Endocr (2007) 31:167–173
DOI 10.1007/s12020-007-0025-8

ORIGINAL PAPER

# Tyrosine receptor kinase B (TrkB) protein expression in the human endometrium

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Received: 22 February 2007 / Accepted: 15 May 2007 / Published online: 12 June 2007


**Abstract** Tyrosine kinase receptor B (TrkB) gene expression, a neurotrophic factor receptor expressed in the brain and ovary, has recently been identified in deep infiltrating endometriosis by gene array. TrkB is thought to be important in resistance to anchorage independent apoptosis (ANOIKIS) and thus could be important in the pathogenesis of endometriosis. However, TrkB protein expression in the eutopic endometrium of women with and without endometriosis is unknown. Therefore, we examined TrKB protein expression in the endometrium by Western blot ($n$ = 50) and immunohistochemistry ($n$ = 17). Immunoblots of endometrial biopsies were prepared from women with endometriosis ($n$ = 21) vs. healthy controls ($n$ = 29) undergoing benign gynecological surgery at McMaster University Medical Centre. TrkB protein expression was significantly higher in immunoblots from women with endometriosis compared to women without endometriosis. In samples of archived paraffin-embedded endometrial tissue TrkB was localized to the cytoplasm of epithelial cells of the eutopic endometrium from women with endometriosis ($n$ = 7) and without endometriosis ($n$ = 10). We conclude that TrkB protein is expressed in human endometrium. Our results also suggest that TrkB expression may be greater in women with endometriosis compared to women without endometriosis.

**Keywords** Tyrosine kinase receptor B · TrkB · Endometriosis · Neurotrophins · Apoptosis

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## Introduction

Endometriosis is an estrogen-dependent disease characterized by the growth of endometrial stromal cells and glands outside of the uterine cavity. Between 1 and 7% of women in the general population [1] and up to 30% of women undergoing laparoscopy for chronic pelvic pain are diagnosed with endometriosis [2]. Although the cause of endometriosis remains an enigma, retrograde menstruation of shed endometrial cells and tissue fragments is thought to be central to the development of this disease [3]. However, regurgitation of menstrual effluent occurs to some degree in all women of which only a fraction develop endometriosis. Hence, factors other than access of endometrial contents to the pelvis via retrograde menstruation are thought to contribute to the pathogenesis of this disease. However, the critical event(s) or biochemical change(s) that ultimately lead to the establishment of endometriosis remains unknown.

Although an estrogen-dependent disease, endometriosis is undoubtedly multifactorial in origin involving features of immune modulation [4], adhesion [5. 6], angiogenesis [7–10], invasion, proliferation, and decreased apoptosis [11–15]. Several distinct lines of evidence suggest that endometrial cells destined to become endometriotic implants are biochemically and functionally distinct from eutopic endometrium of women without endometriosis. For example, endometrial cells from women with endometriosis

survived transplantation in athymic nude mice for months, whereas normal proliferative endometrium from women without disease did not implant and proliferate [16]. Furthermore, the endometrium of women with endometriosis aberrantly expresses cell adhesion molecules such as integrins [17–20] and cadherins [21]. Dysregulation of the endometrial growth inhibitory factor interleukin-6 and its soluble receptor has been identified in ectopic endometrium [22]. Moreover, aromatase is expressed in eutopic and ectopic endometrium from women with endometriosis but not in endometrium from women without disease, thus providing a mechanism by which the ectopic endometrium can synthesize its own estrogen to promote implant survival [23]. We further suggest that an imbalance between proliferation and apoptosis signals in the endometrium of women with endometriosis is potentially important in the development of this disease. Of note, in women with endometriosis, menstruated endometrial cells and tissue fragments resist degradation [24, 25]. Moreover, compared to women without endometriosis, rates of apoptosis are also lower in the eutopic endometrium [13–15, 26, 27]. Differences in the expression of Bcl-2 (pro-survival) and Bax (apoptotic signal) in the endometrium of women with endometriosis compared to healthy controls has been documented [27]. However, no differences in either the amount of apoptosis or Bcl-2 levels between the endometrium of women with and without endometriosis has been reported [28]. The mechanisms regulating apoptosis or promoting cell survival in the endometrium are not known. We propose that the neurotrophin family and their respective receptor tyrosine kinases could be important in endometrial-cell survival [29] and pain perception [30].

The tyrosine receptor kinases (TrkA, TrkB, and TrkC) are the functional receptors for neurotrophins. Specifically, TrkA is the receptor for nerve growth factor (NGF), whereas brain-derived neurotrophic factor (BDNF) and neurotrophin-4/5 (NT-4/5) are the ligands for TrkB, and neurotrophin-3 (NT-3) binds with TrkC. Of the tyrosine receptor kinases, TrkB is a neurotrophic receptor best known for its role in the nervous system where it regulates cellular proliferation, differentiation and survival [31]. Moreover, TrkB expression in epithelial cancer cells was shown to be obligatory for matrix-free escape from caspase-associated apoptosis [29]. Aberrant tyrosine receptor kinase gene expression has been described for the endometrium of women with endometriosis [30, 32] but not ovarian endometriosis [32]. Therefore, we propose that TrkB expression could be important in the survival of regurgitated endometrial cells and tissue fragments and thus its expression may promote the development of endometriosis. TrkB is expressed in many tissues throughout the human body including the ovary [33–35]. While increased TrkB expression has been documented in epithelial cells of deep infiltrating endometriosis [30, 32], gene array studies are viewed by some as sophisticated hypothesis generating experiments involving complicated analyses and frequently poor reproducibility. Specific limitations of gene array studies include the lack of specificity in gene expression, insight to the mechanisms regulating gene expression, and inability of the technique to demonstrate that gene transcription is correlated with an increase in translation to functional protein. Thus changes in gene expression demonstrated by gene array techniques may not predict increased protein expression in target tissues. Therefore, the objective of the present study was to investigate the expression of TrkB protein in archived paraffin-embedded endometrial tissue samples from women with and without endometriosis. In addition, immunoblots prepared from eutopic endometrium of women with and without endometriosis were studied for differences in TrkB expression.

## Results

To examine TrkB protein expression in the endometrium of women with and without endometriosis ethics approval was obtained from the Faculty of Health Sciences Research Ethics Board at McMaster University to collect archived endometrial tissue for immunohistochemistry. Immunohistochemical localization of TrkB expression was investigated in archived paraffin-embedded endometrial tissue samples from women with and without endometriosis. Endometrial samples from both the proliferative and secretory phases of women with a diagnosis of endometriosis as well as healthy controls were evaluated (Fig. 1). TrkB protein expression was evident as a brown flocculent precipitate in the cytoplasm of glandular epithelial cells in both cycle phases of women with and without endometriosis (Fig. 1). While the majority of glands had staining in all cells, some tissues had glands in which 50% or fewer cells stained positively for TrkB. Although there was a trend for increased numbers of TrkB positive cells in the endometrium of women with endometriosis in the secretory phase, no significant difference in the H-score was found across the menstrual cycle (Table 1). Specifically, comparison of H-score values for cycle stage ($P = 0.18$), endometriosis diagnosis ($P = 0.56$), or for interaction between stage and diagnosis ($P = 0.29$) failed to reveal a significant difference.

TrkB protein expression was further examined by Western blot in endometrial samples collected from a second group of women ($n = 50$). Specifically, endometrial samples were collected from women between the ages of 18 and 45 undergoing hysterectomy for benign gynecological reasons. Two distinct immunoreactive TrkB bands

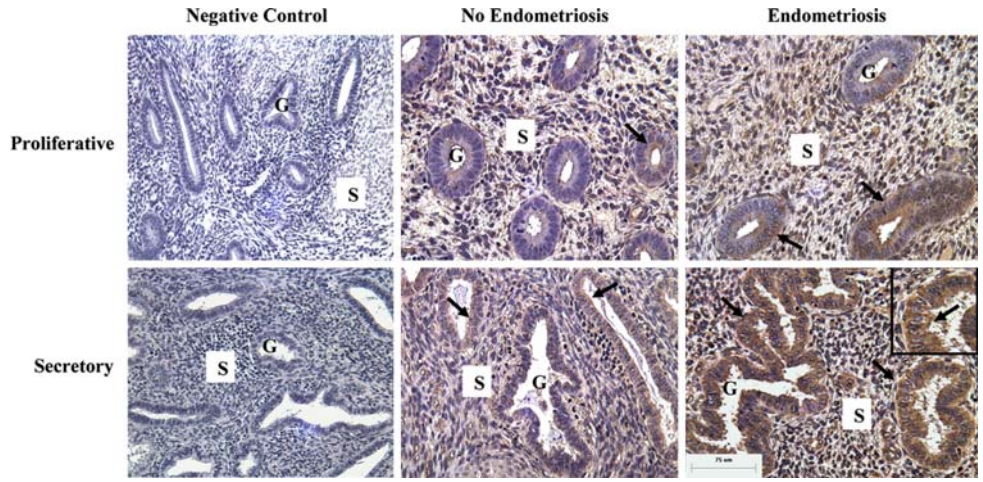


**Fig. 1** Archived paraffin-embedded samples from eutopic endometrium collected from the proliferative and secretory phases of the menstrual cycle of reproductively aged women with and without endometriosis. Sections were incubated overnight at 4°C with polyclonal rabbit anti-human TrkB antibody (sc-8316; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) diluted 1:100 (2 μg/mL) in PBS with 1% BSA. Negative controls were incubated with non-immune rabbit $IgG_1$ serum (1:100). TrkB protein was localized by diaminobenzidine as the chromagen which appeared as a flocculent brown precipitate in the cytoplasm of epithelial cells (arrows) of endometrial glands (G) but not the stroma (S). Insert demonstrates the cytoplasm specific localization of TrkB in epithelial glands of the endometrium

**Table 1** Mean H-scores for TrkB protein expression in eutopic endometrium over the menstrual cycle in women with and without endometriosis. No significant difference was observed upon comparison of H-score values for cycle stage ($P = 0.18$), endometriosis diagnosis ($P = 0.56$), or for interaction between stage and diagnosis ($P = 0.29$) by 2-way ANOVA

| Cycle stage | *N* | Endometriosis Dx | H-Score |
|---|---|---|---|
| Proliferative | 4 | No | 1.3 ± 0.1 |
| Proliferative | 4 | Yes | 1.2 ± 0.1 |
| Secretory | 6 | No | 1.4 ± 0.1 |
| Secretory | 3 | Yes | 1.6 ± 0.3 |

were found on the immunoblots corresponding to the full-length receptor (TrkB-FL; 145 kD) and the other representative of the truncated isoforms (TrkB-T1 and T-shc; 95 kD). TrkB protein was abundantly expressed in endometrial biopsies of women with endometriosis ($P < 0.05$) compared to samples from a reference population without endometriosis (Fig. 2). Interestingly, TrkB protein levels in samples from women with menorrhagia ($n = 6$) with no evidence endometriosis were comparable to those of women with the disease (Fig. 2).

## Discussion

Endometriosis is an estrogen-dependent disease that is thought to arise from retrograde menstruation of endometrial tissue. However, it is unclear why some women with retrograde menstruation develop endometriosis, whereas others do not. Results of the present study demonstrate that TrkB, a neurotrophin receptor important in regulating resistance to ANOIKIS, attachment-free cell survival, is expressed in the endometrium. Specifically, immunohistochemical analysis of archived paraffin-embedded endometrial tissue samples revealed TrkB protein in the cytoplasm of endometrial epithelial and, to a lesser extent, stromal cells. Moreover, TrkB protein was expressed at higher levels in the endometrium of women with endometriosis compared to women without endometriosis. Indeed in women without endometriosis TrkB protein expression was either not detectable or expressed at lower levels compared to endometrial tissue from women with endometriosis. To our knowledge this is the first study to demonstrate that TrkB protein is expressed in the human endometrium and to suggest a possible association with endometriosis.

TrkB gene expression in human endometrium was suggested previously on the basis of a gene array study examining deep infiltrating endometriosis obtained via

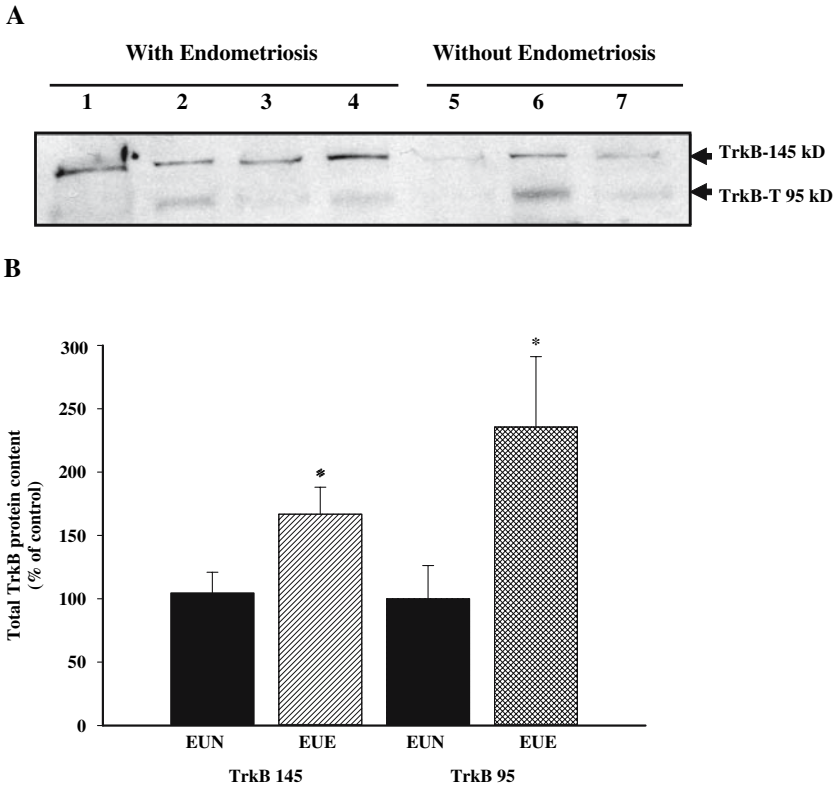


**Fig. 2** A representative immunoblot demonstrating full-length (145 kD) and truncated TrkB (95 kD) protein expression in eutopic endometrium of reproductively aged women with endometriosis (lanes 1–4) and without endometriosis (lanes 5 and 7). The sample in lane 6 was from a woman with menorrhagia but with no evidence of endometriosis (**A**). Total full-length (145 kD) and truncated TrkB (95 kD) protein content (**B**) in the eutopic endometrium of women with endometriosis (EUE; $n = 21$) compared to eutopic endometrium of women without endometriosis (EUN; $n = 29$). Data were compared by unpaired t-test with $P < 0.05$

laser capture microdissection [30]. However, TrkB protein expression in the endometrium has not been demonstrated previously. In the present study, TrkB immunostaining was localized to the cytoplasm of primarily epithelial cells of endometrial glands. In the only other study to immunohistochemically examine eutopic endometrium for this protein, TrkB expression was not found [36]. Reasons for the divergent results may be explained by differences in the immunohistochemical methods and tissues used in these studies. For example, in the prior study [36], the authors screened multiple organs and tissues for tyrosine receptor kinase expression using immunohistochemical methods optimized for this purpose. In the present study, the procedures used were optimized specifically for the endometrium where TrkB expression may be lower than in other tissues and accordingly the primary antibody dilution used was lower than in the previous study. Our results expand those of a previous gene array study [32] in which higher levels of TrkB mRNA expression were observed in deep infiltrating endometriosis lesions only. In this prior study TrkB expression was found in epithelial cells of endometriotic implants, with higher levels seen in samples obtained from women in the secretory phase of the menstrual cycle [32]. However, no TrkB expression was detected in ovarian endometriosis [32]. Therefore, cycle stage and source of ectopic endometrium (ovarian vs. peritoneal endometriosis) could be important in detecting TrkB protein by immunoshistochemistry. Another factor that could affect TrkB expression in the target tissue is the extent and duration of endometriosis. For example, *FOS* expression was found to decline during the progression of endometriosis in a baboon model [37]. Therefore, biochemical changes documented in women with endometriosis relative to healthy controls may not be stable over the course of the disease.

Results from our immunoblots confirm our immunohistochemical investigations and extend the findings of gene array studies [30, 32] revealing increased TrkB protein expression in the endometrium of women with endometriosis. In the current study, two distinct bands were found on the immunoblots, one representative of the full-length receptor (TrkB-FL; 145 kD) which signals through an intracellular tyrosine kinase domain and the other of two truncated isoforms (TrkB-T1 and T-shc; 95 kD) generated through alternate splicing of TrkB pre-mRNA [38, 39]. Ligand binding to TrkB-FL results in the activation of up to three signaling pathways involving phosphatidylinositol 3-kinase, mitogen-activated protein kinase and/or phospholipase C$\gamma$. While the truncated receptors are identical to the full-length form but lack the intracellular kinase domain, they are still able to initiate signaling [40]. Immunoblot detection of TrkB-FL and the truncated isoforms in our study suggests that multiple neurotrophin signaling pathways may be important in the pathogenesis of endometriosis.

While the role of TrkB in the endometrium is currently unknown, previous studies have suggested that its

expression could be associated with pain perception [30]. Alternatively, we propose that endometrial TrkB expression could play an important role in the development of endometriosis through the promotion of a pro-survival signal. In a recent study [29], TrkB expression was shown to be obligatory for malignant epithelial cell resistance to ANOIKIS in culture, a phenomenon that was increased further in the presence of the TrkB ligand, brain-derived neurotrophic factor (BDNF). Therefore, we speculate that TrkB expression is an early step in the progression of changes leading to the development of endometriosis. Apoptosis is known to be attenuated in the endometrium of women with endometriosis and thus alterations in the regulation of this process have been suggested to play a role in the pathobiology of this disease [13, 15, 41].

Regulation of TrkB expression in the endometrium is unknown. In the brain, levels of TrkB mRNA are enhanced following limbic seizure [42, 43] and long-term potential-induced tetanic stimulation [44], phenomena linked to the entry of calcium through voltage-gated channels which activates calcium-response elements in the promoter region of the gene [45]. Interestingly, rapid surface expression of TrkB may occur without protein synthesis in response to neuronal stimulation, as receptor recruitment to the plasma membrane can take place from intracellular stores [46]. This activity-dependent insertion of the TrkB receptor appears to occur independently of ligand binding [47], although acute exposure to BDNF can also rapidly increase TrkB surface expression while chronic treatment leads to decreased levels [48]. Expression of BDNF itself is induced via two adjacent calcium-response elements, one of which is regulated by cAMP-response element binding protein (CREB) [49, 50]. Thus, calcium and cAMP appear to play important roles in the expression of TrkB and its ligand. Several studies have indicated that estradiol treatment alters the expression of TrkB and/or its ligand, BDNF, in various types of neural tissue [51–53]. However, at the current time, BDNF expression in the endometrium and the mechanisms regulating TrkB expression are unknown.

Interpretation of our results is limited by the retrospective nature of the present study. Since site of tissue collection [32] and duration of disease [37] could be factors important in the expression of TrkB, prospective assessment of TrkB expression over the course of the menstrual cycle is essential. Furthermore, the relationship between TrkB expression and severity of endometriosis is unknown. Therefore, the relationship between TrkB expression and severity of endometriosis according to American Fertility Society classification of endometriosis [54] and scores of pain perception in women with endometriosis is needed.

In summary, results of the present study demonstrate that TrkB protein is expressed in human endometrium and thus extend the results of previous gene array studies. Moreover, our results suggest that TrkB expression is greater in women with endometriosis compared to healthy controls.

## Materials and methods

### Study subjects and tissue sample collection

Approval to collect archived endometrial tissue for immunohistchemistry was obtained from the Faculty of Health Sciences Research Ethics Board at McMaster University. Archived samples of formalin-fixed, paraffin-embedded endometrium from 17 female patients of reproductive age (42 ± 2.5 years) were obtained from the Department of Pathology at McMaster University Medical Centre. The diagnosis of endometriosis was confirmed by review of pathology reports.

Endometrial biopsies were obtained from a second group of women ($n$ = 50) between the ages of 18 and 45 undergoing benign gynecological surgery at McMaster University Medical Centre. All women were cycling normally and had not received hormonal therapy for at least 1 month prior to surgery. Informed consent was obtained prior to surgery and all procedures were conducted in accordance with McMaster University Research Ethics Board approval.

### Routine histology and immunohistochemistry

A 5 μm paraffin sections were stained with hematoxylin and eosin to identify the reproductive cycle stage (proliferative or secretory) of each patient at the time of surgery. While blinded to patient diagnosis (i.e., endometriosis or endometriosis-free), immunohistochemical staining was undertaken to identify the presence of TrkB. Sections were deparaffinized using xylene, brought to water through graded ethanol solutions, rinsed in phosphate buffered saline (PBS), then incubated in a 1% solution of $H_2O_2$ in methanol for 30 min to inhibit endogenous peroxidases. Non-specific binding was blocked for 1 h using 1.5% normal goat serum (Vectastain ABC kit, Vector Laboratories Inc., Burlingame, CA, USA) in PBS with 1% bovine serum albumin. Sections were then incubated overnight at 4°C with polyclonal rabbit anti-human TrkB antibody (sc-8316; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) diluted 1:100 (2 μg/mL) in PBS with 1% BSA. Negative controls were incubated with non-immune rabbit $IgG_1$ serum (1:100). Sections were washed three times with PBS followed by incubation in biotinylated secondary antibody for 2 h at room temperature. The slides were washed and incubated with Vectastain ABC Reagent (avidin DH and biotinylated horseradish peroxidase H;

Vector Laboratories Inc.) for 2 h at room temperature. Diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich Chemical Co., St. Louis, MO, USA) was used as the chromagen for color development, followed by counterstaining with Carazzi's hematoxylin. Sections were dehydrated through graded ethanol solutions, cleared in xylene, and mounted with Permount for bright-field microscopy. Images were acquired digitally using an Olympus microscope coupled to an image analysis system (Image Pro Plus, Media Cybernetics, Silver Spring, MD, USA).

To quantify the immunohistochemical staining for TrkB, H-scores were determined for each section by one investigator who was blinded to the endometriosis diagnosis of each patient. Approximately 1000 glandular cells were counted per section using an orthogonal gridmask placed over images captured at 40x magnification using Image Pro Plus. The following equation was used to calculate the H-scores: $H = \Sigma\ Pi\ (i + 1)$, where $i$ is the staining intensity of the cells (with 0, 1, 2, and 3 representing zero, weak, moderate and strong staining, respectively) and $Pi$ is the percentage of cells stained at each intensity (0–100%).

### Tissue sample preparation and Western blotting

Endometrial biopsy samples from women with endometriosis ($n = 21$; $39.0 \pm 1.8$ years) and women without endometriosis ($n = 29$; $40.5 \pm 0.9$ years) were immediately frozen and stored at –85°C until use. Proteins were extracted from tissues by homogenization in RIPA buffer containing 1% Triton-X, 3.5 mM SDS, 0.2 M NaCl, 0.2 M Tris–HCl, 0.01 M deoxycholic acid sodium salt (Sigma Aldrich, Oakville, Ont., Canada) and Complete Mini protease inhibitor (1 tablet per 10 mL; Roche Diagnostics, Laval, Que., Canada). Homogenates were centrifuged at $2{,}000\times g$ for 15 min at 4°C. The supernatants were collected and protein concentration was determined using Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Hercules, CA, USA) and the Bradford method [55]. Proteins (15 μg) were electrophoresed by 10% SDS-PAGE under reducing conditions and transferred onto nitrocellulose membranes. Equal loading in each lane was confirmed by staining the blots with 2% Ponseau S. Non-specific binding was blocked by incubation with phosphate-buffered saline (PBS) containing 0.5% Tween-20 with 5% nonfat powdered milk overnight at 4°C. Membranes were incubated for 1 h with TrkB polyclonal antibody (sc-8316; Santa Cruz Biotechnology Inc.) diluted 1:200 in blocking buffer. This antibody recognizes both full-length (145 kDa) and truncated (95 kDa) forms of the receptor. Following incubation with the primary antibody, membranes were washed with PBS/Tween and incubated with horseradish peroxidase conjugated secondary antibody (1:5000 anti–rabbit IgG; Amersham Biosciences Inc., Piscataway, NJ, USA) in blocking buffer for 1 h. Enhanced chemiluminescent detection (ECL; Amersham Biosciences) was used to visualize the protein bands.

### Statistical analyses

Summary statistics and group comparisons were made by 2-way analysis of variance (ANOVA) using SigmaStat (Systat Software, Inc., Richmond, CA, USA). A $P$ value of $<0.05$ was considered significant.

**Acknowledgments** The authors would like to sincerely thank the members of McMaster's Department of Pathology for their help in obtaining endometrial biopsy samples and retrieving archived embedded samples. The technical assistance of Ms Stephanie Bulk for running the immunoblots in this study is gratefully appreciated. Financial support for this project was provided by the Natural Sciences and Engineering Research Council of Canada (261905-03 to WGF). Salary support for Dr. Foster from the CIHR and the Ontario Women's Health Council is gratefully acknowledged.

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