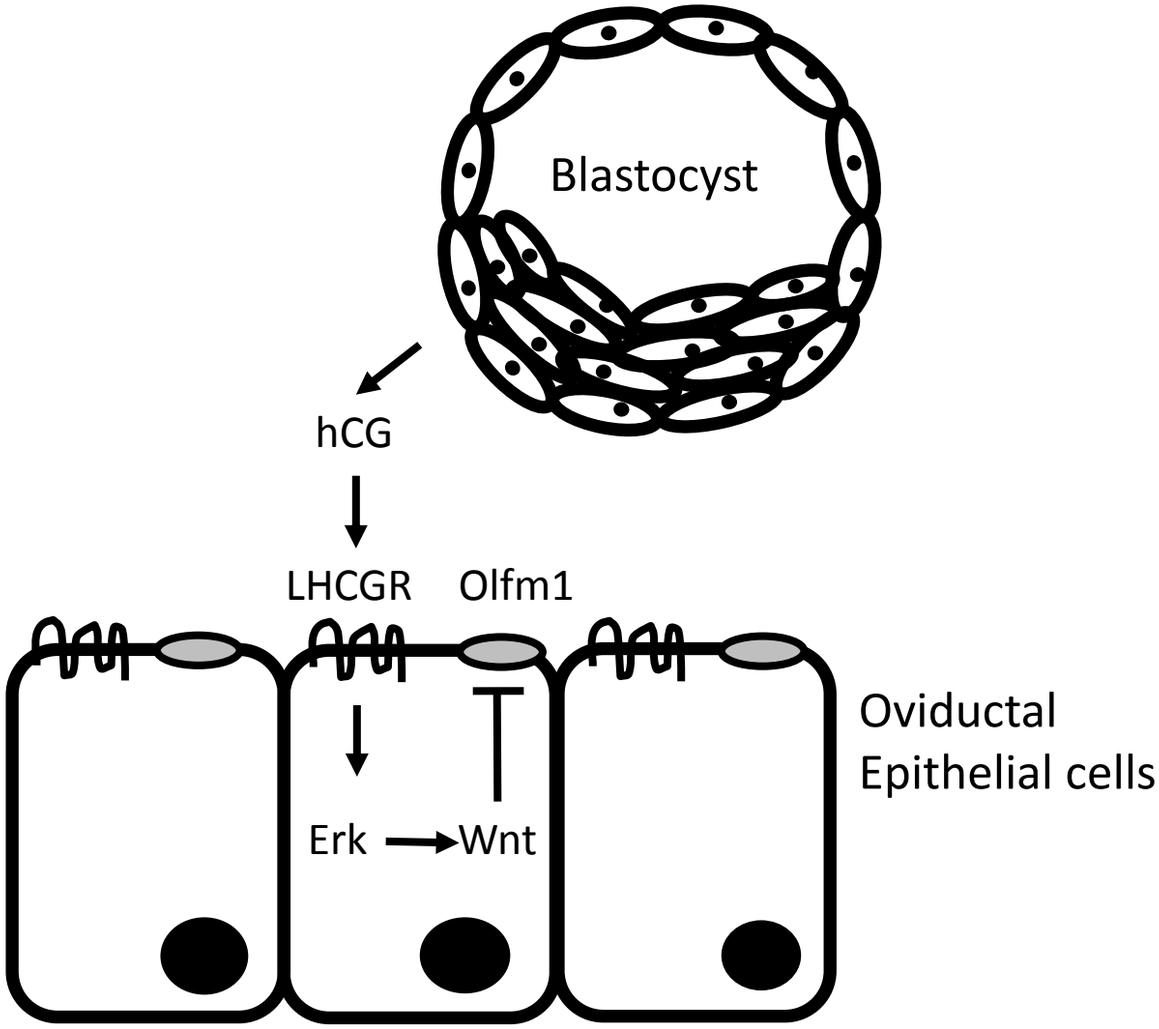
Figure 2. hCG down-regulated Olfm-1 and Axin2 but up-regulated active β-catenin expression in OE-E6/E7 cells. (A) hCG (2.5 and 25 IU/mL) down-regulated Olfm1 expression but not LHCGR expression in OE-E6/E7 cells. (B) hCG (25 IU/mL) down-regulated Olfm1 expression in primary Fallopian epithelial cells isolated from Fallopian tissue. (C) β-catenin is expressed in epithelial cells of normal Fallopian tube (top). Embedded image in the photo was negative control without primary antibodies. Stm: Stromal, Lmn: Lumen, Epi: epithelium. The expression of active β-catenin is up-regulated by hCG at 25 IU/mL in primary Fallopian epithelial cells isolated from Fallopian tissue (middle and bottom). (D) hCG down-regulated Axin2 and

584 up-regulated active-β-catenin in OE-E6/E7 cells. No changes in total β-catenin
585 and β-actin were observed (total β-catenin and β-actin were used as controls).
586 (E) hCG (25 IU/mL) increased TOP/FOP Flash luciferase signaling in
587 OE-E6/E7 cells indicating activation of the Wnt/β-catenin signaling pathway. (*
588 p<0.05 compared to control)
589
590 **Figure 3. hCG activated both Erk and β-catenin signaling pathways in**
591 **OE-E6/E7 cells.** (A) hCG (2.5 and 25 IU/mL) up-regulated phospho-Erk in
592 OE-E6/E7 cells. No changes in phospho-JNK and phospho-p38 were
593 observed (total-Erk, total-JNK, total-p38 and β-actin were used as controls). (B)
594 hCG increased the spheroid attachment rate, activated Erk and β-catenin
595 signaling pathways, and down-regulated Olfm1 expression. Treatment with
596 U0126 Erk signaling pathway inhibitor decreased the spheroid attachment rate,
597 reduced Erk and β-catenin signaling pathways, and up-regulated Olfm1
598 expression. Treatment with XAV939 Wnt/β-catenin signaling inhibitor
599 decreased the spheroid attachment rate, suppressed the β-catenin but not Erk
600 signaling pathway, and up-regulated Olfm1 expression. hCG did not reverse
601 the suppressive effect on spheroid attachment by XAV939 and U0126. (*
602 p<0.05 compared to control)
603
604 **Figure 4. The role of hCG in tubal ectopic pregnancy.** Embryonic hCG
605 activated Erk and Wnt/β-catenin signaling pathways in Fallopian tube epithelial
606 cells through LHCGR causing down-regulation of Olfm1. Aberrant high levels
607 of hCG may promote attachment of the embryo on Fallopian tube epithelial
608 cells leading to tubal ectopic pregnancy.
609


A
Follicular
Periovulatory
Luteal
Olfm1
LHCGR
Lmn
Stm
Epi
B
Olfm1
H Score
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Follicular
Peri-ovulatory
Luteal
LHCGR
H Score
*
Follicular
Peri-ovulatory
Luteal
C
D
OE-E6/E7
Spheroid attachment rate (%)
100
80
60
40
20
0
*
0
0.25
2.5
25
hCG (IU/mL)
JAR
Spheroid attachment rate (%)
0
0.25
2.5
25
hCG (IU/mL)
OE-E6/E7 & JAR
Spheroid attachment rate (%)
*
*
0
0.25
2.5
25
OE-E6/E7
0
25
25
25
25
JAR
hCG (IU/mL)


A
hCG receptor
Olfm-1
β-actin
hCG receptor/β-actin
Relative Expression Level (%)
200
150
100
50
0
Olfm-1/β-actin
0
2.5
25
hCG (IU/mL)
C
Stm
Lmn
Epi
Active β-catenin
Total β-catenin
β-actin
Active β-catenin/ Total β-catenin
250
0
25
D
Axin2
Active-β-catenin
Total β-catenin
β-actin
Axin2/β-actin
0.25
B
Olfm1
β-actin
Olfm-1/β-actin
120
80
60
40
20
E
Relative Luciferase Level (%)
160
140


A
phospho-Erk
Total-Erk
phospho-Jnk
Total-Jnk
phospho-p38
Total-p38
β-actin
phospho-Erk/Total-Erk
Relative expression level (%)
200
150
100
50
0
*
*
phospho-JNK/Total-JNK
Relative expression level (%)
200
150
100
50
0
phospho-p38/Total-p38
Relative expression level (%)
200
150
100
50
0
0
0.25
2.5
25
hCG (IU/mL)
B
Active-β-catenin
Total-β-catenin
phosphor-Erk
Total-Erk
Olfm-1
β-actin
Active beta-catenin / Total beta-catenin (%)
200
150
100
50
0
*
**
**
**
**
p-Erk / Erk (%)
140
120
100
80
60
40
20
0
*
**
**
*
Olfm1 / beta-actin (%)
400
300
200
100
0
**
*
*
*
Spheroid attachment rate (%)
80
60
40
20
0
*
**
**
**
**
-
+
-
+
-
+
hCG
-
-
+
+
-
-
U0126
-
-
-
-
+
+
XAV939


Blastocyst
hCG
LHCGR
Olfm1
Erk
Wnt
Oviductal
Epithelial cells