Original Article

Reproductive Sciences
2017, Vol. 24(8) 1176-1186

DOI: 10.1177/1933719116681515
journals.sagepub.com/home/rsx
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Decreased Endometrial Expression of Leukemia Inhibitory Factor Receptor Disrupts the STAT3 Signaling in Adenomyosis During the Implantation Window

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Abstract

Background: Adenomyosis was found to have negative impacts on embryo implantation. Leukemia inhibitory factor (LIF), proposed to be a molecular marker for endometrial receptivity, works through the LIF receptor (LIFR) on both the embryo and the endometrium. We aimed to evaluate the endometrial expression of LIF and LIFR and its subsequent signaling in patients with adenomyosis during the window of implantation (WOI). **Methods:** Endometrium was obtained during the WOI from patients with adenomyosis (age <45 years) who underwent hysterectomy and from age-matched controls who had no endometriosis or adenomyosis. The LIF and LIFR expressions were measured by polymerase chain reaction for messenger RNA expression, immunohistochemistry for protein intensity and localization, and immunofluorescent staining for colocalization. The ratio of signal transducer and activator of transcription 3 (STAT3) to extracellular signal-regulated kinase (ERK) phosphorylation was measured by Western blot of both the endometrium and the isolated human endometrial stromal cells (ESCs). **Results:** Patients with adenomyosis showed significantly and parallelly reduced LIF and LIFR expressions in the eutopic endometrium during WOI as compared with the control women and subsequently with remarkably reduced activation of STAT3 and ERK signaling. The significantly increased STAT3 and ERK phosphorylation induced by the LIF treatment in the cultured ESCs supported the linkage between the LIF–LIFR reaction and the signaling cascade. **Conclusion:** Significant reduction in LIFR expression and the reduced activation of subsequent signaling strongly suggest a working model of how the implantation markers, LIF, may affect the endometrium of patients with adenomyosis. These molecular changes supported the declined implantation rates reported in patients with adenomyosis.

Keywords

adenomyosis, endometrium, leukemia inhibitory factor (LIF), window of implantation (WOI), signal transducer and activator of transcription (STAT) 3

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Introduction

Adenomyosis is manifested by the diffuse or aggregated presence of endometrial glands and stroma within the hypertrophic and hyperplastic myometrium.[1] Although it is generally considered benign, conservative treatments[2,3] may not be satisfactory for symptomatic patients and the definitive treatment is hysterectomy.[4,5]

Although the association of adenomyosis and subfertility was controversial,[6] recent studies using gonadotropin-releasing hormone antagonist in vitro fertilization (IVF) protocol have found a negative impact of adenomyosis on embryo implantation,[7-9] with positive correlation between adenomyosis and abnormal uterotubal contractility.[10] Statistically, women with adenomyosis displayed a 28% reduction in pregnancy rate at IVF/intracytoplasmic sperm injection (ICSI) clinics,[9] with a remarkable increased miscarriage rate in comparison with women without adenomyosis (31.9% vs 14.1%, respectively).[9]

The endometrial receptivity for the blastocyst is only for a limited period from days 6 to 9 following the luteinizing hormone surge or days 19 to 23 of a normal 28-day cycle known as window of implantation (WOI) in the menstrual mid-secretory phase.[11] Coinciding with the WOI, the endometrial expression of leukemia inhibitory factor (LIF) was observed to be low in the proliferative phase, with a substantial increase in the mid-secretory phase.[12,13] Accumulated evidence indicates that LIF plays a critical role in orchestrating a cross talk between the embryo and the receptive endometrium,[14] as evidenced by the blastocyst of the LIF-knockout mice failing to attach to the endometrial luminal epithelium.[15] Human blastocysts have also been shown to express messenger RNAs (mRNAs) for LIF receptor (LIFR) and glycoprotein (gp) 130.[16] Also, infertile women produce significantly less endometrial LIF compared with fertile controls during WOI.[16-19] Therefore, LIF has been proposed to be a molecular marker for receptive endometrium.[20,21]

Leukemia inhibitory factor signals through the heterodimeric transmembrane proteins consisting of LIFR-α and gp130 as well as downstream molecules. The activation of the Janus kinase/signal transducer and activator of transcription 3 (JAK/STAT3) predominates this signaling transduction pathway, while the Src homology 2-domain containing tyrosine phosphatase (SHP-2)/Ras/extracellular signal-regulated kinase (ERK) signaling[22-24] acts as a redundant pathway.

As endometrium is the main stage for embryo implantation, the current study aims to characterize the molecular changes in the endometrium for the subfertility in patients with adenomyosis. The endometrial expression of LIF and LIFR as well as the associated signaling(s) during the WOI was evaluated between patients with and without pathologically confirmed adenomyosis. In addition, cultured endometrial stromal cells (ESCs) treated with or without LIF were analyzed to complement in situ observations for relevant signaling pathway(s).

Materials and Methods

Patients and Tissue Specimens

This study was previously approved by the human investigation review board at the Chang Gung Memorial Hospital, Taiwan (#97-1654A3, #97-2460A3, #98-3685B, #100-4660A3, and #104-8835C), and all patients who underwent surgery and donated tissue gave their written informed consent. All of the endometrial tissues of adenomyosis were obtained from women ($n = 5$ with diffuse adenomyosis and $n = 14$ with focal adenomyosis) of reproductive age who underwent laparoscopically assisted vaginal hysterectomy (LAVH)[25] during the WOI (19th to 23rd day of the menstrual cycle) based on the women's menstrual history and confirmed by the histological examination.[26] Patients with adenomyosis were diagnosed preoperatively with clinical symptoms and ultrasonogram and confirmed postoperatively by pathologists. Control endometria were collected from age- and menstrual phase–matched patients of uterine leiomyoma ($n = 16$ with intramural or associated with subserosal and $n = 3$ with submucosal location) who underwent LAVH ($n = 14$) or myomectomy ($n = 2$ laparoscopically and $n = 3$ hysteroscopically) and were pathologically proved to be without endometriosis or adenomyosis (Table 1). All included patients were not taking any hormonal medication for at least 3 months. Specimens were either snap frozen and stored in liquid nitrogen for RNA and protein extraction or embedded in paraffin for immunohistochemistry (IHC).

Quantitative Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated from 50 to 100 mg of endometrium using TRI Reagent (Sigma-Aldrich, St. Louis, Missouri). For complementary DNA synthesis, 2 μg of total RNA was reverse transcribed with 1 μg of oligo-deoxythymidine primers using a reverse transcription kit (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions.

Endometrial expression of target genes was analyzed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The mRNA levels were measured in triplicate using the SYBR Select Master Mix for CFX (Applied Biosystems, Thermo Fisher Scientific, Waltham, Massachusetts) and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), according to the manufacturer's protocol, on a CFX Connect 384 (Bio-Rad Laboratories, Waltham, Massachusetts).

Gene-specific primer sets (Table 2) were selected with Primer3-BLAST. The PCR product identity was demonstrated by sequencing. All the primer pairs span exon–intron junctions and are located in different exons. In selected experiments, PCR products were run on 3% agarose gel to confirm the product size. For each RNA specimen, a negative control was prepared by omitting the reverse transcriptase.

Amplifications were carried out at 95°C for 5 minutes, followed by 10 seconds at 95°C, and 30 seconds at 60°C for 40

Table 1. Characteristics of the Women Who Provided Uterine Tissue Samples.

	Control (n = 19)	Adenomyosis (n = 19)	*P* Value
Age (years)	41.2 ± 0.5	39.8 ± 0.8	NS
BMI (kg/m^2)	23.5 ± 0.8	26.3 ± 1.2	NS
Parity, median (range)	2 (0-3)	2 (0-3)	NS
Menstrual day	20.3 ± 0.4	21.4 ± 0.4	NS
Type of lesion	IML or with SSL (solitary: n = 7, multiple: n = 9), SML (solitary: n = 3)	DA: n = 5, FA: (posterior: n = 11; fundal: n = 1; anterior: n = 1)	–
CA-125 value (U/mL), (range)	25.6 ± 3.5 (6.6-66.7)	145.1 ± 36.3 (42.5-688.8)	.001
Type of surgery	Hysterectomy (LAVH: n = 13; LSH: n = 1), Myomectomy (LM: n = 2; HM: n = 3)	Hysterectomy (LAVH: n = 18; LSH: n = 1)	–

Abbreviations: BMI, body mass index; DA, diffuse adenomyosis; FA, focal adenomyosis; HM, hysteroscopic myomectomy; (Lesion) IML, intramural leiomyoma; (Surgery) LAVH, laparoscopic assisted vaginal hysterectomy; LM, laparoscopic myomectomy; LSH, laparoscopic subtotal hysterectomy; NS, not significant; SML, submucosal leiomyoma; SSL, subserosal leiomyoma.

Table 2. Primers for Reverse Transcription-Polymerase Chain Reaction.

Gene	Sense (5′ → 3′)	Antisense (5′ → 3′)	Size (bp)
LIF	TGG ACG TGA CCT ACG GCC CTG	TGC AGG TCC AGC CAT CAG ACC	886
LIFR	AGT TAC CAC CTG GTC TTG CG	TGC TTG AGG CTG ATA CAT CG	539
GAPDH	TGGTATCGTGGAAGGACTCATGAC	ATGCCAGTGAGCTTCCCGTTCAGC	189

Abbreviations: bp, base pair; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *LIF*, leukemia inhibitory factor; *LIFR*, LIF receptor; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction.
[a]Twenty-five cycles of PCR amplification were performed at 94°C for 30 seconds, 58°C for 1 minute, and 72°C for 2 minutes.

cycles, with fluorescence detection at the end of each extension step. For each run, melting curve analysis was used to confirm the specificity of the amplified products and the absence of primer–dimer formation. Final results of the target gene expression were expressed as fold differences in gene expression relative to the normalized calibrator, calculated by the $\Delta\Delta Ct$ method as follows: n-fold $= 2^{-(\Delta Ct[\text{sample}] - \Delta Ct[\text{calibrator}])}$, where ΔCt values of the sample and calibrator were determined by subtracting the average threshold cycle (Ct) value of the transcript under investigation from the average Ct value of the human GAPDH gene for each sample.

Immunohistochemistry

Full-thickness endometrium (1 × 1 cm) and a piece of adenomyotic lesion obtained from the upper half of the uterine corpus were fixed with formalin and embedded in paraffin. Sections (5 μm) were deparaffinized and rehydrated in a graded ethanol series and washed in Tris-buffered saline (TBS, 20 mM Tris–HCl, 150 mM NaCl, pH 7.6). Antigen was retrieved by boiling the slides in sodium citrate buffer (10 mmol/L, pH 6.0) for 15 minutes, blocking the endogenous peroxidase by rinsing in 3% hydrogen peroxide (in 50% methanol/50% distilled water) for 5 minutes, and then the slides were incubated at room temperature with blocking serum (5%) in a humidified chamber for 30 minutes. The slides were incubated with either goat polyclonal antihuman LIF antibody (Santa Cruz [N-18]: SC-1336) or rabbit polyclonal antihuman LIFR antibody (Santa Cruz [H-220]: SC-20752), both in 1:100 dilution, overnight at 4°C in a humidified chamber. Normal goat or rabbit immunoglobulin G (IgG) isotypes were used as negative controls. Using the biotinylated secondary antibody (Vector Laboratories, Inc, Burlingame, California), the antigen–antibody complex was detected by the avidin–biotin–peroxidase complex (Vectastain ABC Kit; Vector Laboratories) and 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) with light hematoxylin counterstaining.

Immunoreactivity was evaluated with HSCORE in an area of around 100 cells (×400), using the formula HSCORE $= \Sigma P_i \times$ (intensity score), where the intensity score is graded as 0 for none, 1 for weak but detectable, 2 for moderate or distinct staining, or 3 for strong staining; and P_i is the corresponding percentage value (range 0-100) of the positively stained cells in each category and hence yielded a HSCORE value ranging between 0 and 300.[27] The final average scores were from 5 fields/slide that were evaluated by 2 investigators blinded to the tissue source.

Histologically, the endometrium is divided into layers of the upper functionalis, which occupies the upper two-third thickness of the secretory phase endometrium and contains glands loosely held together by supportive stroma, and the lower basalis, which consists of dense stroma and branching glands.[28,29] Therefore, we evaluated each slide spatially on the luminal and its adjacent half thickness of endometrium as the scores of functionalis and on the endometrium within 1-mm distance from the endometrial–myometrial junction as the scores of basalis.[30,31] In addition, the ectopic endometrium of the adenomyotic nodules among the myometrium was also scored.

Immunofluorescent Staining

Deparaffinized formalin-fixed sections (5 μm) were incubated with 5% normal horse serum in a humidified chamber for 30 minutes at room temperature. The slides were then treated with either goat polyclonal antihuman LIF antibody (Santa Cruz [N-18]: SC-1336) or rabbit polyclonal antihuman LIFR antibody (Santa Cruz [H-220]: SC-20752), both in 1:100 dilutions, overnight at 4°C; followed by either donkey phycoerythrin-conjugated anti-goat IgG (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) or chicken fluorescein isothiocyanate-conjugated anti-rabbit IgG (1:50; Santa Cruz Biotechnology). After staining with 4′,6-diamidino-2-phenylindole (Santa Cruz Biotechnology), the slides were examined using Olympus/IX71 (Olympus Corporation, Tokyo, Japan) fluorescent microscope equipped with OLYMPUS DP2-BSW software (ver.2.1).

Endometrial Stromal Cell Isolation and Culture

Endometrial tissue was minced and digested in Hank's Balanced Salt Solution (HBSS) (Sigma-Aldrich) containing collagenase B (1 mg/mL, 15 U/mg; Roche, Indianapolis, Indiana), deoxyribonuclease I (0.1 mg/1 mL, 1500 U/mg; Roche), penicillin (200 U/mL), and streptomycin (200 mg/mL) for 60 minutes at 37°C, with gentle agitation. Human ESCs were separated by a wire sieve (40-μm-diameter pore; BD, Bioscience, Bedford, MA) and cultured in Dulbecco Modified Eagle Medium/Ham F-12 (1:1 vol/vol; Sigma-Aldrich) containing fetal bovine serum (10% vol/vol; Gibco-BRL, Rockville, Maryland) until confluence. Endometrial stromal cells after first passage were assayed immunocytochemically and previously found to contain 0% to 3% epithelial cells, no detectable endothelial cells, and 0.2% macrophages.[32,33]

Then, the confluent, leukocyte-free ESCs (passage 3-5) were primed with 10^{-8} M estradiol (Sigma-Aldrich) plus 10^{-7} M medroxyprogesterone acetate (Sigma-Aldrich) in serum-free basal medium (BM) with 100 U/mL penicillin, 100 mg/mL streptomycin, and 0.25 mg/mL fungizone for 48 hours before being cultured in BM supplemented with 10% charcoal-stripped calf serum (BMS) with or without recombinant human LIF (50 ng/mL; R&D, Minneapolis, MN) for the indicated times in time-course experiments. The cells were harvested and lyzed for further use.

Western Blotting

Total protein was extracted from 50 to 100 mg of endometrium and ESCs with cell lysis buffer (Sigma-Aldrich) containing 3 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Sigma-Aldrich). Protein concentration was determined by a detergent-compatible protein assay (Bio-Rad Laboratories, Hercules, California). A 60 μg of total protein was separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis before being electroblotted onto a polyvinylidene difluoride (PVDF) Hybridization Transfer Membrane (PerkinElmer, Boston, Massachusetts). After blocking with 10% nonfat dry milk in TBS containing 0.1% Tween 20 for 1 hour, the membrane was incubated overnight at 4°C with antihuman STAT3 (79D7) rabbit monoclonal (Cell Signaling, Danvers, MA), phospho-STAT3 (pSTAT3, Tyr705) (D3A7) rabbit monoclonal (Cell Signaling), phospho-ERK (pERK, Thr204) goat polyclonal (Santa Cruz Biotechnology, Santa Cruz, California), ERK 2 (C-14) rabbit polyclonal (Santa Cruz), or α-tublin rabbit polyclonal antibody (Cell Signaling) in proper dilutions as per the manufacturer's recommendations with gentle agitation. Primary antibodies depict the STAT3 and the pSTAT3 proteins at both 79 and 86 kDa, the ERK and the pERK protein at 42 and 44 kDa, respectively. After a wash, the membranes were incubated with horseradish peroxidase-conjugated donkey anti-goat (Santa Cruz) or goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) for 1 hour. Chemiluminescent detection was performed with enhanced chemiluminescence substrate (Amersham Biosciences, Buckinghamshire, England). Autoradiography of the membranes was processed using hyperfilm-ECL (Amersham). Densitometry was used to compare the differences in LIF and LIFR mRNA expressions between different groups of patients.

Statistics

Continuous variables such as age and body mass index values were presented as mean ± standard error of the mean, whereas parity was presented as median value and range. Normality test of data was performed with Kolmogorov-Smirnov test. Data with normal distribution were then analyzed with Student *t* test or 1-way analysis of variance (ANOVA), with post hoc Bonferroni test for pairwise comparisons between groups. Data in abnormal distribution were presented in median value associated with the 25th to 75th percentile range and were analyzed with Mann-Whitney rank sum test or Kruskal-Wallis nonparametric 1-way ANOVA on ranks followed by post hoc Dunnett test. Statistical calculation was performed using SPSS for Windows (release 17.0.0/2008; IBM-SPSS, Inc, Chicago, Illinois). Statistical significance was defined as $P < .05$.

Results

Demographics of patients with specimens demonstrated in this article are summarized in Table 1. There were no differences in age, parity, and menstrual phase between both groups. Levels of CA-125 were significantly increased in women with adenomyosis ($P < .001$).

Expression of LIF and LIFR in Eutopic Endometrium

The LIF and LIFR mRNA expressions were demonstrated by end point (Figure 1A) and the qRT-PCR (Figure 1B). The expression of LIF mRNA in the endometrium from patients with adenomyosis was significantly lower (0.15 fold) than that of the control patients, and the LIFR mRNA level in the endometrium from patients with adenomyosis was also significantly lower (0.3 fold) than that of the control patients (n = 10; both *P*s < .01; Figure 1B).

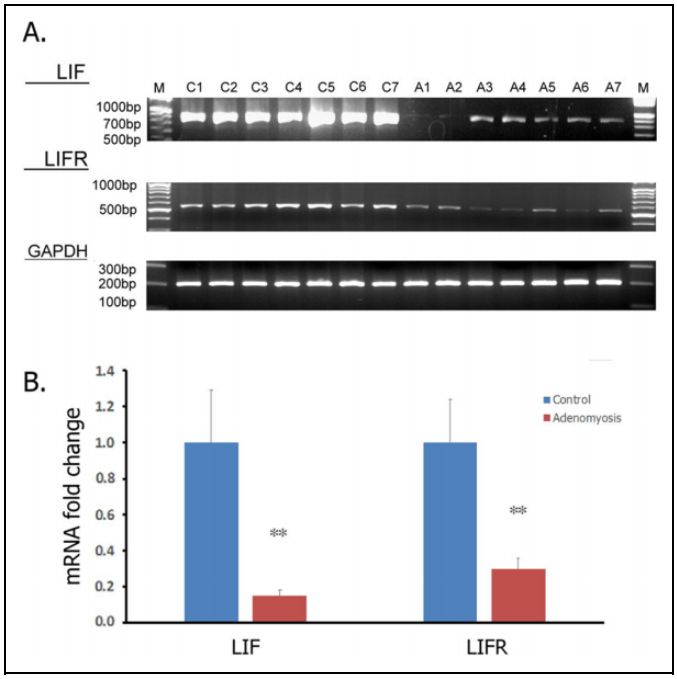

Figure 1. The messenger RNA (mRNA) expression of leukemia inhibitory factor (LIF) and LIF receptor (LIFR) in eutopic endometrium of the controls and patients with adenomyosis in mid-secretory phase. A, Agarose gel stained with ethidium bromide shows the electrophoresis of end point reverse transcription-polymerase chain reaction (RT-PCR) products (n = 7) for LIF and LIFR obtained from patients between menstrual days 19 and 23 in each group. B, Quantitative real-time RT-PCR (n = 10), where the relative mRNA expression of LIF and LIFR were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and ordinate represents data in fold changes. Results are expressed as mean ± standard error of the mean (SEM). C1-7 indicates control patient; A1-7, patient with adenomyosis; M, marker. $^{**}P < .01$.

Spatial Evaluation of LIF and LIFR Protein Intensity in Endometrium

Immunostaining demonstrated the localization of LIF and LIFR proteins in the eutopic endometrium and in the adenomyotic nodules (Figure 2A, B.a-d). The HSCORE was calculated by measuring 12 patients in each group (Figure 2A, B.e).

In general, LIF immunostaining was exclusively cytoplasmic and significantly stronger in the gland than in the stroma in both the control ($P < .001$; Figure 2A.a and e) and adenomyosis groups ($P < .05$; Figure 2A.b and e). Spatial difference in LIF staining was also significant, especially in the control women, with greater LIF immunointensity in the luminal and functional layer than the basal layer of endometrium in both the glandular (231.8 ± 6.0 vs 98.2 ± 3.2, $P < .001$; Figure 2A.c) and stromal cells (135.4 ± 4.7 vs 55.8 ± 5.4, $P < .001$; Figure 2A.e). However, the LIF expression of patients with adenomyosis (Figure 2A.b) was significantly decreased in the functional endometrium than that of the control in both glandular (61.5 ± 5.6 vs 231.8 ± 6.0, $P < .001$) and stromal cells (40.7 ± 4.3 vs 135.4 ± 4.7, $P < .001$; Figure 2A.e). The spatial difference in LIF expression in patients with adenomyosis was also significant in the gland (functional layer: 61.5 ± 5.6 vs basal layer: 32.8 ± 4.0, $P < .05$) but not in the stroma (Figure 2A.e). The immunointensity of LIF in the adenomyotic lesions was as low as that of the basal layer of eutopic endometrium of the same patients (Figure 2A.d, inset).

Stronger LIFR immunostaining was observed in glandular cells than that in stromal cells in both functional ($P < .001$) and basal layers (48.3 ± 8.4 vs 29.9 ± 3.5, $P < .05$) in control groups (Figure 2B.a and c). Significant spatial difference was also noted, with more prominent LIFR expression at the luminal epithelium and functional layer than the basal layer of endometrium (in both the glandular and stromal cells, $P < .001$). However, in patients with adenomyosis, the LIFR was significantly lower than that in the control group in both glandular cells (43.6 ± 3.1 vs 92.3 ± 7.3, $P < .001$) and stromal cells (27.3 ± 2.3 vs 57.7 ± 5.9, $P < .001$; Figure 2B.e).

Linking Role of LIFR and the Subsequent Signaling

Immunofluorescent staining was performed to colocalize LIF (red) and LIFR (green) in the functional layer of eutopic endometrium (Figure 3). The LIF and LIFR in the eutopic endometrium were found to be exclusively colocalized outside the nuclei in both groups. Consistent with IHC, the expression of LIF and LIFR was more prominent in glandular cells than in stromal cells, with parallelly lower immunointensity observed in patients with adenomyosis.

The activation status of the subsequent signaling of LIFR such as JAK/STAT3 and mitogen-activated protein kinase (MAPK)/ERK pathways was assessed by Western blot analysis in eutopic endometrium of patients with adenomyosis. The phosphorylation of STAT3 (pSTAT3) and ERK (pERK) was reduced in the eutopic endometrium from patients with adenomyosis without significantly different changes in total protein levels (Figure 4A). With the densitometry measurement, the phosphorylation ratio in the eutopic endometrium of patients with adenomyosis significantly declined in both pSTAT3/STAT3 (0.22 ± 0.04 vs 0.94 ± 0.10, $P < .01$) and pERK/ERK (0.46 ± 0.03 vs 1.20 ± 0.18, $P < .05$), compared with controls (Figure 4B).

In Vitro Testing of the Phosphorylation of STAT3 and ERK

We would further confirm that the STAT3 and ERK pathways were the downstream signaling of LIF–LIFR and tested whether the changes in phosphorylation ratio could be affected by the synchronous downregulation of LIF/LIFR. Confluent, ESCs were first synchronized with a 48-hour starvation (time 0) and then the time course was started by renourishing the cells with BMS and dividing the groups by treating with or without the recombinant human LIF (Figure 5A). The phosphorylation of STAT3 (pSTAT3) was strikingly increased in LIF-treated ESCs but almost undetectable in the nontreated ESCs, and even the renourishing of the starved cells did not elicit the activation of STAT3 pathway in the nontreated group. The pSTAT3/STAT3 ratio was increased in all time points,

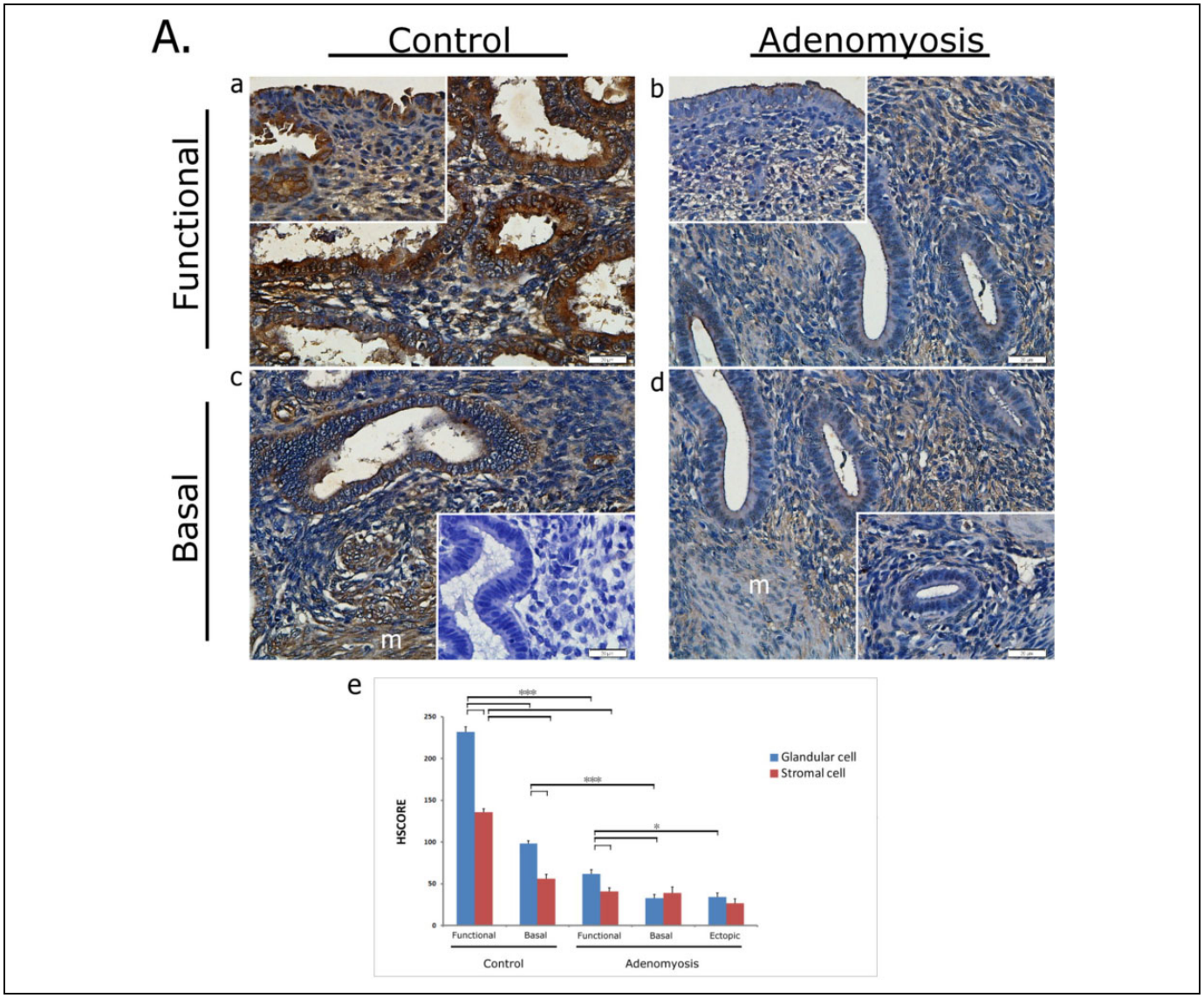

Figure 2. The localization and intensity measurement of leukemia inhibitory factor (LIF) and LIF receptor (LIFR) proteins in the endometrium from control and patients with adenomyosis during window of implantation (WOI). The expression of (A) LIF and (B) LIFR in the endometrium was evaluated by immunohistochemistry (IHC, ×400) using diaminobenzidine as chromogen and hematoxylin for nucleus counterstain. (a) and (b) The functional layers (inset, endometrial luminal layer). (c) and (d) The basal layer. Inset in (c) shows the negative control of monoclonal antibody using mouse isotype staining, and inset in (d) shows the staining of ectopic endometrium within the myometrium in adenomyotic specimens (×400, scale bar: 20 μm). (e) Histology Score (H-score) of LIF expression in glandular and stromal cells. Results are expressed in mean ± standard error of the mean (SEM; n = 12; **P* < .05; ****P* < .001).

including 5 minutes ($P < .05$), 15 minutes, 30 minutes (both Ps $< .01$), and 60 minutes ($P < .05$; Figure 5B.a). Phospo-STAT3 became undetectable at 120 minutes (data not shown). In contrast, the basal level of phosphorylated ERK is greater than STAT3 in the nontreated ESCs (Figure 5A) after the renourishing of the starved cells. Nevertheless, the LIF treatment also elicited borderline increase in pERK at 5 minutes ($P = .057$) and the phosphorylation of ERK was significantly increased after 15 minutes of treatment ($P < .05$, Figure 5B.b). However, the effect of LIF treatment on ERK phosphorylation disappeared after 60 minutes (Figure 5B.b).

Discussion

Although a previous study has described the decreased LIF secretion in the endometrium and uterine flushing fluid of patient with adenomyosis having infertility during WOI,[34] this study is the first to extendedly reveal the changes in LIFR expression and its consequences in the endometrium, as the receptor is undoubtedly the first step in which the cells could react to the cytokine stimuli. Besides, the current study also demonstrates the reduced activation of the LIFR-mediated downstream phosphorylation cascades during the WOI, which

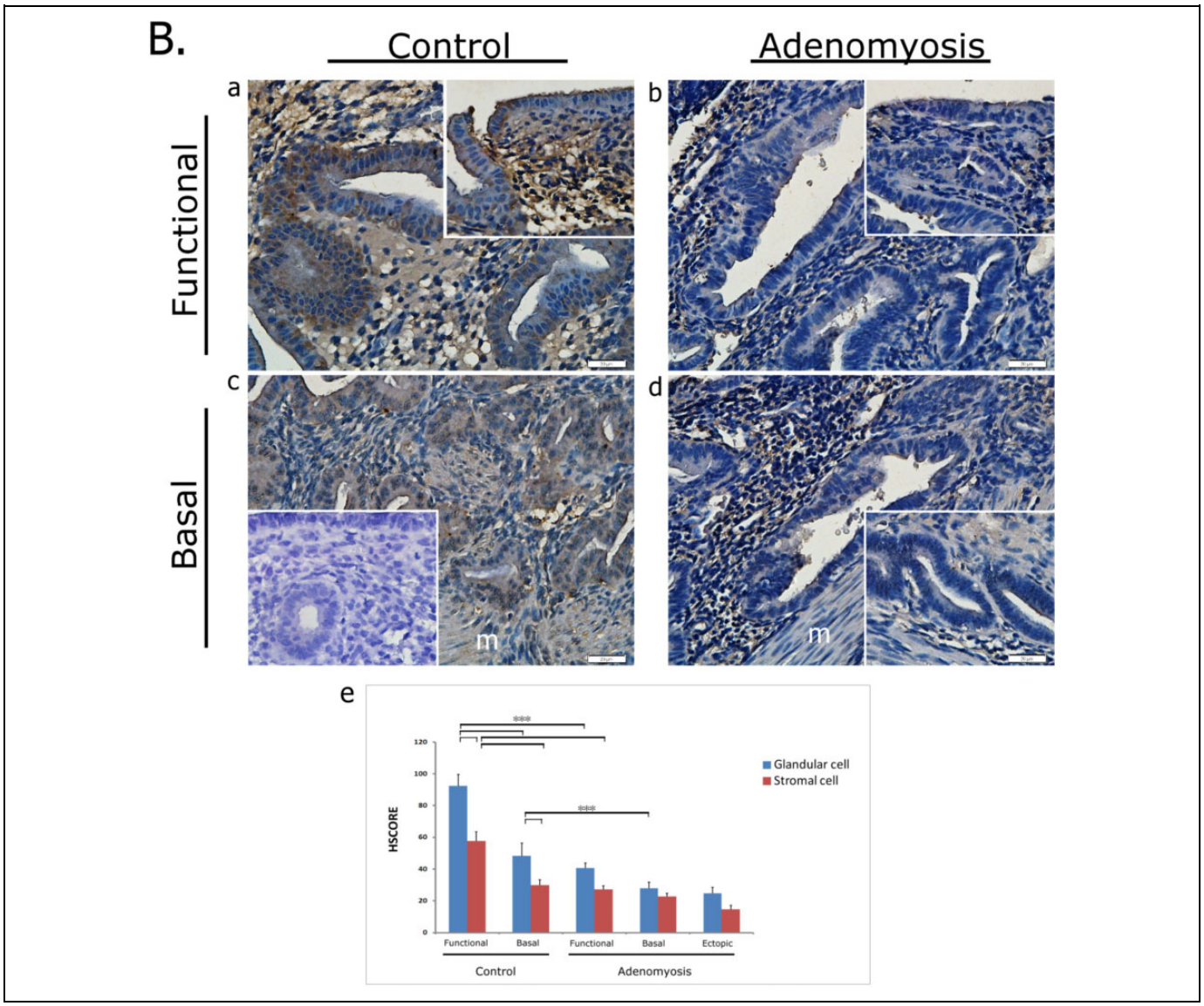

Figure 2. (continued).

implies the impaired signaling in the endometrium of patients with adenomyosis. Linking between the knowledge that LIF is a key modulator of implantation[20,21] and is reduced in infertile patients,[16,17,35] in association with the recent reports of patients with adenomyosis and declined implantation rates,[7-10] our results provide novel cellular and molecular mechanisms responsible for the adenomyosis-mediated impairment of implantation.

Because the functional layer is the major platform of implantation, the predominant LIF and LIFR expression in the functional layer of control women is physiologically significant. However, as the stem-like cells are thought to exist within the basal layer that initiates the regeneration after menstruation,[36] what we noted about the spatial difference in the LIF and LIFR expression could suggest novel mechanisms of endometrial differentiation during the regeneration or decidualization of the functional layer from the basal layer.[37] Thus, we speculate that losing spatial difference in LIF/LIFR expression in the endometrium of patients with adenomyosis could be a consequence of impaired endometrial differentiation during decidualization. Besides, the endometrial molecular characteristics of adenomyosis has been noted to be different from the normal control. Microarray analyses of endometrium have identified several differentially expressed genes between women with and without adenomyosis.[38-40] Our previous study also revealed that patients with adenomyosis expressed significantly higher levels of endometrial myostatin and follistatin, a member of the transforming growth factor beta (TGF-β) superfamily and its inhibitor, than the control.[41]

In the current series, we noted significantly increased cancer antigen (CA)-125 values in women with adenomyosis comparing to controls (Table 1), which echoes to the findings in previous studies.[42,43] CA-125 was also immunolocalized on the

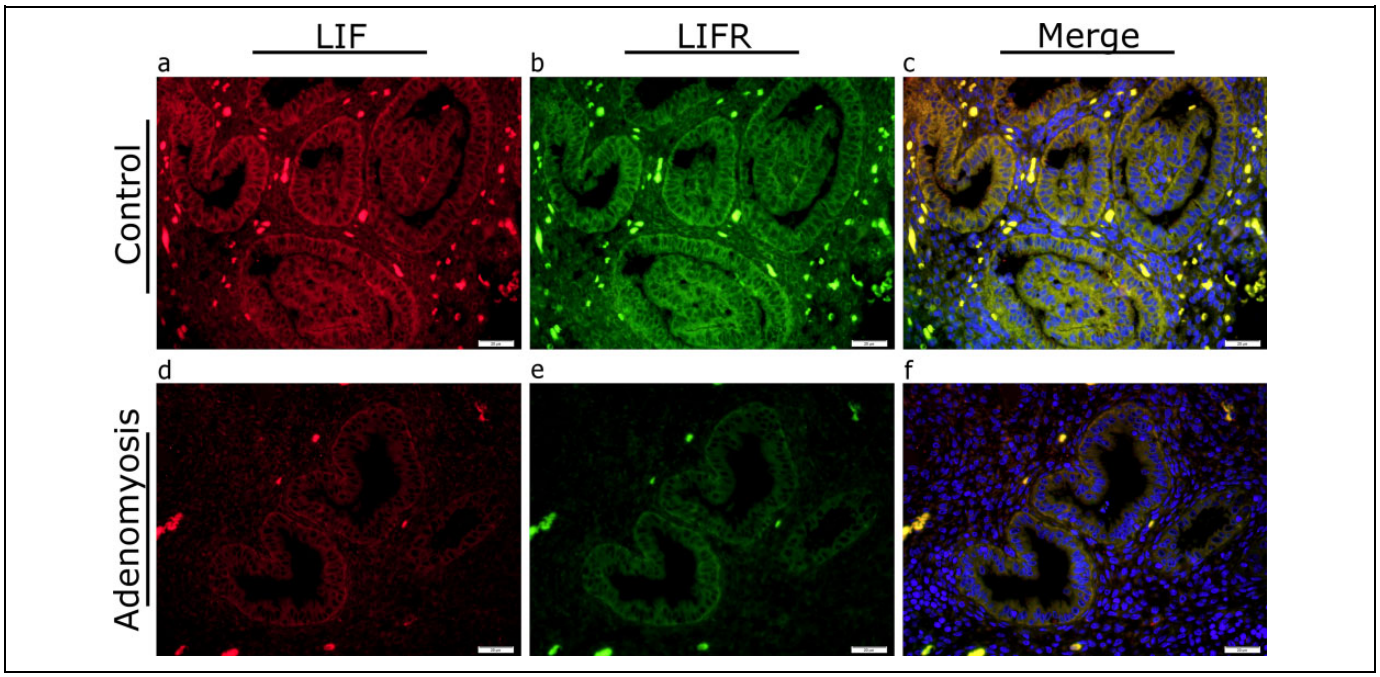

Figure 3. The colocalization of leukemia inhibitory factor (LIF) and LIF receptor (LIFR) proteins. Immunofluorescent staining showing the colocalization of LIF and LIFR in the functional layer of eutopic endometrium, in which the LIF was stained by phycoerythrin (PE)-conjugated antibody (red) and the LIFR stained with fluorescein isothiocyanate conjugate (FITC)-labeled antibody (green), respectively. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) and shown in blue (×400, scale bar = 20 μm, m, myometrium). (The color version of this figure is available online.)

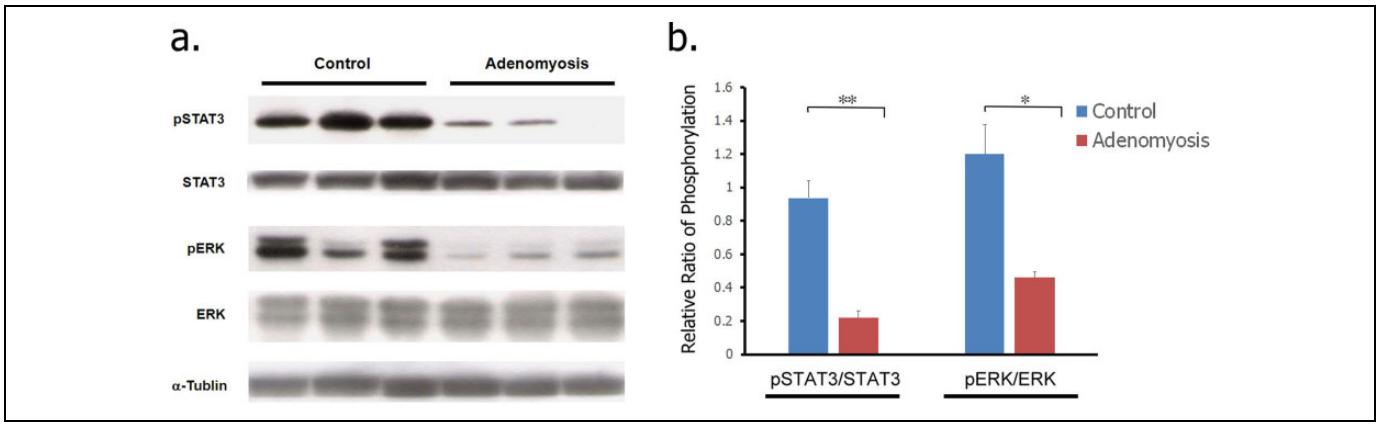

Figure 4. Phosphorylation of signal transducer and activator of transcription 3 (STAT3) and extracellular signal-regulated kinase (ERK), using the eutopic endometrial tissues in the window of implantation (WOI). A, Western blot of the phosphorylation of STAT3 and ERK. B, Densitometry represents semi-quantitative analysis of Western blot. Results are expressed as mean ± standard error of the mean (SEM; n = 3; *$P < .05$; **$P < .01$).

glandular epithelium of the adenomyotic lesions in the myometrium,[44] and ultrasound-guided microwave ablation of adenomyotic lesions could significantly decrease the serum CA-125 levels.[45] The expression of LIF and LIFR immunostaining in the adenomyotic nodule also resembles the basal layer of endometrium (Figure 2A.d and B.d). Although the pathogenesis of adenomyosis is poorly understood, the invagination of the basal endometrium into the inner layer of the myometrium, known as junctional zone, is the most widely accepted theory.[4,46,47] A mouse model of tamoxifen-induced adenomyosis suggested that highly proliferating endometrial cells could break through the inner myometrial layer of the uterus.[48,49] Moreover, the resemblance of the expression pattern of estrogen receptor beta (ER-β), progesterone receptor (PR)-A, and progesterone receptor (PR)-B between the basal layer of endometrium and adenomyotic foci[50] provides additional evidence for this invagination theory.

Compared to controls, significantly decreased LIFR expression and hence the subsequent phosphorylation of both STAT3 and ERK in eutopic endometrium were noted from patients

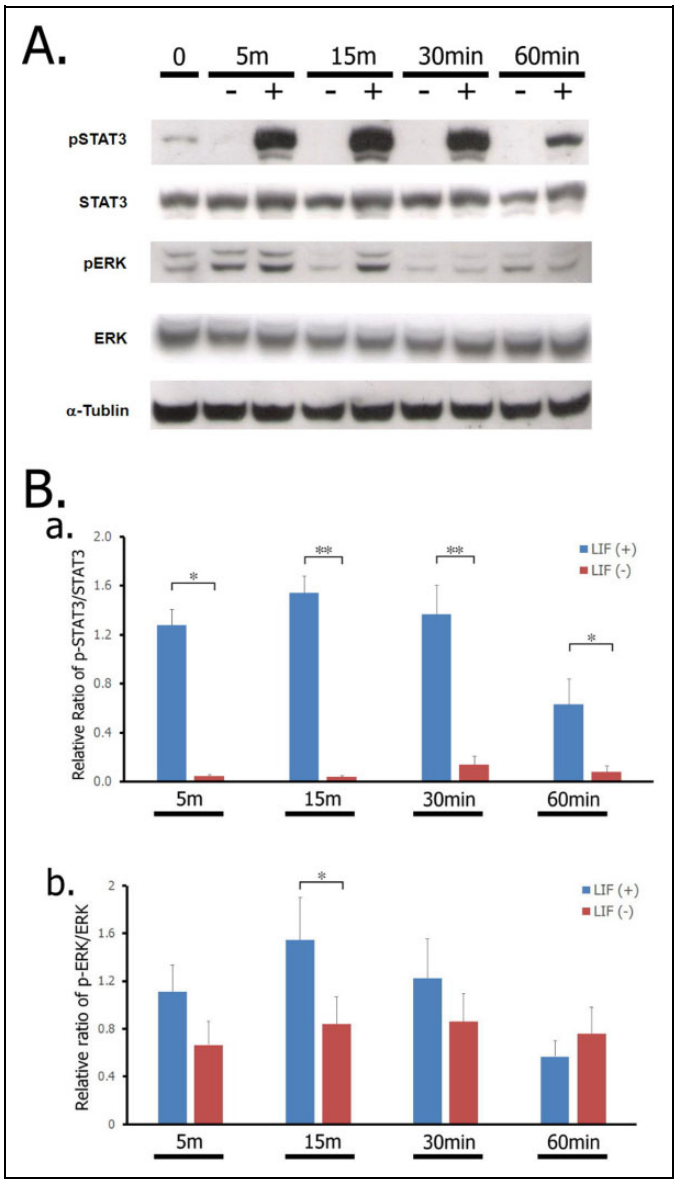

Figure 5. Phosphorylation of signal transducer and activator of transcription-3 (STAT3) and extracellular signal-regulated kinase (ERK), using the confluent endometrial stromal cells (ESCs) with in vitro treatment. Confluent, leukocyte-free ESCs were incubated in serum-free basal medium (BM) with 10^{-8} M estradiol + 10^{-7} M medroxyprogesterone acetate for 48 hours and then switched to BM with corresponding steroids supplemented with 10% charcoal-stripped calf serum (BMS) with/without recombinant human leukemia inhibitory factor (LIF, 50 ng/mL) for 0, 5, 15, 30, or 60 minutes. A, Western blot analysis of the phosphorylation of STAT3 and ERK in ESCs treated with or without LIF. B, The relative ratios of phosphorylated and total (a) STAT3 and (b) ERKs were calculated. Results are expressed as mean ± standard error of the mean (SEM; n = 5, *$P < .05$, **$P < .01$).

with adenomyosis in WOI with similar constitutive levels (Figure 4A). The LIF treatment also elicited phosphorylation of STAT3 and ERK in cultured ESCs (Figure 5A), which is compatible to the findings of previous literature.[14,22] It is quite interesting that the pSTAT3 in the nontreated ESCs were almost undetectable, even after the renourishing of the starved cells, but highly elicited by the LIF treatment. It could imply that the STAT3 pathway works more specifically under the LIF/LIFR system and is not involved much in other cellular metabolism, so that the adding of serum on the starved ESCs did not activate its phosphorylation. In contrast to pSTAT3, pERK was seen with higher basal levels after the renourishing of the starved ESCs in the nontreated group (Figure 5), suggesting that the ERK pathway may be not as specific to the LIF/LIFR system as the STAT3 pathway and should involve in other basal cellular metabolic functions, though the LIF treatment also elicited significantly more pERK. Thus, the decreased activation of signaling cascade in eutopic endometrium is linked to the decreased LIF–LIFR expression in patients with adenomyosis. In a conditional knockout mouse model, the activation of STAT3 in glandular cells was shown to be responsible for the disruption of the cell–cell contact of the endometrial luminal epithelium to enhance the implantation process.[51] Therefore, the significant reduction in STAT3 activation could fail to loosen the contacts among endometrial cells and hinder the process of embryo implantation.

Synchronous upregulation of LIF–LIFR in the mid-secretory phase indicates its potential autocrine/paracrine effects.[13,16,52] Previous study also demonstrated that the conditional deletion of STAT3 in the glandular cells impairs the proliferation and decidualization of stromal cells.[51] Therefore, LIF is proposed to act via STAT3 signaling in endometrial glandular cells to promote decidualization of stromal cells in a paracrine manner. A recent in vitro study of depleting LIF by adding the LIF antagonist found significantly decreased expression of gp130 in the endometrial tissue culture.[53] Furthermore, LIFRs were found to be expressed on trophoblasts.[54,55] Taken together, these evidence suggested that the decreased LIF expression in WOI could synchronously inhibit LIFR/gp130 expression and subsequently disrupt STAT3 signaling, and ultimately, these interactions between the endometrium and embryo would impair the process of implantation and contribute to the subfertility of patients with adenomyosis.

Based on our results, a paracrine effect is suggested to play a crucial role in regulating the stromal cell function. Since the LIF is predominantly expressed in endometrial glands, further studies using primary glandular cells will verify the role of glandular LIF–LIFR signaling in altered endometrium from patients with adenomyosis. Using cancer cell lines will not be appropriate in studying the fertility and signaling transduction.

In conclusion, the present study reveals that the reduced expression of LIF–LIFR impairs the subsequent activation of STAT3 and MAPK/ERK signaling pathways in the endometrium of patients with adenomyosis during the WOI. Significantly reduced activation of STAT3 signaling strongly implies the disrupted endometrial receptivity at the cellular (ie, abnormal trophoblast invasion) and/or molecular (aberrant intracellular signaling) levels in patients with adenomyosis during the WOI. These findings strongly suggest that the decreased

LIF–LIFR expression with subsequent inhibition of STAT3 signaling plays crucial roles in the subfertility of patients with adenomyosis and could open a new avenue for future studies aimed at developing effective infertility treatment.

Authors' Note

Hsing-Shih Wang and Umit A. Kayisli shared co-corresponding authorship. This study was presented in part at the 62nd Annual Scientific Meeting of the Society for Reproductive Investigation, San Francisco, California, March 25 to 28, 2015. This study is part of the PhD dissertation of C. F. Yen.

Acknowledgments

The authors thank Tzu-Ti Shaw and Pei-Chia Yang for their great contributions in technical assistance. The authors would also like to thank Abhishek Mangeshikar for his assistance with the review of the language of the manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported in part by the Chang Gung Memorial Hospital research grants CMRPG3B1123 (H.-S. Wang) and CMRPG3C0673 (C.-F. Yen) and the Department of Health of Taiwan grant DOH100-TD-C-111-008 (S.-K. Liao).

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