Leptin receptor is induced in endometriosis and leptin stimulates the growth of endometriotic epithelial cells through the JAK2/STAT3 and ERK pathways

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ABSTRACT: Leptin acts as a potential growth stimulator in several normal and neoplastic cells. Recent studies have shown the presence of increased levels of leptin in the peritoneal fluid of patients with endometriosis, implicating leptin in the pathogenesis of endometriosis. However, the specific function of leptin in the induction of mitogenesis in endometriosis is not known. This study investigated the expression of the leptin receptor (ObR) in endometrioma tissues and immortalized endometriotic cells, and the effect of leptin on cell growth. ObR expression was higher in endometriomas than in the normal endometrium, and it was detected in 74% of epithelial and 30% of stromal endometrioma tissues. In addition, human endometriotic epithelial cells (11Z and 12Z) showed a high level of ObR when compared with endometrial cells and endometriotic stromal cells (22B). Furthermore, leptin treatment stimulated the growth of 11Z and 12Z cells, but not that of 22B cells. Knockdown of the ObR in 11Z and 12Z cells impaired the ability of leptin to induce cell growth. Leptin induced the activation of Janus Kinases 2 (JAK2), signal transducers and activators of transcription 3 (STAT3) and extracellular signal-regulated kinase (ERK) in endometriotic epithelial cells. Moreover, pretreatment with the JAK2/STAT3 inhibitor AG490 and the ERK inhibitor PD98059 significantly inhibited leptin-induced cell growth. The present results show that the ObR is induced in endometriosis, and that leptin stimulates the growth of endometriotic epithelial cells through the JAK2/STAT3 and ERK pathways.

Key words: endometriosis / leptin receptor / leptin / JAK2/STAT3 / ERK

Introduction

Endometriosis is a common chronic disease characterized by the ectopic implantation and growth of endometrial tissue. The disorder is a common cause of pelvic pain, and accounts for over 20% of all cases of infertility in women. Its defining feature is the presence of endometrial implants outside the uterus, primarily on the pelvic peritoneum and ovaries. Accumulating evidence suggests that the concentrations of various substances such as cytokines, chemokines, prostaglandins and growth factors in the peritoneal fluid are aberrant in women with endometriosis and these molecules are associated with the pathogenesis of the disease (Kyama et al., 2003).

Although discrepancies exist in the measured leptin serum levels in endometriosis patients (Matarese et al., 2000; Somigliana et al., 2006), multiple lines of evidence have suggested that leptin levels are significantly elevated in the peritoneal fluid of patients with endometriosis (Matarese et al., 2000; Mahutte et al., 2003; Bedaiwy et al., 2006; Barcz et al., 2008; Alviggi et al., 2009). Leptin, a product of the obese (ob) gene, is predominantly secreted by adipocytes and shows a strong positive correlation with total body fat and body mass index (BMI). The effects of leptin are mediated by the transmembrane leptin receptor (ObR), which belongs to the cytokine receptor superfamily. In human tissues, at least four isoforms of ObR with different COOH-terminal cytoplasmic domains exist, including a long
form and a short form. The long form of ObR (ObRb) activates classical cytokine Janus Kinases 2 (JAK2)/signal transducers and activators of transcription 3 (STAT3) pathways (Yamashita et al., 1998) as well as the ras/extracellular signal-regulated kinase (ERK) (Yamashita et al., 1998; Catalano et al., 2004) and phosphatidylinositol 3’-kinase (PI3K)/Akt (O’Rourke et al., 2001) pathways.

Although the primary function of leptin is the regulation of food intake and energy consumption through its effects on the brain, leptin is known to play multiple roles in various cell systems, including reproduction, angiogenesis and immune homeostasis (La Cava et al., 2004). Interestingly, recent studies have demonstrated that this hormone stimulates growth, migration, invasion and angiogenesis in tumor cell models, suggesting that leptin is capable of promoting an aggressive cancer phenotype (Lang and Ratke, 2009). The similarity between these phenotypes and the pathogenesis of endometriosis led to the question of whether leptin could play a role in endometriosis. To address this, the expression of the ObR was examined in endometrioma tissues and the potential effect of leptin on cell growth and its underlying molecular mechanism in immortalized endometriotic cells were investigated.

Materials and Methods

Patient subjects

Ovarian endometriosis tissue samples were obtained from 44 women of reproductive age (24–47 years old) who underwent laparoscopic surgery for ovarian endometriomas at the Department of Obstetrics and Gynecology, Daegu Catholic University Medical Center in Daegu, Korea. The study was approved by the Institutional Review Board of Daegu Catholic University Medical Center. Endometriosis was classified according to American Society for Reproductive Medicine classification. The patients have not received any hormonal therapy before the surgery. The mean BMI was 20.7 ± 3.0 (kg/m²). Nineteen women were in the proliferative phase and 25 were in the secretory phase. Normal endometrial tissues from disease-free patients of reproductive age undergoing hysterectomy for leiomyoma or ovarian pathology (n = 40) were also collected at the time of laparoscopy. BMI was 23.3 ± 3.4 (kg/m²). Twenty-four out of 40 women were in the proliferative phase and 25 were in the secretory phase. Normal ovaries were also collected at the time of laparoscopies. The histo- logical slides of the excised ovarian endometriomas were evaluated by one pathologist (H.K.O.), who was blind to the clinical variables of the patient. Routine hematoxylin and eosin staining was used to diagnose endometriosis.

Materials

Recombinant leptin was purchased from Sigma-Aldrich (Oakville, Ontario, Canada). PD98059 [2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one], a MAPK/ERK kinase (MEK) inhibitor, and AG490 [a-cyanoo-3,4-dihydroxy-N-benzyl-cinnamid]e, a JAK2/STAT3 inhibitor, and wortman- nin, a PI3K inhibitor, were purchased from New England Biolabs (Beverly, MA, USA) and Calbiochem (La Jolla, CA, USA), respectively. Antibodies against ObR (rabbit polyclonal) and β-actin (goat polyclonal) were purchased from Cell Signaling Technology (Beverly, MA, USA).

Cell culture

Immortalized human endometriotic epithelial cells (11Z and 12Z) and stromal cells (22B) (Zeitvogel et al., 2001) and immortalized human endometrial surface epithelial cells (HES) and stromal cells (HESC) (Desai et al., 1994; Krikun et al., 2004) were maintained in Dulbecco’s modified Eagle’s medium (DMEM)/F12 medium supplemented with 5% fetal bovine serum, 100 U/ml penicillin G and 100 µg/ml streptomycin (Life Technologies, Grand Island, NY, USA) in a humidified atmosphere of 5% CO2—95% air at 37°C. The endometriotic cells were generously provided by Dr Starzinski-Powitz (Johann-Wolfgang-Goethe-Universitaet, Germany). The endometrial cells HES, recently established by Dr Krikun (Yale University, New Haven, CT, USA), and HESC were kindly provided by Dr Asgi Fazleabas of University of Illinois at Chicago. MCF7 cells (Korean Cell Line Bank, Seoul, Korea) were maintained in DMEM.

MTT and clonogenic assay

Cell growth was estimated using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; Sigma-Aldrich) assay. Cells were seeded in 96-well plates at a density of 5 x 10^3 cells per well and incubated for 24 h. To examine the growth stimulatory effect of leptin, cells were treated with leptin for 48 h. On the day of collection, 50 µl of MTT solution was added to the medium and the cells were incubated at 37°C for 4 h. The MTT-containing medium was removed and the cells were solubilized in DMSO (100 µl) for 30 min. The absorbance at 490 nm was determined using a microplate spectrophotometer (Fisher Scientific Ltd., Ottawa, Ontario, Canada). To determine long-term effects, clonogenic assay was performed. Cells were seeded in 6-well plates at a cell density of 5000 cells per well and treated with leptin at various concentrations. Cells were allowed to form colonies for 14 days, which were counted after staining with crystal violet (Sigma). The density of colony in the well was quantified in pixels using Scion Image Software (Scion Corp, Frederick, MD, USA).

Immunoblot assay

The cells were washed twice with ice-cold phosphate buffered saline and lysed in ice-cold RIPA buffer [150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris [pH, 7.5], 1 mM phenylmethylsulphonyl fluoride, 10 µg/ml leupeptin and 100 µg/ml aprotinin]. The extracts were placed on ice for 15 min and centrifuged to remove cellular debris. The protein concentration of supernatants was determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Thirty micrograms of total protein was run on 10% SDS–polyacrylamide gels and electrotransferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Oakville, Ontario, Canada). The membranes were blocked in 5% non-fat dry milk for 1 h, rinsed and incubated with specific antibodies against ObR, p-JAK2, p-STAT3, p-ERK and β-actin (1:1000–2000) in Tris-buffered saline (TBS) containing Tween 20 (0.1%) overnight at 4°C. Primary antibody was removed by washing the membranes three times in TBS-T, and incubated for 1 h with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000). Following three times of washing in TBS-T, immunopositive bands were visualized by enhanced chemiluminescence and exposed to X-ray film (Amersham Pharmacia Biotech). Quantification of the immunoblots was performed on Scion image software (Scion Corp., Frederick, MD, USA). Intensities of interested protein bands were scanned and quantified by density plot.

Reverse transcription–polymerase chain reaction

Total RNA was extracted from immortalized endometrial and endometrio- tic cells using the TRIzol reagent (Invitrogen Canada, Burlington, Ontario,
Immunohistochemistry

Immunohistochemistry was carried out on 40 normal endometrium and 44 endometrioma tissue sections and 2 well-differentiated hepatocellular carcinoma tissue sections for positive control using the Bond Polymer Intense Detection System (Leica microsystems, VIC, Australia) according to the manufacturer’s instruction with minor modifications. In brief, the 5 µm sections of formalin-fixed and paraffin-embedded tissues were deparaffinized by Bond Dewax Solution (Leica microsystems), and an antigen retrieval procedure was done using Bond ER Solution (Leica microsystems) for 30 min at 100°C. The endogenous peroxidase was quenched by incubation with hydrogen peroxide for 5 min. Sections were incubated for 15 min at ambient temperature with a rabbit polyclonal Leptin receptor antibody (ab60042, 1:150; Abcam, Cambridge, UK) using biotin-free polymeric HRP-linker antibody conjugate system in a Bond-Max automatic slide stainer (Leica microsystems). Hepatocellular carcinoma (ObR) tissues, obtained from patient who had undergone surgical resection at Daegu Catholic University Medical Center, were used as a positive control (Wang et al., 2006). A negative control was included by using non-specific bovine serum instead of primary antibody. The expression of ObR in samples was scored separately in epithelial and stromal cells by using a four-point scale: 0, negative (score 0) or positive (score 1–3).

Transfection of siRNA

ObR and control small interfering RNAs (siRNAs) were synthesized by Bioneer technology (Daejon, South Korea). Cells were transfected with siRNA at a final concentration of 20 nmol/l using lipofectamine (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s suggested protocol. Briefly, cells were plated in 6-well culture dishes and allowed to attach and grow 24 h before transfection. Each transfection mixture was prepared by mixing up the siRNA and lipofectamine in serum-free Opti-MEM (Invitrogen) and incubating for 15 min at room temperature. The transfection mixture was slowly added to the cells, which were allowed to recover for an additional 24 h before experimental treatments.

Data analysis

Statistical analysis for cell growth data were performed by one-way analysis of variance (ANOVA). Relationships between staining intensity of ObR in endometrioma and clinical parameters (size of endometrioma, BMI, stage of disease, menstrual phase and age) and correlation between epithelial and stromal expression of ObR were evaluated by calculation of Spearman’s correlation coefficient. χ2-test was used for correlation between ObR expression and endometriosis. In all statistical analysis used in this study, P < 0.05 was considered statistically significant.

Results

Expression of the leptin receptor in endometrioma tissues

In this study, the levels of ObR were evaluated in 40 normal endometria from endometriosis-free patients and 44 endometrioma tissues from patients with endometriosis by immunohistochemical analysis. In endometrioma tissues, ObR immunoreactivity was found in 32 of 44 (72.8%) epithelial and 14 of 44 (31.8%) stromal tissues, while only 18 of 40 (45%) epithelial and 5 of 40 (12.5%) stromal tissues of normal endometrium were ObR positive (Table I). Notably, ObR expression in endometrioma epithelium was significantly more extensive and stained more intensely than in the epithelial compartment of the normal endometrium (P < 0.01). In endometrioma tissues, ObR expression in the epithelium was significantly correlated with the expression of the receptor in the stroma (Supplemental data, Table SI). Figure 1A is the representative staining of endometrioma tissue to show the strong epithelial and relatively mild stromal staining. In

| Table I | The epithelial and stromal expression of ObR in normal endometrium and endometrioma. |
|-----------------|---------------------------------|---------------------------|----------------]|---------------------------------|-----------------|
| **Tissues**     | **Staining intensity** | **Endometrium (n = 40)** | **Endometrioma (n = 44)** | **P-value** | **Endometrium versus endometrioma** |
| Epithelial cells | 0    | 22 | 12 | 0.009* |
|                 | +1   | 18 | 24 |       |
|                 | +2   | 0  | 7  |       |
|                 | +3   | 0  | 1  |       |
| Stromal cells   | 0    | 35 | 30 | 0.065** |
|                 | +1   | 5  | 11 |       |
|                 | +2   | 0  | 3  |       |
|                 | +3   | 0  | 0  |       |

*χ2 test to compare the difference in staining intensity of ObR between epithelial cells of normal endometrium and that of ovarian endometriomas.

**χ2 test to compare the difference in staining intensity of ObR between stromal cells of normal endometrium and stromal cells of ovarian endometriomas.
the representative staining of normal endometrium (Fig. 1B), very weak ObR expression was observed in the epithelial cells. Endometrioma tissue with no ObR expression is shown as a negative control (Fig. 1C) and hepatocellular carcinoma tissue is shown as a positive control (Fig. 1D; Wang et al., 2006). The expression of ObR did not correlate with disease stage, size of the endometrioma, menstrual phase (proliferative versus secretory phase), age or BMI (Supplementary data, Tables SII and SIII).

The expression of ObR was also assessed in immortalized human endometriotic epithelial and stromal cells. As seen in Fig. 2A, human endometriotic epithelial cells 11Z and 12Z showed high levels of ObR protein (~130 kDa) when compared with endometrial cells (HES and HESC) and endometriotic stromal cells (22B). In parallel with ObR protein levels, the expression of ObR, mRNA was significantly enhanced in 11Z and 12Z cells (Fig. 2B). MCF7 cells have been used as a positive control for ObR expression (Yin et al., 2004; Garofalo et al., 2006). This observation is consistent with our findings in primary tissues showing that ObR expression is increased in endometrioma tissues compared with normal endometrium (Table I).

**Effect of leptin on cell growth in endometriotic cells**

Although the primary function of leptin is the regulation of food intake and energy consumption, leptin also promotes cell growth in various cell systems. To evaluate the effect of leptin on cell growth, endometriotic epithelial and stromal cells were treated with leptin and cell growth was assessed by MTT assay. Leptin treatment (0.05, 0.2, 1 μg/ml) stimulated the growth of endometriotic epithelial cells 11Z and 12Z but did not induce significant changes in endometriotic stromal cells 22B (Fig. 3A). Knockdown of the ObR in 11Z cells impaired the ability of leptin to induce cell growth (Fig. 3B), suggesting that leptin-induced cell growth is mediated through the ObR. In addition, a clonogenic assay was performed to assess the long-term effect of leptin treatment. As shown in Fig. 4, clonogenic survival of both 11Z and 12Z cells was increased in a dose-dependent manner after exposure to leptin (0.2 and 1 μg/ml).

**Involvement of the JAK2/STAT3 and ERK pathways in leptin-induced cell growth in endometriotic cells**

Leptin signaling in normal endometrial and endometriotic cells has not been studied to date. In other types of cells including adipocytes, leptin stimulates multiple pathways, including the JAK2/STAT3, ERK and PI3K/Akt pathways. To test the effect of leptin on the activation of JAK2/STAT3, ERK and PI3K/Akt signaling cascades, phosphorylated forms of JAK2, ERK, Akt were detected by immunoblot analysis.

**Effect of leptin on endometriotic cell growth**

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**Figure 1** Immunohistochemical staining for ObR in human normal endometrium and endometrioma tissues. (A) Endometrioma tissues show strong cytoplasmic expression in epithelial cell (EP, arrowhead) and a few stromal cells (ST, double arrows). (B) Normal proliferative endometrium with weak expression. (C) Endometrioma tissue with no ObR expression. (D) Hepatocellular carcinoma tissue section show strong cytoplasmic expression as positive control. The insert in (D) shows a representative microphotograph of a section stained with non-specific bovine serum instead of primary antibody. Original magnification ×400.

**Figure 2** Expression of ObR in endometrial and endometriotic cell lines. The protein (A) and mRNA expression (B) of ObR was investigated by western blot and real-time RT–PCR, respectively. HES, human immortalized endometrial epithelial cells; HESC, human immortalized endometrial stromal cells; 11Z and 12Z, human endometriotic epithelial cells; 22B, human endometriotic stromal cells; MCF7, human breast cancer cells. Values are the mean ± SD for three individual experiments (n = 3).
Figure 3  Effect of leptin on cell growth in endometriotic cells. (A) Human endometriotic epithelial cells (11Z and 12Z) and stromal cells (22B) were treated with different concentrations of leptin (0.05, 0.2, 1.0 μg/ml) for 2 days. Cell growth was measured using MTT assay. (B) Cells were transiently transfected with ObR siRNA (20 nM) or control (con) siRNA, and treated with leptin (1.0 μg/ml) for 2 days. Cell growth was measured using MTT assay. Values are the mean ± SD for three individual experiments (n = 5). *P < 0.05 versus untreated control; **P < 0.05 versus 1.0 μg/ml leptin-treated group; significant differences between groups were determined using one-way ANOVA.

Figure 4  Effect of leptin on clonogenic survival of endometriotic epithelial cells 11Z and 12Z. Cells were seeded in 6-well plates at a cell density of 5000 cells per well and treated with leptin (0.2 and 1.0 μg/ml) for 2 weeks. The colony formation was analyzed as described in Material and Method section. Values are the mean ± SD for three individual experiments (n = 5). *P < 0.05 versus untreated control; significant differences between groups were determined using one-way ANOVA.
As shown in Fig. 5, leptin significantly increased the activation of JAK2, STAT3 and ERK in 12Z, but not that of Akt. To further investigate the specific signaling pathways involved in the stimulation of growth by leptin in endometriotic epithelial cells, the effect of specific signal transduction inhibitors on leptin-induced cell growth was examined. The results showed that AG490, a JAK2/STAT3 inhibitor and PD98059, an ERK inhibitor, markedly blocked leptin-stimulated cell growth of endometriotic cells, whereas the PI3K/Akt inhibitor wortmannin did not cause a substantial inhibition (Fig. 6). In 11Z cells, leptin also induced the activation of JAK2/STAT3 and ERK and pre-treatment with the AG490 and PD98059 significantly inhibited leptin-induced cell growth (Supplemental data, Fig. S1). These results indicate that leptin may increase 11Z and 12Z cell growth by activating the JAK2/STAT3 and ERK signaling pathway.

Discussion

Over the past few years, several lines of evidence have demonstrated that the peritoneal fluid concentration of leptin is increased in women with endometriosis (Matarese et al., 2000; Mahutte et al., 2003; Bedaiwy et al., 2006; Barcz et al., 2008; Alviggi et al., 2009). However, whether this aberrant high level of leptin is due to obesity is still unclear, as the association between obesity and endometriosis is controversial (Ferrero et al., 2005; Yi et al., 2009). In addition to body fat as a cellular source of peritoneal leptin, other mechanisms responsible for aberrant expression of leptin have been studied. For example, Wu et al. (2002) suggested that ectopic endometriotic lesions may be important sites of leptin production, thereby elevating leptin concentration in the peritoneal fluid of women with endometriosis. These authors also showed that expression of leptin is induced by hypoxia in endometriosis (Wu et al., 2007). Whether the exact origin of peritoneal leptin is fat, endometriotic tissues or other cell types remains to be elucidated.

Accumulating evidence indicates that leptin has specific biological functions in the human normal endometrium and plays a role in embryo implantation and remodeling of the endometrium (Cervero et al., 2004; Tanaka and Umesaki, 2008). Leptin signaling appears to be essential for endometrial cell adhesion, proliferation, survival and migration (Carino et al., 2008; Tanaka and Umesaki, 2008). However, few studies on the functional role of leptin in endometriosis exist to date. A study using a murine endometriosis model showed that disruption of leptin signaling by i.p. injection of a pegylated leptin peptide receptor antagonist impairs the establishment of endometriosis-like lesions and results in a reduction of viable organized glandular epithelium, vascular endothelial growth factor-A expression and mitotic activity (Styer et al., 2008). Despite these observations, the role of leptin in endometriotic cell biology and in the pathogenesis of endometriosis remains to be fully elucidated and the exact mechanism of the response to leptin is not clearly understood.

ObR is expressed in various tissues including the peripheral reproductive system, such as placenta and ovary (Cioffi et al., 1997). It is also expressed in the human normal endometrium and in endometrial cancer (Kitawaki et al., 1999; Gonzalez-Robayna et al., 2000). One study showed that ObR is expressed in epithelial and stromal cells in the endometrium and stromal cells in endometriotic tissues (Ness et al., 2002). However, among 25 endometriotic tissues tested in this study, the number of samples showing ObR immunoreactivity and the tissue immunolocalization of ObR were not clearly described. Significant differences in ObR expression in the eutopic endometrium between women with endometriosis and controls were not detected (Lima-Couy et al., 2004). In the present study, ObR expression and
immunolocalization were assessed in 44 endometrioma tissue samples and compared with ObR expression in 40 normal endometrium tissues. Although ObR expression was not significantly correlated with the stage of the disease, size of the endometrioma, menstrual phase, age or BMI, endometrioma tissues expressed higher levels of ObR than normal endometrium. Interestingly, ObR immunoreactivity was more frequently observed in epithelial cells than in stromal cells of both the normal endometrium and that of endometriosis patients. The present finding of strong epithelial ObR staining is consistent with a previous study done in endometrial tissues. Lima-Couy et al. (2004) demonstrated that a clear positive signal is observed in the luminal and glandular epithelium of the endometrium compared with stromal cells. Other studies showed ObR protein expression in the glandular and luminal epithelium of the human endometrium and in cultured endometrial epithelial cells (Kitawaki et al., 1999; Gonzalez-Robayna et al., 2000). In this study, the increased expression of ObR in endometriotic tissues, which are likely exposed to a high leptin environment from peritoneal fluid, suggested that the potential role of leptin in the development and progression of endometriosis required further investigation.

In addition to its known role in metabolism, and cardiovascular and renal function, there is convincing evidence that leptin functions as a mitogen in various cancers, including breast, prostate and gastrointestinal cancer (Lang and Ratke, 2009). Despite this, it is unclear whether leptin plays a role in promoting the growth of endometriotic cells, and the exact molecular mechanism of the response to leptin has yet to be investigated. To investigate the effect of leptin on the cell growth of endometriotic cells, immortalized endometriotic cells were used as the experimental model in the present study. The source and availability of primary tissues and the ethical concerns in obtaining endometriotic tissues are among the major obstacles for study on endometriosis. In addition, there have been no appropriate in vivo and in vitro models available for studying the characteristics of the active phase of endometriosis in humans. Immortalized endometriotic cells 11Z, 12Z and 22B used in this study were established from active endometriotic lesions from women with endometriosis, and these lines retained the phenotypic characteristics and several in vivo properties of the active phase of endometriosis (Banu et al., 2008; Grund et al., 2008). The results show that leptin significantly increased cell growth and clonogenic survival of the endometriotic epithelial cell lines 11Z and 12Z, while no significant effect was observed in stromal 22B cells. As shown in Fig. 2, endometriotic epithelium and immortalized endometriotic epithelial cells (11Z and 12Z) expressed significantly higher levels of ObR than their normal and stromal counterparts. These data support the potential role of leptin in the pathogenesis of endometriosis via the regulation of endometriotic epithelial cell growth.

The activity of leptin as a signaling molecule is known to be mediated by multiple pathways, including the STAT3 (Yamashita et al., 1998), ERK (Yamashita et al. 1998; Catalano et al., 2004) and PI3K/Akt (O’Rourke et al., 2001) pathways. The concomitant activation of multiple signaling pathways by leptin was observed in certain types of cells (Bedaiwy et al., 2006; Gao et al., 2009). For example, in hepatocellular carcinoma cells, the concomitant activation of JAK2/STAT3, ERK and PI3K/AKT signaling is associated with leptin-mediated promotion of invasion and migration (Zeleznik et al., 2003). However, there are no reports in the current literature describing leptin signaling pathways in endometriotic cells. In the present study, activation of both the JAK2/STAT3 and ERK pathways correlated with leptin-induced proliferation in epithelial endometriotic cells. The molecular mechanisms underlying the interaction between these two pathways leading to the induction of cell growth remain to be elucidated.

The previously reported elevated levels of leptin found in the peritoneal fluid of women with endometriosis (Matarese et al., 2000; Mahutte et al., 2003; Bedaiwy et al., 2006; Barcz et al., 2008; Alviggi et al., 2009), together with the presence of ObRs in endometriotic tissues revealed in this study suggest that leptin may play a role in the development and/or progression of endometriosis. We found that leptin stimulates the growth of endometriotic epithelial cells through the JAK2/STAT3 and ERK pathways in this study. Despite these findings, the role of leptin in endometriosis-related pelvic pain and infertility remains to be elucidated. In fact, recent studies suggested that leptin in the peritoneal fluid is associated with chronic pelvic pain and inflammation, but not infertility, in endometriosis patients (Wertel et al., 2005; Bedaiwy et al., 2006; Milewski et al., 2008). Leptin-induced cell growth revealed in this study may accelerate the peritoneal inflammation and chronic pelvic pain. In addition, leptin-induced activation of the JAK/STAT and ERK pathway may be involved in the pelvic pain by regulating the expression of pro-inflammatory cytokines. For example, the STAT3 signaling was shown to regulate the expression of tumor necrosis factor-α and IL-1β (Pang et al., 2010), which are well documented to play a role in chronic pain (Sommers and Kress, 2004; Marchand et al., 2005). More studies are needed to evaluate the exact role of leptin in pelvic pain and inflammation.

This study demonstrated for the first time that epithelial expression of ObR is significantly greater in the endometrioma than in the endometrium. This study also revealed that leptin is a potent mitogenic factor in endometriotic epithelial cells, but not in endometriotic stromal cells. These data suggest that elevated leptin levels in the peritoneal environment, which have been reported before, can be a stimulating factor of cell growth in ObR positive endometriosis, thus emphasizing the need to assess ObR and leptin status in endometriosis patients. Furthermore, targeting the ObR and its signaling pathway could result in potent anti-proliferative effects in endometriotic epithelial cells and become a new strategy for the treatment of endometriosis.

**Supplementary data**

Supplementary data are available at http://molehr.oxfordjournals.org/.

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**Authors’ roles**

H.K.O. performed the immunohistochemical analysis and participated in study design, manuscript writing and critical discussion. Y.S.C. participated in study design, analysis, manuscript writing and critical discussion. R.H., T.K., T.O. and S.Y. participated in study design, analysis, manuscript writing and critical discussion. Y.S.C. performed the experiments. H.K.O. and S.Y. participated in the interpretation of the data. F.Y. and T.O. participated in the interpretation of the data. H.K.O. and S.Y. participated in study design, analysis, manuscript writing and critical discussion. Y.S.C. participated in study design, analysis, manuscript writing and critical discussion. H.K.O. and S.Y. participated in the interpretation of the data. F.Y. and T.O. participated in the interpretation of the data. H.K.O. and S.Y. participated in study design, analysis, manuscript writing and critical discussion.

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Conflict of interest
None declared.

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