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

2

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

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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.

36 **Capsule**

37 hCG increases the attachment of trophoblastic spheroids on human Fallopian

38 tube epithelial cells through activation of Erk and Wnt/β-catenin signaling

39 pathways leading to down-regulation of Olfm1 expression.

40

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.

90 Functionally and biologically active LHCGRs are expressed in several
91 tissues, including the female reproductive tract from the Fallopian tubes to the
92 uterus (11-13). However, some studies have reported that hCG can carry out
93 its action independent of LHCGR (14, 15). LHCGR is a G-protein coupled
94 receptor that is capable of acting through multiple signal transduction
95 pathways, including phospholipid-specific phospholipase C and adenylyl
96 cyclase, which activate the mitogen-activated protein kinase (MAPK) pathway
97 (16, 17). The MAPK pathway contains many components including
98 extracellular signal-regulated kinase (Erk), c-Jun N-terminal kinase (JNKs),
99 and p38. It has been reported that hCG can stimulate production of
100 prostaglandin in endometrial epithelial cells through the phosphatidylinositol
101 3-kinase-extracellular regulatory kinase pathway (18). Recent studies have
102 suggested that Erk can crosstalk and regulate the Wnt/β-catenin signaling
103 pathway, which is essential for implantation (19-21).
104
105 In the Wnt/β-catenin signaling pathway, the Wnt ligand binds to the cell
106 surface Frizzled (Fz) receptor, which is a seven-transmembrane domain
107 receptor (22). Interaction between the Wnt ligand and Fz receptor activates
108 Dishevelled (Dvl), which leads to inactivation of glycogen synthase kinase-3β
109 (GSK-3β) (23). GSK-3β is one of the core components of the β-catenin
110 destruction complex, which consists of GSK-3β, phosphorylated adenomatous
111 polyposis coli (APC), and Axin (24). Inactivation of any of the molecules in the
112 β-catenin destruction complex allows β-catenin to escape proteasomal
113 degradation, resulting in cytoplasmic accumulation of β-catenin that can enter
114 the nucleus for gene activation (25).
115

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

126

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

136

137 We hypothesized that hCG secreted from human pre-implantation
138 embryos can enhance embryo attachment onto Fallopian tube epithelial cells
139 through down-regulation of Olfm1 expression in the tube. It is likely that hCG
140 secreted from the embryo accompanied with embryo retention in the Fallopian
141 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

146 **Materials and Methods**

147 **Study Participants**

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

158

159 **Immunohistochemistry and Histological Scoring**

160 Fallopian tube biopsies were fixed in 4% paraformaldehyde followed by
161 70% ethanol. The biopsies were embedded in paraffin wax and sectioned at a
162 thickness of 5 µm and mounted on polylysine-coated slides. Tissue sections
163 were deparaffinized and rehydrated, and then subjected to antigen retrieval
164 using Target Retrieval Solution (Dako Cytomation, Carpinteria, CA, USA).
165 Ampullary region of Fallopian tube tissues sections were incubated with
166 anti-β-catenin (1:200, BD610153, BD Biosciences, San Jose, California, USA),
167 anti-LHCGR (1:200; ab125214, Abcam, Cambridge, MA, USA) and anti-Olfm1
168 (1:50; ab71540, Abcam) antibodies for 18 h. Fallopian tube sections were
169 incubated in 3,3'-diaminobenzidine (DAB substrate chromogen, Dako
170 Cytomation) and the nucleus was counter-stained with Hematoxylin. Images
171 were captured under a light microscope with a digital camera (Axioscop, Zeiss,

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

177

178 **Western Blotting**

179 For Western blot analysis, total protein extraction was performed by
180 dissolving cell lysates from Fallopian tube epithelial cell line (OE-E6/E7 cells)
181 or isolated primary Fallopian epithelial cells (37) in radioimmunoprecipitation
182 assay (RIPA) buffer solution [1x phosphate-buffered saline (PBS), 1% Nonidet
183 P-40, 0.5% sodium deoxycholate, 0.1% SDS] containing protease inhibitors
184 (Calbiochem, Darmstadt, Germany). The membrane was probed with
185 anti-Olfm1 (1:400, ab71540, Abcam), anti-active-β-catenin (1:1000, 05-665,
186 Millipore, Billerica, MA, USA), anti-β-catenin (1:1000, BD610153, BD
187 Biosciences), anti-Axin2 (1:1000, ab32197, Abcam), anti-phospho-Erk (1:1000,
188 9910, Cell Signaling Technology Inc., Danvers, MA, USA), anti-Erk (1;1000,
189 9926, Cell Signaling Technology Inc.), anti-phospho-JNK (1:1000, 9910, Cell
190 Signaling Technology Inc.), anti-JNK (1:1000, 9926, Cell Signaling Technology
191 Inc.), anti-phospho-p38 (1:1000, 9910, Cell Signaling Technology Inc.), and
192 anti-p38 (1:1000, 9926, Cell Signaling Technology Inc.) antibodies in blocking
193 solution overnight at 4°C. The membrane was washed five times in PBST for 5
194 min and incubated with anti-rabbit or anti-mouse secondary antibody
195 conjugated with horseradish peroxidase (1:5000, GE Healthcare, Pittsburgh,
196 PA, USA) for 1 h. After washing five times in PBST for 5 min, the signal was
197 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°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°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

224 prepared for the luciferase activity assay 24 h post-transfection according to
225 the manufacturer's instructions (Promega).
226
227 **Treatment with hCG, MAPK Inhibitor or Wnt Inhibitor**
228 Both OE-E6/E7 and JAr cells were treated with hCG (0, 0.25, 2.5 and 25
229 IU/mL) for 24 h. For the MAPK inhibitor study, OE-E6/E7 cells were treated
230 with 0.07 μM U0126 Erk inhibitor for 15 min. For the Wnt inhibitor study,
231 OE-E6/E7 cells were treated with 0.1 μM XAV939 Wnt inhibitor for 24 h.
232
233 **Statistical Analysis**
234 Statistical analysis was determined by non-parametric ANOVA on Rank
235 test. Non-parametric Mann Whitney U test or parametric Student's t-test were
236 used where appropriate as the post-test. All data were presented as mean ±
237 standard error mean (S.E.M.). Each experiment was repeated in triplicate. A
238 p-value < 0.05 was considered statistically significant.
239
240

241 **Results**

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

244 Olfm1 and LHCGR proteins were strongly expressed in the cytoplasm of
245 the human Fallopian epithelial cells as detected by immunohistochemistry (Fig.
246 1A). Olfm1 expression was significantly lower during the luteal phase of the
247 cycle compared to the follicular and peri-ovulatory phases (Fig. 1B). In contrast,
248 LHCGR expression in Fallopian tube epithelial cells was significantly higher
249 during the luteal phase compared to the follicular and peri-ovulatory phases
250 (Fig. 1B). Olfm1 and LHCGR were weakly expressed in the stromal cells of the
251 Fallopian tube throughout all menstrual phases.

252

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

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

265

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

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

268 OE-E6/E7 cells express hCG receptor, when OE-E6/E7 was treated with
269 hCG (2.5 and 25 IU/mL), there was no effect on hCG receptor, but significantly
270 decreased the Olfm1 expression (Fig. 2A). Similarly, hCG (25 IU/mL)
271 down-regulated Olfm1 expression in primary Fallopian epithelial cells isolated
272 from Fallopian tissue (Fig. 2B). β-catenin was localized at the epithelium of
273 human Fallopian tube and hCG up-regulated active β-catenin expression in
274 primary Fallopian epithelial cells isolated from Fallopian tissue (Fig. 2C). hCG
275 down-regulated Axin2 expression, but increased the ratio of
276 active-β-catenin/total β-catenin expressions in the OE-E6/E7 cells (Fig. 2D).
277 The effect of hCG on Wnt-signaling activation was confirmed using the
278 TOP/FOP Flash luciferase reporter assay. Treatment with 25 IU/mL hCG
279 significantly increased luciferase activity in the transfected OE-E6/E7 cells (Fig.
280 2E).

281

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

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

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

297 **Discussion**

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

304

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

315

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

323 h (45) and the trophoblastic spheroid can secrete <0.01 IU/mL hCG in 6 days
324 of culture (46). We found that a high hCG level in the local microenvironment
325 favored embryo attachment on epithelial cells in the Fallopian tubes. The
326 upper dosage of hCG (25 IU/mL) used in this experiment was comparable to
327 various studies, which range from 1 to 50 IU/ml (8-10). Assuming that the
328 volume of Fallopian tube to be 1-10 μl, the concentration of hCG encountered
329 by pre-implantation embryo in the blocked tube which could reach 617.5 ~ 61.8
330 IU/ml (9). Results from this study may shed light on the underlying mechanism
331 on the role of hCG in regulating Olfm1 in ectopic pregnancy.
332
333 LHCGR is a G-protein coupled receptor that activates many downstream
334 signaling pathways such as MAPK pathway (16, 17). Accumulating evidence
335 has suggested that the MAPK pathway cross-talks with the Wnt/β-catenin
336 signaling pathway through regulation of the β-catenin destruction complex,
337 which consists of Axin2, APC and GSK3β (47). We previously reported that
338 Wnt-activation down-regulated Olfm1 in Fallopian tube epithelial cells and
339 promoted spheroid attachment (30). To elucidate the molecular mechanism
340 involved in the hCG-mediated attachment process, we examined whether hCG
341 regulated the Wnt/β-catenin signaling pathway. We found that β-catenin was
342 localized in the epithelial cells of the Fallopian tube and similar expression
343 pattern was reported in mouse oviduct (48). hCG treatment up-regulated
344 active-β-catenin and down-regulated Axin2 expressions. This suggests that
345 Axin2 down-regulation could allow β-catenin to enter the nucleus for the
346 activation of gene transcription (49). Similarly, microarray profiling has also
347 confirmed that Wnt-related genes could be activated by hCG in pre-ovulatory
348 ovarian follicles (50).

349

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

359

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

368

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

375 to focus on the role of hCG in regulating Erk and Wnt/β-catenin expressions in
376 patients with TEP.
377
378 **Acknowledgements**

379 This study was supported in part by grants from the Committee on
380 Research and Conference grant, The University of Hong Kong and General
381 Research Fund (HKU770813M), Hong Kong Research Grant Council to KFL.
382
383

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556

557

558 **Figure Legend**

559

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

573

574 **Figure 2. hCG down-regulated Olfm-1 and Axin2 but up-regulated active**
575 **β-catenin expression in OE-E6/E7 cells.** (A) hCG (2.5 and 25 IU/mL)
576 down-regulated Olfm1 expression but not LHCGR expression in OE-E6/E7
577 cells. (B) hCG (25 IU/mL) down-regulated Olfm1 expression in primary
578 Fallopian epithelial cells isolated from Fallopian tissue. (C) β-catenin is
579 expressed in epithelial cells of normal Fallopian tube (top). Embedded image
580 in the photo was negative control without primary antibodies. Stm: Stromal,
581 Lmn: Lumen, Epi: epithelium. The expression of active β-catenin is
582 up-regulated by hCG at 25 IU/mL in primary Fallopian epithelial cells isolated
583 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

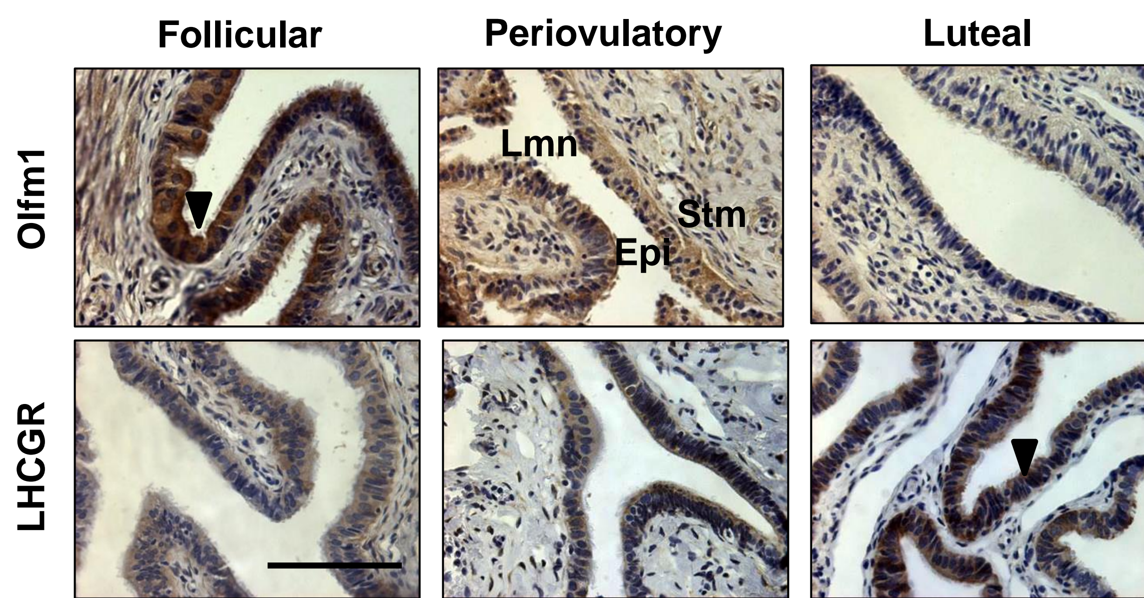
590 **Figure 3. hCG activated both Erk and β-catenin signaling pathways in**
591 **OE-E6/E7 cells.** (A) hCG (2.5 and 25 IU/mL) up-regulated phospho-Erk in
592 OE-E6/E7 cells. No changes in phospho-JNK and phospho-p38 were
593 observed (total-Erk, total-JNK, total-p38 and β-actin were used as controls). (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



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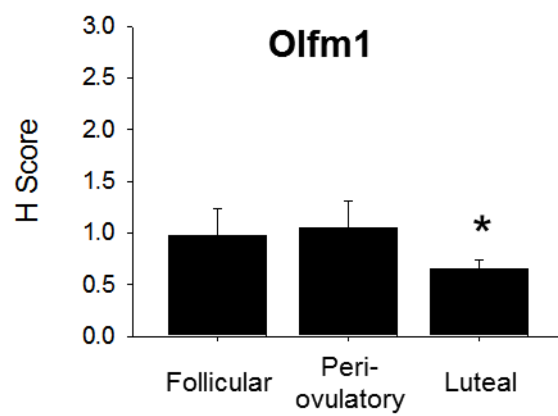

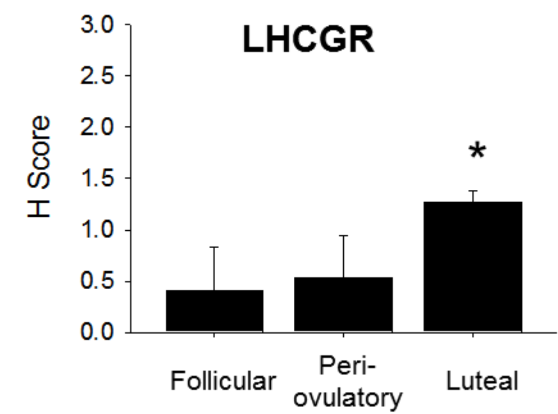

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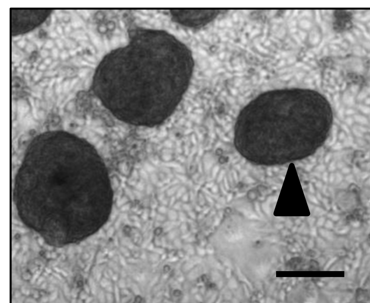

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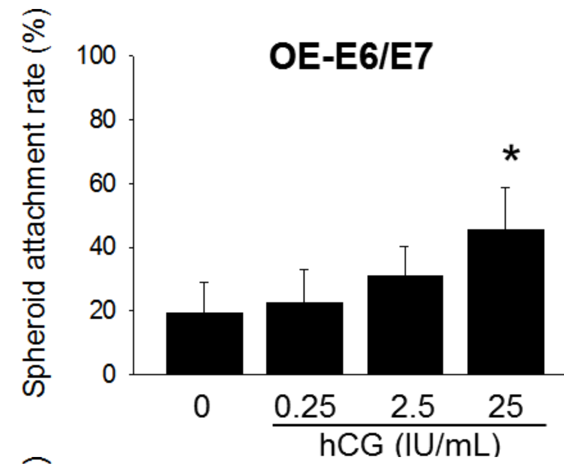

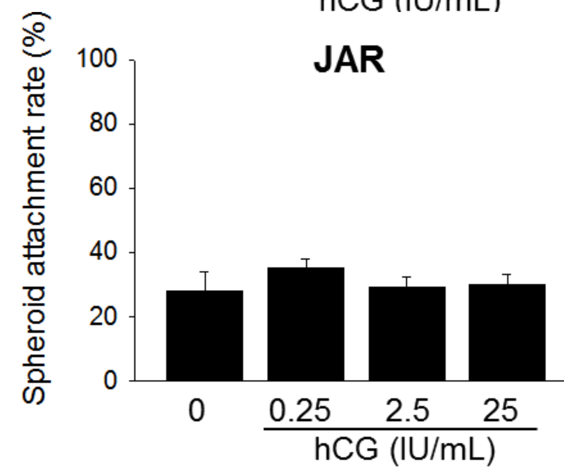

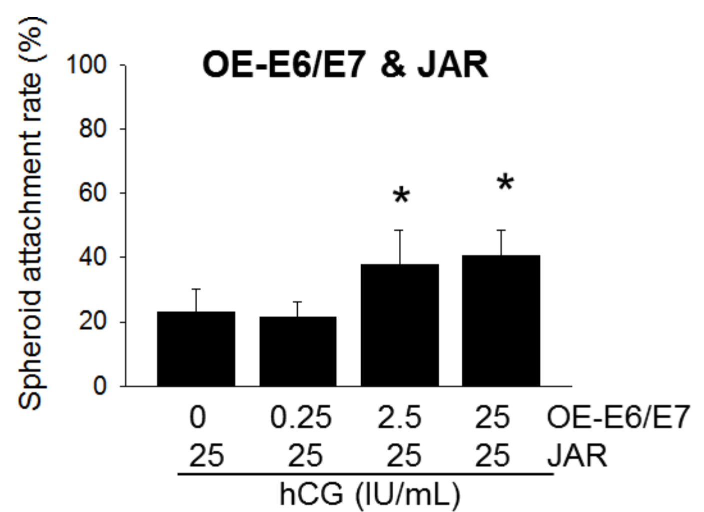

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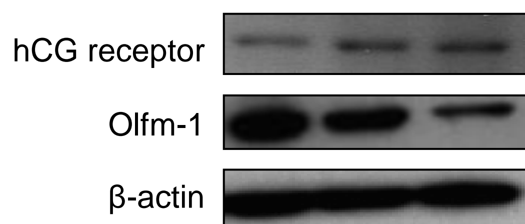

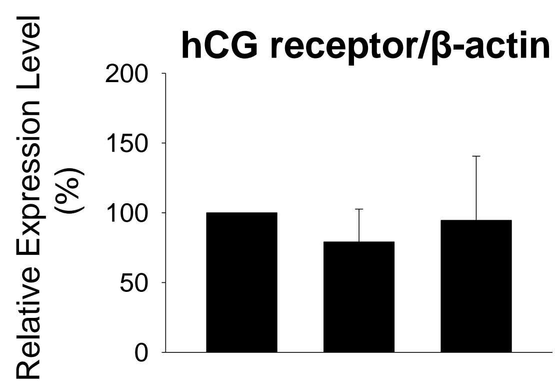

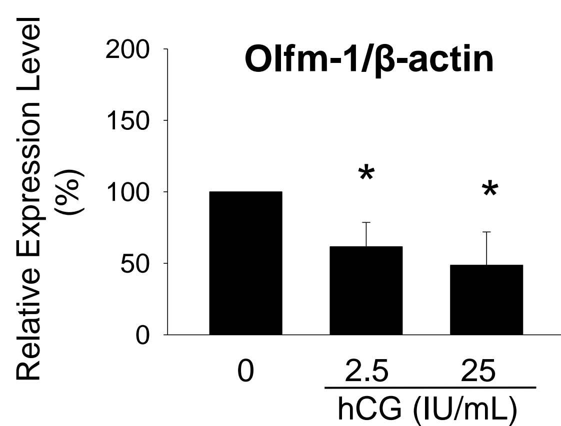

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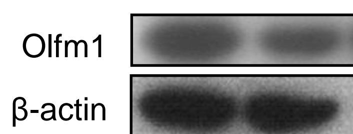

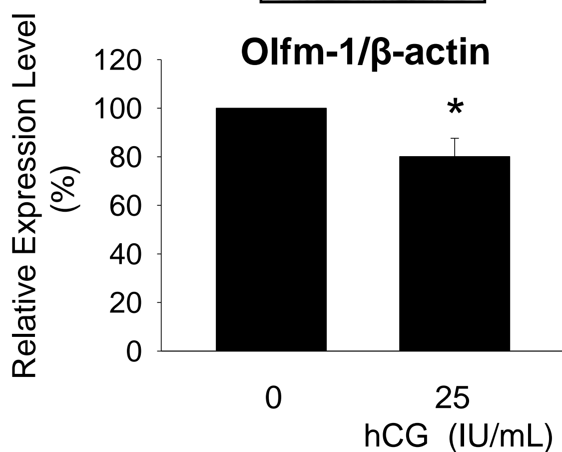

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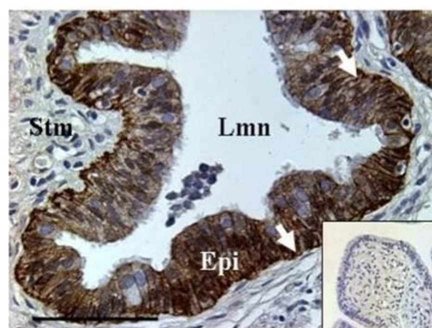

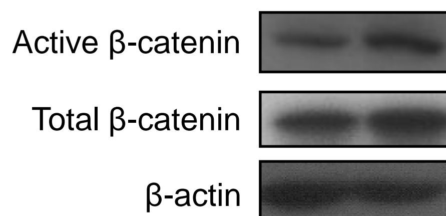

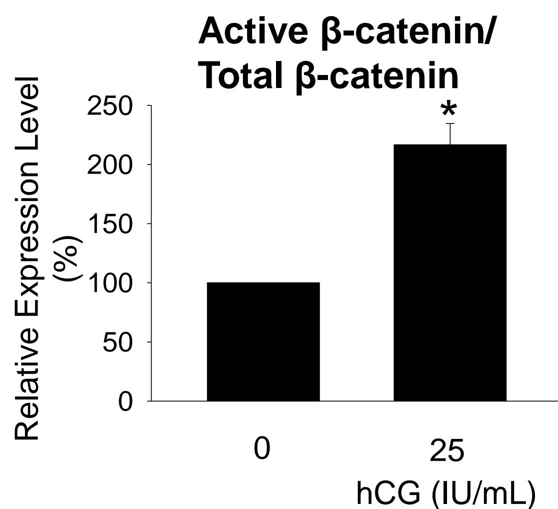

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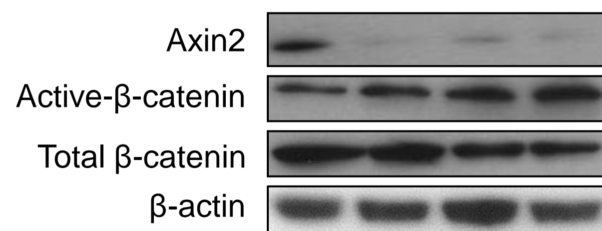

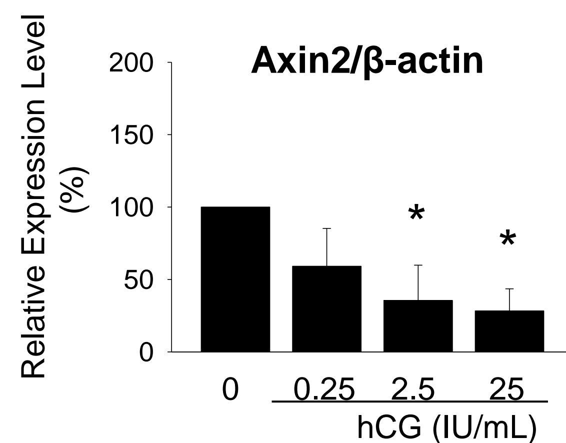

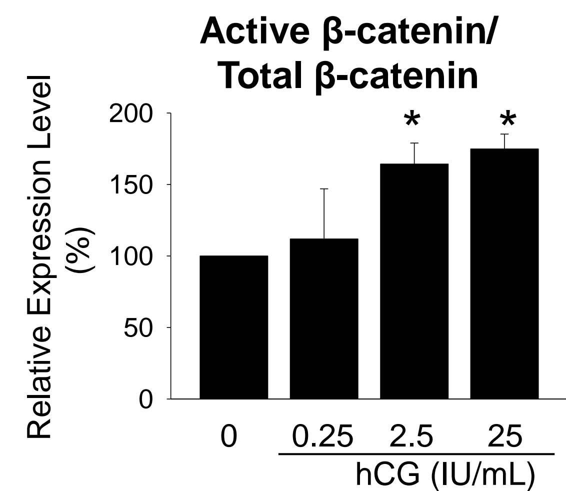

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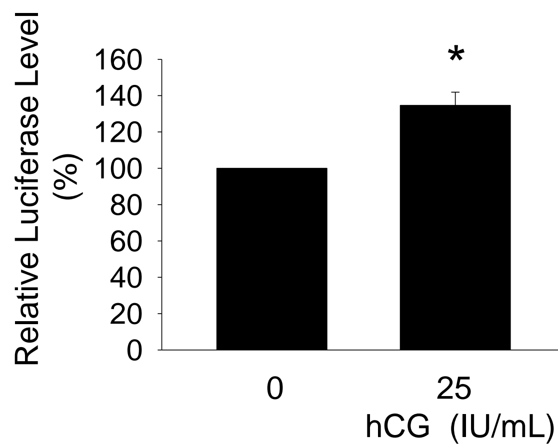
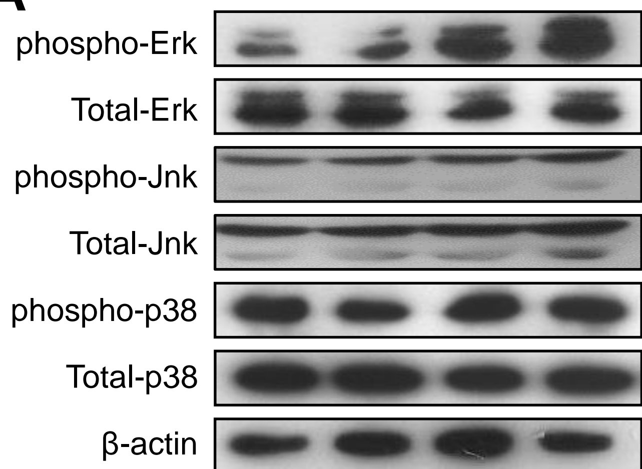
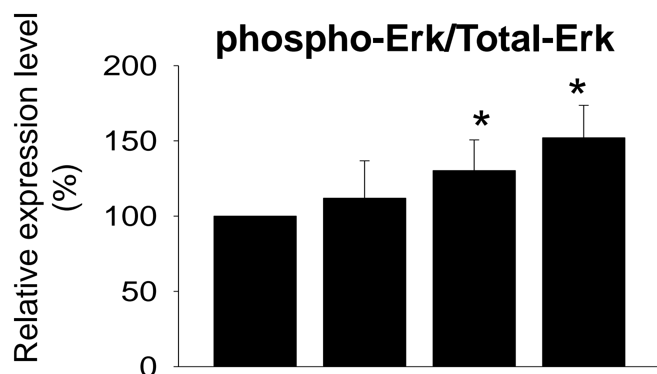
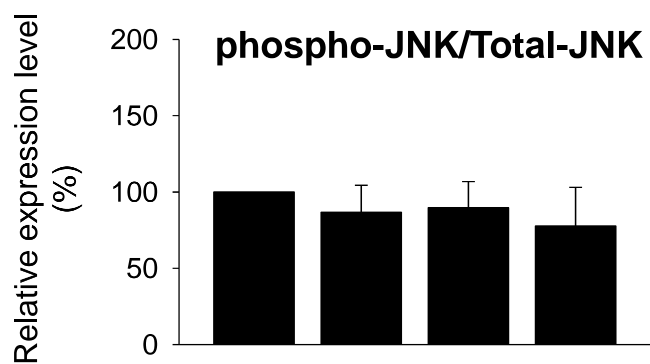
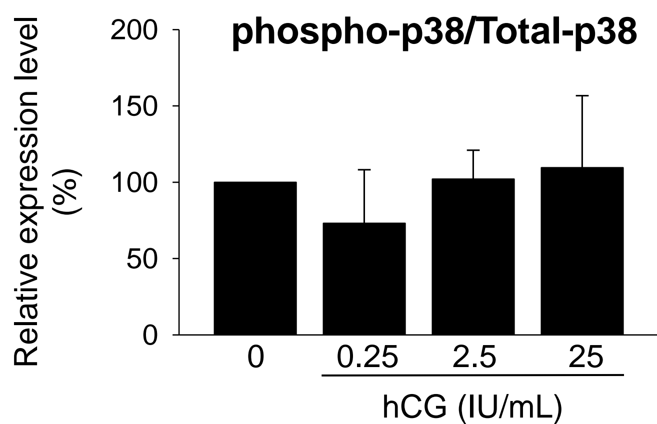
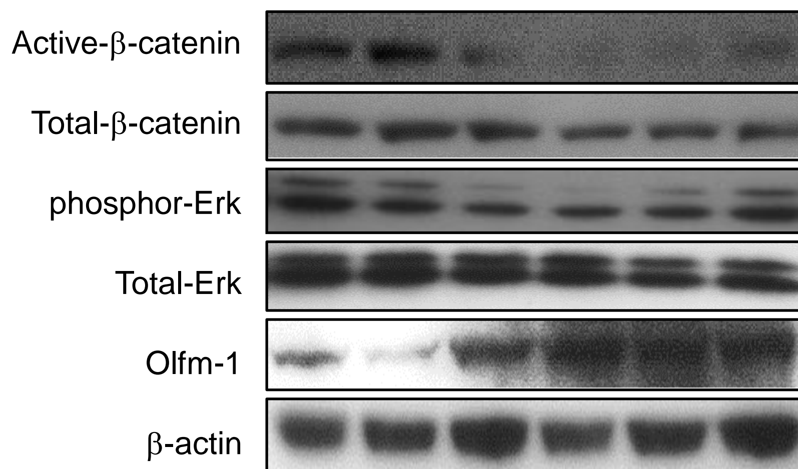


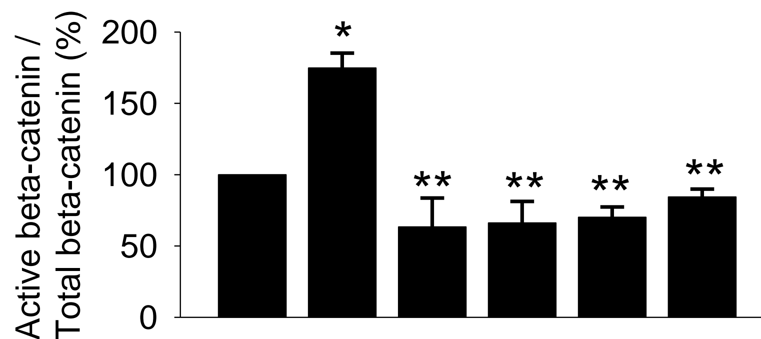

A
phospho-Erk
Total-Erk
phospho-Jnk
Total-Jnk
phospho-p38
Total-p38
β-actin

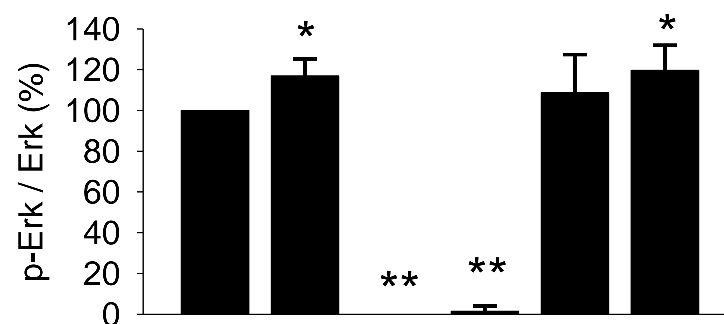

phospho-Erk/Total-Erk
Relative expression level (%)
200
150
100
50
0
*
*

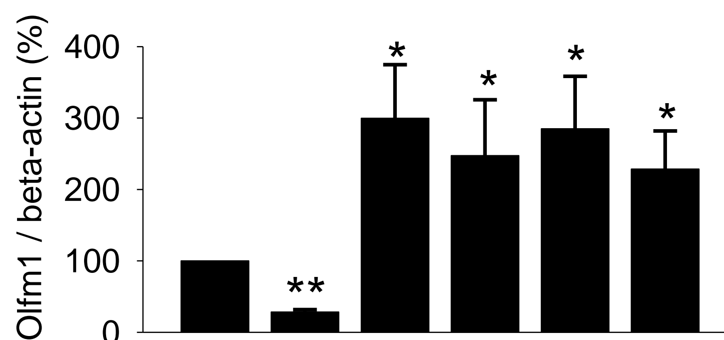

phospho-JNK/Total-JNK
Relative expression level (%)
200
150
100
50
0

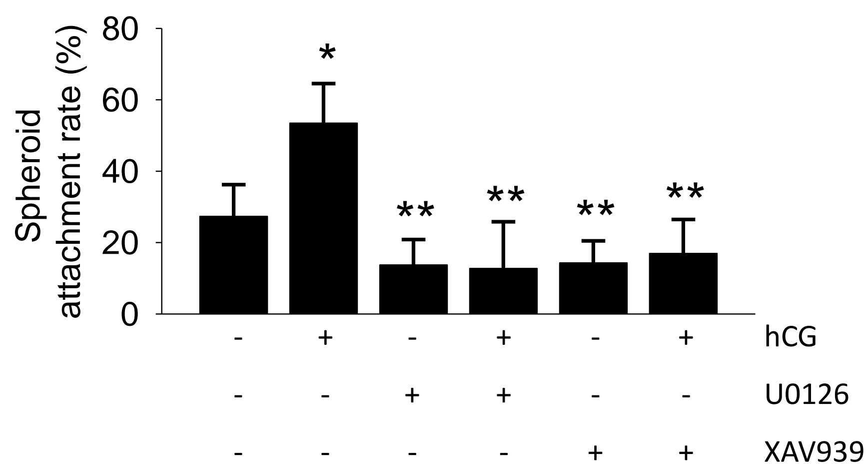

phospho-p38/Total-p38
Relative expression level (%)
200
150
100
50
0
0
0.25
2.5
25
hCG (IU/mL)

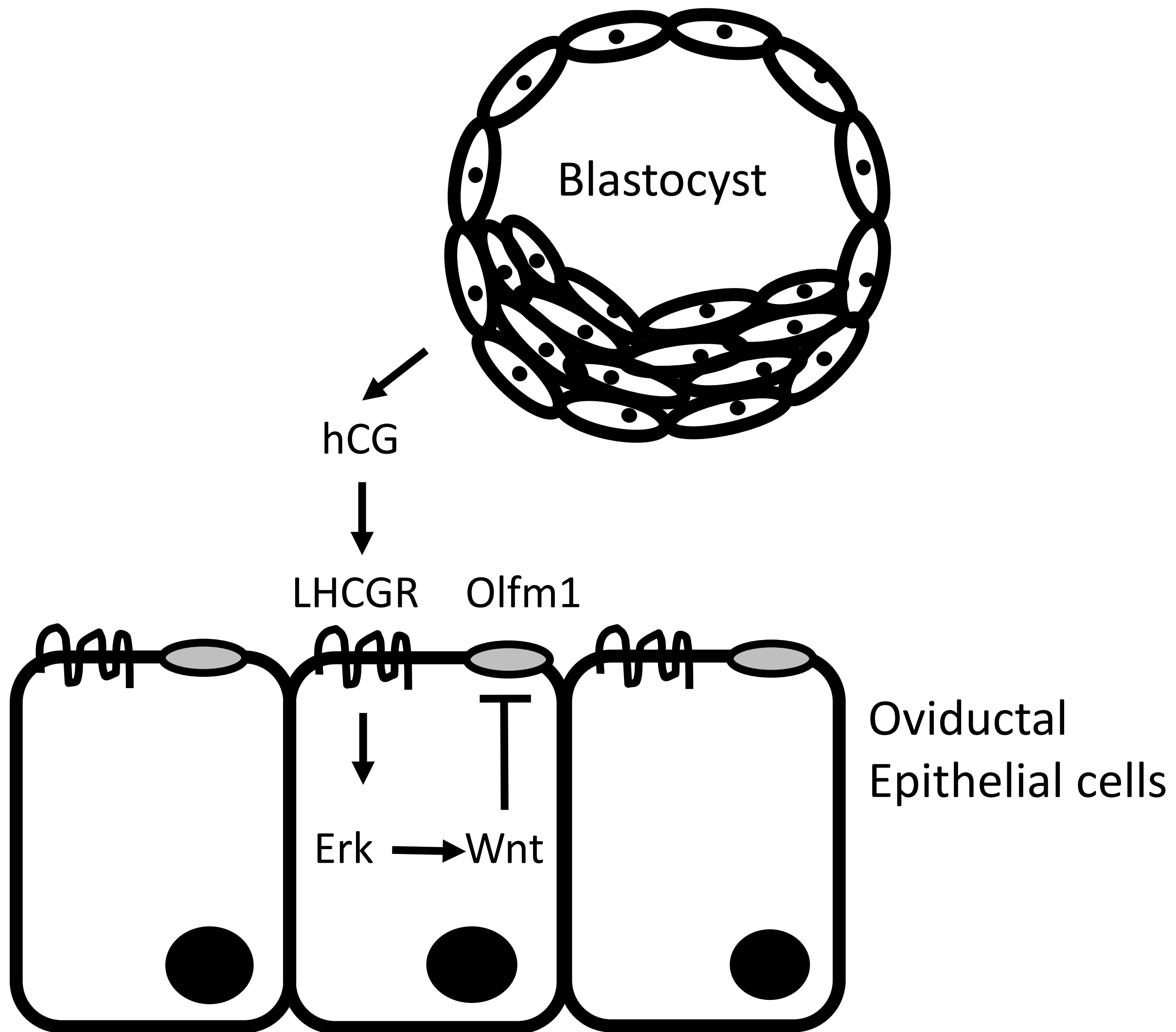

B
Active-β-catenin
Total-β-catenin
phosphor-Erk
Total-Erk
Olfm-1
β-actin


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