Received: 11 January 2017 | Accepted: 21 March 2017

DOI: 10.1111/aji.12690

ORIGINAL ARTICLE

WILEY AJRI American Journal of Reproductive Immunology

Hypoxia-inhibited DUSP2 expression promotes IL-6/STAT3 signaling in endometriosis

Kuei-Yang Hsiao[1] | Ning Chang[1,2] | Jia-Ling Tsai[1] | Shih-Chieh Lin[1] | Shaw-Jenq Tsai[1] | Meng-Hsing Wu[1,2]

[1]Department of Physiology, College of Medicine, National Cheng Kung University, Tainan, Taiwan

[2]Department of Obstetrics and Gynecology, College of Medicine and Hospital, National Cheng Kung University, Tainan, Taiwan

Correspondence
Meng-Hsing Wu, Department of Obstetrics and Gynecology, College of Medicine and Hospital, National Cheng Kung University, Tainan, Taiwan.
Email: mhwu68@mail.ncku.edu.tw

Funding information
The Ministry of Science and Technology, Taiwan, Grant/Award Number: 103-2314-B-006-068-MY2

Problem: How does hypoxia-mediated downregulation of dual-specificity phosphatase-2 (DUSP2) promote the development of endometriotic lesions?

Method of study: The levels of IL-6 and DUSP2 were assessed in eutopic stromal cells with DUSP2 knockdown or hypoxia treatment. Bromodeoxyuridine (BrdU) incorporation was applied for evaluating cell proliferation. The protein levels of DUSP2, cleaved caspase-3, phosphorylated STAT3, and STAT3 were analyzed using immunoblot.

Results: The genomewide analysis of cells with DUSP2 overexpression indicated IL-6 regulates multiple pathways related to inflammation, proliferation, and apoptosis. DUSP2 overexpression significantly suppressed IL-6 expression, while DUSP2 knockdown promoted IL-6 expression. The hypoxia-treated eutopic stromal cells expressed higher levels of IL-6, recapitulating the elevated levels of IL-6 in ectopic stromal cells. The treatment with IL-6 elicited the phosphorylation of STAT3, mimicking the elevated levels of phosphorylated STAT3 in the ectopic stromal cells. The IL-6-treated eutopic stromal cells showed more BrdU incorporation and less cleaved caspase-3, which can be reversed by STAT3 inhibitor.

Conclusion: Hypoxia-induced IL-6 production in endometriotic lesions is mediated via downregulation of DUSP2, which causes aberrant activation of STAT3 signaling pathway and helps the endometriotic cells survive under the ectopic environment.

KEYWORDS
endometriosis, hypoxia, dual-specificity phosphatase-2, interleukin-6, signal transducer and activator of transcription 3

1 | INTRODUCTION

Endometriosis is a common gynecological disorder characterized by the presence of endometrial tissues outside of the uterine cavity with approximate 10%-15% prevalence among women of reproductive age. Although endometriosis is not a life-threatening disease, the symptoms such as chronic pelvic pain, dysmenorrhea, and dyspareunia seriously reduce the quality of life in women with endometriosis.[1,2] In addition, high correlation with infertility also brings to light the fact that endometriosis is a noteworthy health problem in women.[3] Nevertheless, the underlying mechanism contributing to the development of endometriosis is still largely unclear.

Hypoxia, the immediate stress for retrograde tissues, is an important driving force for the development of endometriosis. In response to low oxygen, cells stabilize the hypoxia-inducible factor α subunits (HIF-α) which are dimerized with β subunit. Our previous studies found that the level of HIF-1α was aberrantly elevated in the ectopic endometriotic tissues,[4] suppressing the expression of dual-specificity phosphatase-2 (DUSP2), an important phosphatase that specifically inactivates mitogen-activated protein kinase (MAPK).[5] The downregulation of DUSP2 potentiates the expression of COX-2 in response to cytokine stimulation and induces expression of a potent angiogenic factor.[6,7]

Kuei-Yang Hsiao and Ning Chang contributed equally to this work.

© 2017 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

Endometriosis is a disease of chronic inflammation. It has been widely investigated that COX-2/prostaglandin E_2 pathway plays a crucial role in the development of endometriosis. Studies of other inflammatory cytokines remain largely unclear. Interleukin-6 (IL-6), one of the most important pleiotropic cytokines,[8] was elevated in endometriotic tissues or in peritoneal fluids from patients with endometriosis.[9-11] The elevated level of IL-6 has been linked to endometriosis-associated infertility,[12,13] highlighting the importance to investigate the impacts of IL-6-regulated molecular and cellular functions during the development of endometriosis. Of equal importance is to understand the underlying mechanisms responsible for aberrant expression of IL-6 in ectopic lesions. Although it has been reported that the expression of IL-6 is regulated by few inflammatory factors and sex hormones in endometriotic tissues or primary cultured endometriotic cells,[10,14] whether it is regulated by hypoxia or mediates the effect of hypoxia in endometriosis remains largely uncharacterized. IL-6 signaling is mediated through its receptors, IL-6 receptor (IL-6R) and gp130, and subsequently induces phosphorylation and nuclear translocation of STAT3 to nuclei. In addition, soluble form of IL-6 receptors is released from the cell membrane by either proteolysis or expressed as products of alternative splicing.[8,15] It has been reported that ectopic endometriotic tissues expressed lower level of soluble IL-6R,[9] highlighting the dynamic regulation of IL-6 signaling in the pathogenesis of endometriosis. The transcriptionally active phosphorylated STAT3 has been mostly linked to endometriotic epithelium.[16-19] However, the roles of hypoxia and IL-6 in the STAT3 activation in endometriotic stromal cells are still unclear. Herein, we investigated the molecular and cellular mechanisms of hypoxia-mediated IL-6-induced STAT3 activation in regulation of cell proliferation and anti-apoptosis in the pathogenesis of endometriosis.

2 | MATERIALS AND METHODS

2.1 | Patients and study population

Reproductive age women with or without endometriosis treated at the Department of Obstetrics and Gynecology of National Cheng Kung University Hospital (Tainan, Taiwan) were recruited in this project. This study was approved by the Institutional Review Board at National Cheng Kung University Hospital, and informed consent was obtained from each patient. During the procedure of laparoscopy or operative laparotomy, the eutopic endometrium and the pelvic ectopic endometriotic implants, or ovarian endometrioma was collected from patients with endometriosis according to the revised American Society of Reproductive Medicine classification (1997). All samples of endometriotic lesions were histologically confirmed by pathologists.

2.2 | Stromal cell isolation, primary culture, and treatment

The procedure used to isolate stromal cells from uterine endometrium and ectopic endometriotic tissues was described previously.[20] In brief, the minced tissue samples were digested with type IV collagenase (2 mg/mL) and 10 μg/mL DNAse I in phosphate-buffered saline (PBS) at 37°C 125 rpm for 60 minutes with agitation. Large undigested tissue pieces were removed by passing 380-μm mesh and further filtered through 45-μm mesh to separate larger tissue aggregates from stromal cells. The resultant cells were plated onto culture dishes, and the unattached cells (e.g., epithelial cells and lymphocytes) were removed after 30 minutes. The isolated primary stromal cells were cultured in Dulbecco's modified Eagle's medium/F-12 (DMEM/F-12) (Life Technologies, Carlsbad, CA, USA, Catalog# 12400-024) supplemented with 10% fetal bovine serum at 37°C. Cells undergoing treatments were serum-starved for 16-18 hours. Before treatment with IL-6 (Merck, Kenilworth, NJ, USA, Catalog# 407652), the cells were incubated in DMEM/F-12 supplemented with 1% FBS at 37°C for 12 hours to prevent the activation of phosphorylation responded to serum. For hypoxia treatment, stromal cells were incubated in 1% O_2 for 24 hours after serum starvation for 16-18 hours. Following the pre-treatment with or without 5 μmol/L of STAT3 inhibitor, WP1066 (Santa Cruz Biotechnology Inc., Dallas, TX, USA, Catalog# sc-203282) for 24 hours, stromal cells were treated with camptothecin (10 μmol/L, Sigma-Aldrich, St. Louis, MO, USA, Catalog# C9911) for 48 hours to induce apoptosis.

2.3 | RNA isolation, reverse transcription, and quantitative real-time PCR

Total RNA was isolated from stromal cells directly lysed in TRIsure reagent (Bioline, Taunton, MA, USA). For the tissue RNA, tissues pieces were minced and ground in liquid nitrogen before lysed with TRIsure. The procedure was performed according to the manufacturer's instructions. The concentration of RNA was determined by UV absorption at 260 nm. Total RNA (500 ng) was used for reverse transcription at 42°C for 90 minutes. The resulting complementary DNA then underwent SYBR Green-based quantitative real-time PCR (RT-qPCR) using the Applied Biosystems StepOnePlus™ Real-Time PCR System (Foster City, CA, USA). Sets of primer used for quantitative real-time PCR were listed in the Table S1.

2.4 | Gene knockdown with short hairpin RNA (shRNA) interference

Endometrial stromal cells were transduced with shRNA for gene silencing. Lentiviral-based shRNA expression constructs containing shRNA sequence against DUSP2 (shDUSP2) or LacZ (shLacZ) were purchased from the National RNAi Core Facility (Genomics Research Center, Academia Sinica, Taiwan).[21] The procedure of lentivirus package and production was performed according to the manufacturer's instructions. Briefly, lentiviruses were made by transfecting lentiviral packaging plasmids (pMD.G and pCMVΔR8.91) and plasmid carrying shRNA expression cassette to 293FT cells (Thermo Fisher R70007, Waltham, MA, USA), and the virus-containing media were collected at 48 and 72 hours post-transfection. Endometrial stromal cells were infected with lentiviruses expressing shRNA against DUSP2 or LacZ and selected in 2 μg/mL puromycin for more than 4 days.

16000897, 2017, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/aji.12690 by National Cheng Kung University, Wiley Online Library on [18/06/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

2.5 | Western blot for protein analysis

The treated stromal cells were homogenized under radioimmunoprecipitation assay lysis buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and 150 mmol/L NaCl, 50 mmol/L Tris-Cl) containing protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, and 1 μg/mL pepstatin A) and phosphatase inhibitors (1 mmol/L NaF and 1 mmol/L Na_3VO_4). Protein samples were separated by running on a SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane (Millipore Corp., Bedford, MA, USA). After blocking with 5% non-fat milk in PBST (PBS with 0.05% Tween-20) at room temperature for 1 hour, the membrane was then incubated with primary antibody prepared in PBST at 4°C overnight. Specific primary antibodies were used according to the following dilutions: DUSP2 (1:200; Santa Cruz Biotechnology Inc., Catalog# sc-32776), STAT3 phosphotyrosine 705 (1:1000; Cell Signaling Technology Inc., Danvers, MA, USA, Catalog# 4113), STAT3 phosphoserine 727 (1:500; Santa Cruz Biotechnology Inc., Catalog# sc-8001-R), STAT3 (1:1000; Santa Cruz Biotechnology Inc., Catalog# sc-483), caspase-3 (1:1000; Cell Signaling Technology Inc., Catalog# 9662), β-actin (1:10 000; Sigma-Aldrich, Catalog# A5441). Signals were developed using an enhanced chemiluminescence detection kit (PerkinElmer , Waltham, MA, USA).

2.6 | Enzyme-linked immunosorbent assay (ELISA)

Levels of IL-6 in conditioned media collected from treated stromal cells were assessed using Interleukin-6 EIA Kit (Cayman Chemical, Ann Arbor, MI, USA, 501030) according to the manufacturer's instructions.

2.7 | Bromodeoxyuridine (BrdU) incorporation assay

Proliferation of cells was determined by BrdU incorporation assay as previously described with some modifications.[22] Briefly, 2 hours prior to harvest the cells at the desired time, 10 μmol/L BrdU (Sigma-Aldrich, Catalog# B9285) was added to cultured media, followed by fixation in 4% paraformaldehyde/PBS for 12-15 minutes at room temperature. After permeabilization with 0.2% Triton X-100/PBS for 15 minutes, cells were treated with 2N HCl for 20 minutes at room temperature to denature DNA, followed by incubations with anti-BrdU antibody (1:200 dilution; GE Healthcare, Catalog# RPN20AB) at 4°C overnight, and with fluorescent-conjugated secondary antibody (1:200 dilution; Alexa Fluor 488 goat anti-mouse; Invitrogen, Carlsbad, CA, USA, Catalog# A11001) at room temperature for 1 hour. Finally, signals were visualized using Nikon ECLIPSE *Ti* inverted fluorescence microscope integrated with NIS-Elements BR 3.0, and analyzed by ImageJ software (NIH).

2.8 | Statistical analysis

The results were expressed as mean±SEM. Data were analyzed by paired Student's *t* test and considered significant differences when yielding $P<.05$. All analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc. La Jolla, CA, USA).

3 | RESULTS

3.1 | DUSP2 negatively regulates IL-6 expression

Genomewide gene expression profiling in HeLa cells with DUSP2 overexpression from the previously published dataset (Gene Expression Omnibus: GSE66656) revealed that IL-6 was involved in multiple pathways related to cell proliferation, inflammation, and apoptosis (Figure 1A).[21] To confirm the suppressive effect of DUSP2 on the IL-6 expression in endometrial stromal cells, we first overexpressed DUSP2 in the human endometrial stromal cells and found that IL-6 expression was inhibited 24 hours post-transfection (Figure 1B). In contrast, knockdown of DUSP2 in eutopic stromal cells using shRNA significantly induced expression of IL-6 in both mRNA and protein levels (Figure 1C, D), suggesting that DUSP2 may negatively regulate the level of IL-6.

3.2 | Aberrant expression of IL-6 is mediated by hypoxia-mediated loss of function of DUSP2

Our previous studies reported that expression of DUSP2 was suppressed by hypoxia.[6,20] To determine whether the expression of IL-6 is affected by hypoxia-mediated DUSP2 expression, eutopic endometrial stromal cells were treated with hypoxia for 24 hours. The results demonstrated that hypoxia significantly suppressed the level of DUSP2 (Figure 2A, B) and induced the expression of IL-6 (Figure 2C), recapitulating the higher levels of IL-6 in ectopic tissues and isolated ectopic stromal cells (Figure 2D, E).

3.3 | Hyperactivation of STAT3 is stimulated by IL-6 in endometriotic stromal cells

It has been shown that STAT3 is the downstream pathway of IL-6.[23] We next evaluated whether STAT3 is differentially activated in endometrial and endometriotic stromal cells collected from our clinical patients. Immunoblot results demonstrated that the level of phosphorylated tyrosine 705 (pY705) of STAT3 was significantly elevated in ectopic endometriotic stromal cells compared to that of the eutopic stromal cells collected from the same individuals (Figure 3A, B). However, there was no significant difference for the level of phosphorylated serine 727 (pS727) of STAT3 (Figure 3A, B). To elucidate whether the differential activation of STAT6 is mediated by IL-6, the eutopic stromal cells were treated with IL-6. The results showed that treatment of eutopic stromal cells with IL-6 rapidly induced the levels of pY705, but not pS727 of STAT3 (Figure 3C, D), which is consistent with the aberrant activation of STAT3 observed in ectopic stromal cells (Figure 3A, B).

3.4 | IL-6 promotes endometrial cell proliferation

It has been known that STAT3 involves several critical biological functions including cell growth and cell survival.[24,25] To investigate the role of IL-6-activated STAT3 cell proliferation during the development of

16000897, 2017, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/aji.12690 by National Cheng Kung University, Wiley Online Library on [18/06/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

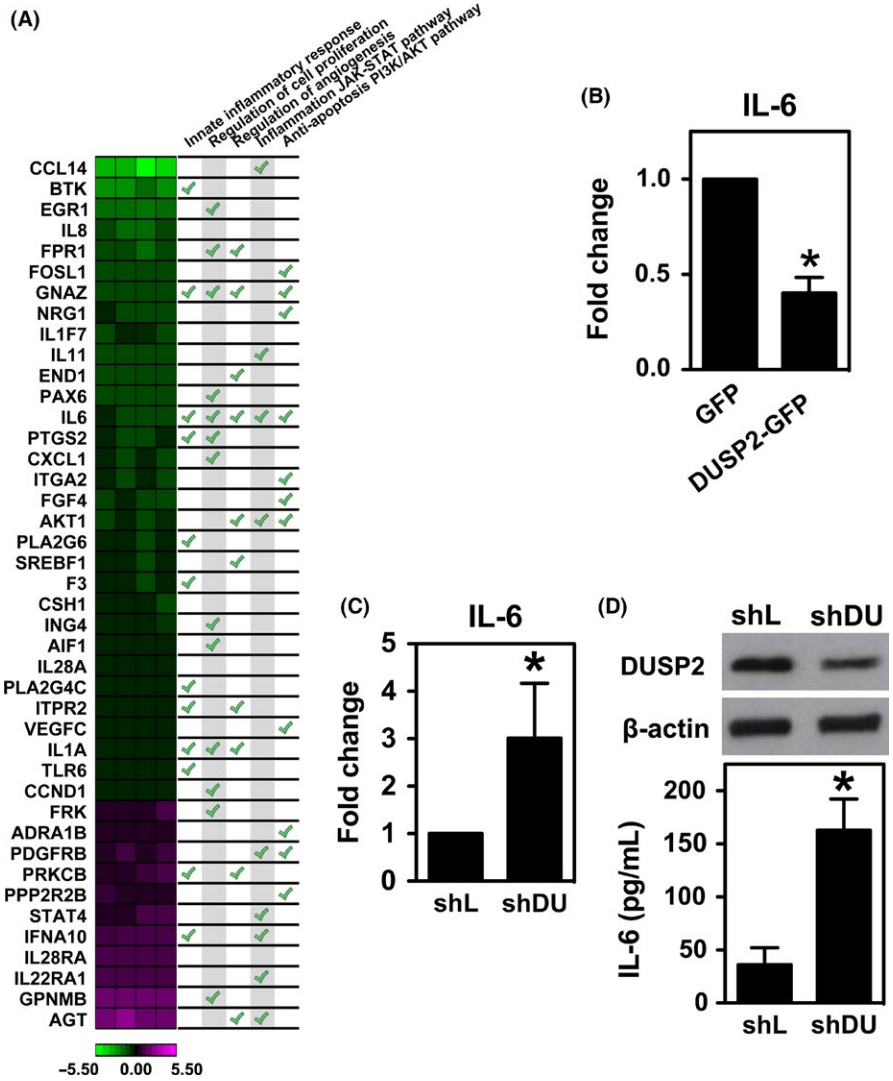

FIGURE 1 DUSP2 negatively regulates IL-6 expression in endometrial and endometriotic stromal cells. A, Expression profiling of genes involved in pathways related to inflammation, cell proliferation, angiogenesis, and apoptosis in DUSP2-overexpressed cells. The ratio of intensities from two colored probes was normalized and color-scaled. Genes are noted in the right table according to their functions. B, Results of RT-qPCR for IL-6 mRNA level in ectopic endometrial stromal cells with DUSP2 overexpression (DUSP2-GFP) and control (GFP) (n=3). C, D, IL-6 mRNA (C) and protein (D) levels in shDUSP2- (shDU) and shLacZ-expressing (shL) eutopic endometrial stromal cells (n=4). *P<.05

endometriosis, the eutopic stromal cells treated with or without IL-6 were incubated with BrdU to assess the DNA synthesis. The results showed that IL-6-treated eutopic stromal cells were labeled more by BrdU, indicating the IL-6 treatment promoted cell proliferation in eutopic endometrial stromal cells (Figure 4A, B).

3.5 | Hypoxia-induced IL-6 protects endometrial stromal cells from apoptosis through activation of STAT3

It has been reported that STAT3 activation plays an important role in IL-6-induced anti-apoptosis in cancer and embryo development.[26,27] Next, we investigated whether IL-6-induced anti-apoptosis was involved in promoting the capacity against genotoxic stress in stromal cells. Treatment with camptothecin (CPT), a well-known compound that inhibits DNA-topoisomerase I complex and induces cell death,[28,29] activated the cascade of caspase-3 cleavage (Figure 5A), while pre-treatment with IL-6 attenuated the CPT-induced caspase-3 cleavage (Figure 5A, B). Furthermore, to demonstrate the role of hypoxia-induced IL-6 in anti-apoptosis, eutopic endometrial stromal cells were pre-treated with or without hypoxia for 24 hours, followed by CPT treatment for another 48 hours to induce cell apoptosis in the absence or presence of STAT3 inhibitor. The results showed that hypoxia prevented stromal cells from CPT-induced caspase-3 cleavage, and this suppressive effect of hypoxia on the level of caspase-3 cleavage was reversed by STAT3 inhibitor (Figure 5C, D), suggesting that hypoxia protects endometrial stromal cells from apoptosis through activation of IL-6/STAT3 pathway.

4 | DISCUSSION

Dysregulation of DUSP2 has been implicated in the establishment and development of endometriosis. Previously, we have reported that hypoxia-inhibited DUSP2 expression causes elevated COX-2 and IL-8 expression, promoting the inflammation and angiogenesis.[6,20] In this report, we showed that loss of DUSP2 leads to hyperactivation of STAT3 through overproduction of IL-6 and ultimately stimulates stromal cell proliferation and anti-apoptosis activity (Figure 5E). Our results demonstrated additional pathological process regulated by hypoxia-mediated DUSP2 downregulation.

16000897, 2017, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/aji.12690 by National Cheng Kung University, Wiley Online Library on [18/06/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

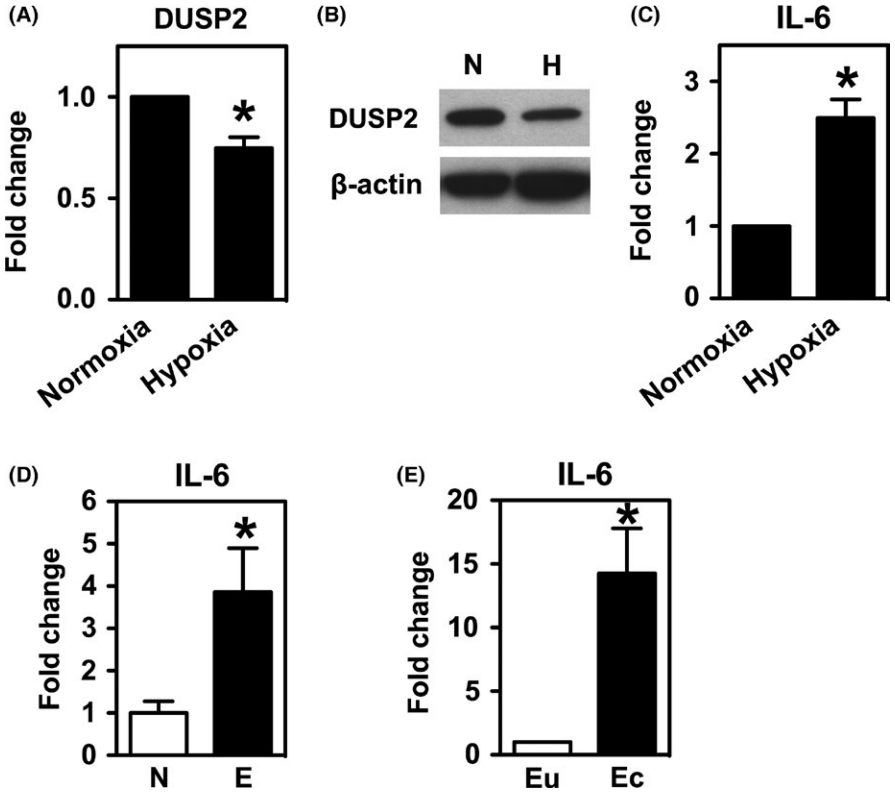

FIGURE 2 Hypoxia-mediated DUSP2 downregulation recapitulates elevated IL-6 expression in ectopic stromal cells. A, Results of RT-qPCR for levels of IL-6 mRNA in eutopic endometrial stromal cells treated with normoxia or hypoxia for 24 h (n=8). B, The representative images of immunoblot show DUSP2 level in eutopic endometrial stromal cells treated with normoxia (N) or hypoxia (H) for 24 h. C, Levels of DUSP2 mRNA in eutopic endometrial stromal cells treated with normoxia or hypoxia for 24 h (n=8). D, Levels of IL-6 mRNA in tissues collected from normal endometrium (N, n=26) and ectopic endometriotic lesions (E, n=42). E, Levels of IL-6 mRNA in paired eutopic endometrial (Eu) and ectopic endometriotic (Ec) stromal cells from the same patients with endometriosis (n=14). *P<.05

The elevated IL-6 has been implicated in the pathogenesis and diagnosis of endometriosis. The levels of IL-6 protein are elevated in the peritoneal fluid from women with endometriosis, ectopic endometriotic tissues, and conditioned medium from endometriotic cells,[14,30,31] making it a potential biomarker for the diagnosis of endometriosis.[32,33] In addition, elevated IL-6 has been linked to suppressed immune surveillance that allows the initial establishment and sustenance of endometriotic lesions, thus making it a potent therapeutic target.[34] The expression of IL-6 was regulated by many microenvironmental factors. Pioneer studies demonstrated that the expression of IL-6 in the isolated primary endometriotic epithelial and stromal cells is induced by hypoxia and various cytokines such as IL-1β, TNF-α, and IFN-γ,[14,35] suggesting that microenvironmental hypoxia and inflammatory stimulation are important cues for the aberrant expression of IL-6 in endometriotic tissues. In this report, we demonstrated that human IL-6 gene without known hypoxia-responsive element in the promoter region was induced through hypoxia-modulated DUSP2 downregulation. Nevertheless, Tsudo et al. reported that treatment with TNF-α induces more IL-6 expression in ectopic stromal cells than that in eutopic stromal cells, indicating that the expression of IL-6 in eutopic and ectopic stromal cells differentially responds to cytokine stimulation.[10] Intriguingly, this differential response in eutopic and ectopic stromal cells was also observed in the regulation of COX-2 in responses to cytokines through hypoxia-mediated DUSP2 downregulation,[6] implying that hypoxia-mediated DUSP2 downregulation may play a central role in the regulation of responses to inflammatory stimulation.

STAT3 pathway is aberrantly activated during the pathogenesis of endometriosis.[36] One report showed that EGF is involved in the STAT3 activation in the immortalized human endometrial epithelial cells,[16] while the other one showed that leptin induced STAT3 activation in the endometriotic epithelial cells.[17] In a similar manner, two more recent studies showed that STAT3 activation is mediated through IL-6 stimulation and potentiated by cytokine-mediated suppression on PIAS1, an inhibitor of STAT3 signaling.[18,19] These studies highlight the role of cytokines in coordination of hypoxia-regulated pathway in the endometriotic epithelial cells. In this current study, we reported that stromal cells, the majority of endometriotic lesions, expressed higher level of IL-6, and this elevated level of IL-6 was induced by hypoxia-mediated DUSP2 downregulation, suggesting that IL-6 and hypoxia form a positive feedback loop to coordinately promote the pathogenesis of endometriotic lesions.

Hypoxia-mediated DUSP2 downregulation results in prolonged ERK and p38 MAPK phosphorylation, promoting inflammation and angiogenesis during the development of endometriosis[6,7,20] (Figure 5E). As endometriosis is an estrogen-dependent disease, it has been reported that estrogen-mediated proliferation, anti-apoptosis, and angiogenesis are pivotal to the establishment and sustenance of endometriotic lesions.[37-39] Similar to the multiple functions of estrogen, in this study, we identified that hypoxia-inhibited DUSP2 expression plays a dual role through IL-6 induction in the regulation of proliferation and anti-apoptosis, both of which are particularly critical for the retrograded tissues to survive in the peritoneal cavity. IL-6 exerts its biological effects through classic and/or trans-signaling pathways. Both membrane-bound and soluble form of gp130 were expressed in the endometrium and regulated by steroid hormone, implying its functional role in endometriosis.[40] The signaling pathways mediated by

16000897, 2017, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/aji.12690 by National Cheng Kung University, Wiley Online Library on [18/06/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

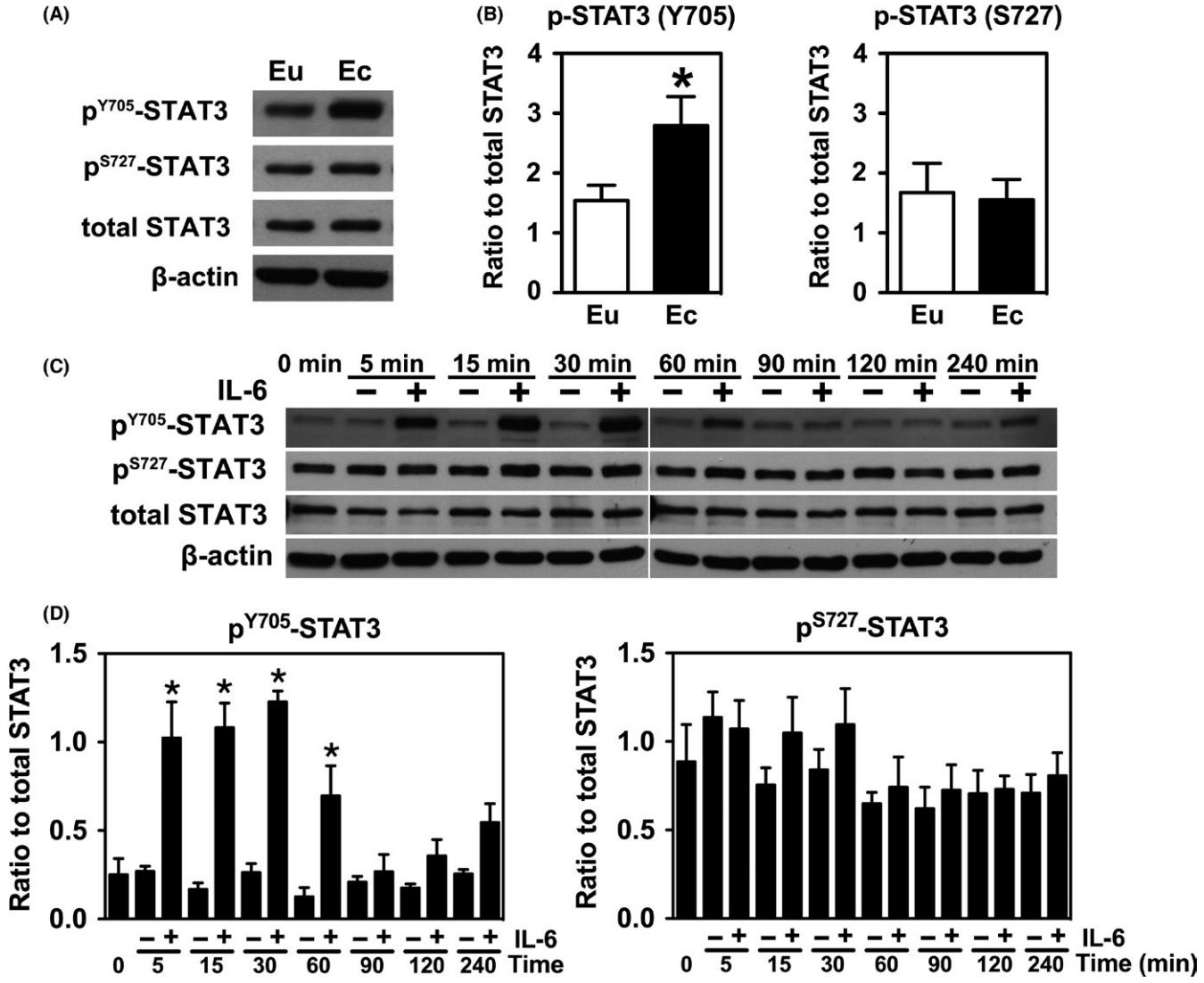

FIGURE 3 IL-6 activates STAT3 in eutopic endometrial stromal cells. The representative images (A) and the quantification results (B) of immunoblot of phosphorylated STAT3 at tyrosine 705 (pY705-STAT3) and serine 727 (pS727-STAT3) in paired eutopic endometrial (Eu) and ectopic endometriotic stromal cells (Ec) from the same patients with endometriosis. The quantification results were analyzed with the ratio to total STAT3 (n=11). C, Representative images of immunoblot of phosphorylated STAT3 at tyrosine 705 (pY705-STAT3) and serine 727 (pS727-STAT3) in eutopic endometrial stromal cells treated with 10 ng/mL IL-6 in a time-course experiment. D, Quantification results analyze the signal intensity of phosphorylated STAT3 at tyrosine 705 (pY705-STAT3) (left panel) and serine 727 (pS727-STAT3) (right panel) normalized to total STAT3 (n=4). *P<.05

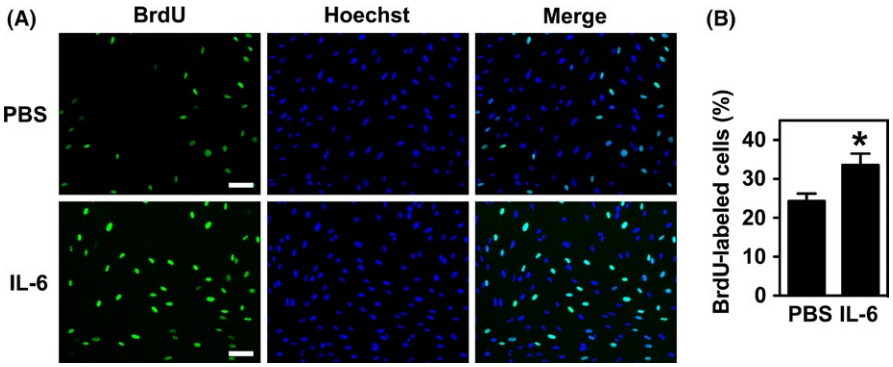

FIGURE 4 IL-6 promotes cell proliferation in endometrial stromal cells. A, Representative micrographs show BrdU-incorporated eutopic endometrial stromal cells treated with vehicle (PBS) or IL-6 (10 ng/mL) for 24 h. Scale bar indicates 100 μm. B, Quantitative results show the percentage of BrdU-labeled cells in IL-6 treatment group and control group (PBS) (n=4). *P<.05

16000897, 2017, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/aji.12690 by National Cheng Kung University, Wiley Online Library on [18/06/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

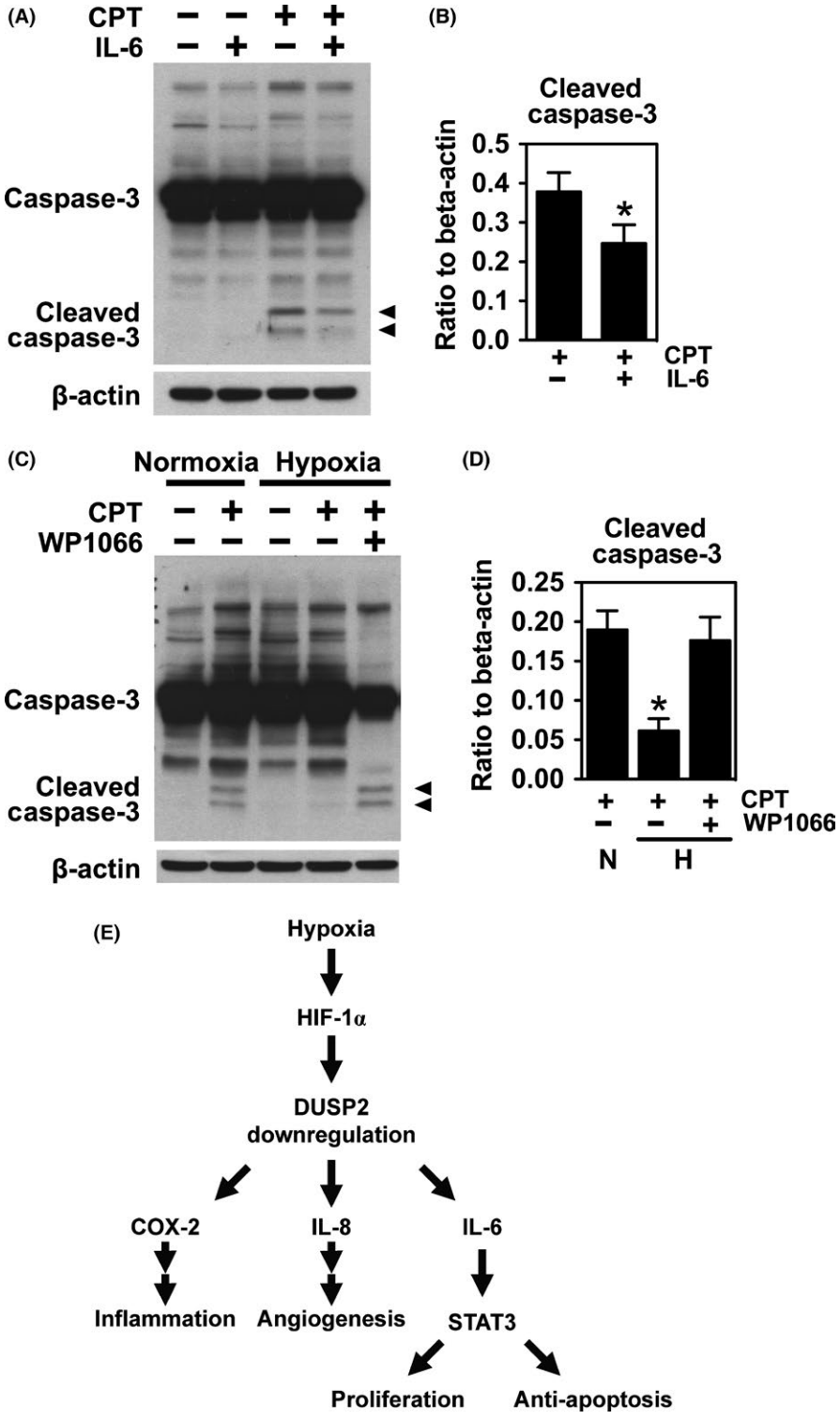

FIGURE 5 Hypoxia-induced IL-6 protects endometrial stromal cells from apoptosis through STAT3 activation. (A, B) Representative images (A) and quantitative results (B) of immunoblot analysis of caspase-3 in eutopic endometrial stromal cells treated with or without IL-6 (10 ng/mL) for 12 h and treated with or without 10 μmol/L camptothecin (CPT) for another 48 h (n=4). (C, D) Representative images (C) and quantitative results (D) of immunoblot analysis of caspase-3 in eutopic endometrial stromal cells treated with normoxia or hypoxia or WP1066 under hypoxia for 24 h and treated with or without 10 μmol/L camptothecin (CPT) for another 48 hours (n=4). *P<.05. (E) Working model

gp130 involve the activation of STAT3. In adherence to the functions of IL-6, hyperactivation of STAT3 is associated with proliferation, anti-apoptosis, and cellular transformation in several types of cancer.[41-43] In this report, we consolidated that IL-6/STAT3 signaling not only promotes proliferation of stromal cells but also prevents endometrial stromal cells from stress-induced apoptosis.

Taken together, hypoxia-mediated DUSP2 downregulation plays a crucial role in induction of IL-6 expression, which further promotes the cell proliferation and cell survival of ectopic endometriotic tissues during the early development of endometriosis.

ACKNOWLEDGMENTS

This work was supported by research grant (103-2314-B-006-068-MY2) to M.H. Wu from the Ministry of Science and Technology, Taiwan.

16000897, 2017, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/aji.12690 by National Cheng Kung University, Wiley Online Library on [18/06/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

DISCLOSURE STATEMENT

The authors have nothing to disclose.

REFERENCES

1. Nnoaham KE, Hummelshoj L, Webster P, et al. Impact of endometriosis on quality of life and work productivity: a multicenter study across ten countries. *Fertil Steril*. 2011;96:366-373, e368.
2. Giudice LC, Kao LC. Endometriosis. *Lancet*. 2004;364:1789-1799.
3. Young K, Fisher J, Kirkman M. Endometriosis and fertility: women's accounts of healthcare. *Hum Reprod*. 2016;31:554-562.
4. Wu MH, Chen KF, Lin SC, Lgu CW, Tsai SJ. Aberrant expression of leptin in human endometriotic stromal cells is induced by elevated levels of hypoxia inducible factor-1alpha. *Am J Pathol*. 2007;170:590-598.
5. Lin SC, Chien CW, Lee JC, et al. Suppression of dual-specificity phosphatase-2 by hypoxia increases chemoresistance and malignancy in human cancer cells. *J Clin Invest*. 2011;121:1905-1916.
6. Wu MH, Lin SC, Hsiao KY, Tsai SJ. Hypoxia-inhibited dual-specificity phosphatase-2 expression in endometriotic cells regulates cyclooxygenase-2 expression. *J Pathol*. 2011;225:390-400.
7. Lin SC, Wang CC, Wu MH, Yang SH, Li YH, Tsai SJ. Hypoxia-induced microRNA-20a expression increases ERK phosphorylation and angiogenic gene expression in endometriotic stromal cells. *J Clin Endocrinol Metab*. 2012;97:E1515-E1523.
8. Hunter CA, Jones SA. IL-6 as a keystone cytokine in health and disease. *Nat Immunol*. 2015;16:448-457.
9. Rier SE, Zarmakoupis PN, Hu X, Becker JL. Dysregulation of interleukin-6 responses in ectopic endometrial stromal cells: correlation with decreased soluble receptor levels in peritoneal fluid of women with endometriosis. *J Clin Endocrinol Metab*. 1995;80:1431-1437.
10. Tsudo T, Harada T, Iwabe T, et al. Altered gene expression and secretion of interleukin-6 in stromal cells derived from endometriotic tissues. *Fertil Steril*. 2000;73:205-211.
11. Wickiewicz D, Chrobak A, Gmyrek GB, et al. Diagnostic accuracy of interleukin-6 levels in peritoneal fluid for detection of endometriosis. *Arch Gynecol Obstet*. 2013;288:805-814.
12. Deura I, Harada T, Taniguchi F, Iwabe T, Izawa M, Terakawa N. Reduction of estrogen production by interleukin-6 in a human granulosa tumor cell line may have implications for endometriosis-associated infertility. *Fertil Steril*. 2005;83(Suppl 1):1086-1092.
13. Yoshida S, Harada T, Iwabe T, et al. A combination of interleukin-6 and its soluble receptor impairs sperm motility: implications in infertility associated with endometriosis. *Hum Reprod*. 2004;19:1821-1825.
14. Akoum A, Lemay A, Paradis I, Rheault N, Maheux R. Secretion of interleukin-6 by human endometriotic cells and regulation by proinflammatory cytokines and sex steroids. *Hum Reprod*. 1996;11:2269-2275.
15. Wolf J, Rose-John S, Garbers C. Interleukin-6 and its receptors: a highly regulated and dynamic system. *Cytokine*. 2014;70:11-20.
16. Klein R, Stiller S, Gashaw I. Epidermal growth factor upregulates endometrial CYR61 expression via activation of the JAK2/STAT3 pathway. *Reprod Fertil Dev*. 2012;24:482-489.
17. Oh HK, Choi YS, Yang YI, Kim JH, Leung PC, Choi JH. Leptin receptor is induced in endometriosis and leptin stimulates the growth of endometriotic epithelial cells through the JAK2/STAT3 and ERK pathways. *Mol Hum Reprod*. 2013;19:160-168.
18. Yoo JY, Jeong JW, Fazleabas AT, Tayade C, Young SL, Lessey BA. Protein inhibitor of activated STAT3 (PIAS3) is down-regulated in eutopic endometrium of women with endometriosis. *Biol Reprod*. 2016;95:11.
19. Kim BG, Yoo JY, Kim TH, et al. Aberrant activation of signal transducer and activator of transcription-3 (STAT3) signaling in endometriosis. *Hum Reprod*. 2015;30:1069-1078.
20. Hsiao KY, Chang N, Lin SC, Li YH, Wu MH. Inhibition of dual specificity phosphatase-2 by hypoxia promotes interleukin-8-mediated angiogenesis in endometriosis. *Hum Reprod*. 2014;29:2747-2755.
21. Lin SC, Hsiao KY, Chang N, Hou PC, Tsai SJ. Loss of dual specificity phosphatase-2 promotes angiogenesis and metastasis via upregulation of interleukin-8 in colon cancer. *J Pathol*. 2017;241:638-648.
22. Chen HM, Lin YH, Cheng YM, Wing LY, Tsai SJ. Overexpression of integrin-beta1 in leiomyoma promotes cell spreading and proliferation. *J Clin Endocrinol Metab*. 2013;98:E837-E846.
23. Braun DA, Fribourg M, Sealfon SC. Cytokine response is determined by duration of receptor and signal transducers and activators of transcription 3 (STAT3) activation. *J Biol Chem*. 2013;288:2986-2993.
24. Kisseleva T, Bhattacharya S, Braunstein J, Schindler CW. Signaling through the JAK/STAT pathway, recent advances and future challenges. *Gene*. 2002;285:1-24.
25. O'Shea JJ, Gadina M, Schreiber RD. Cytokine signaling in 2002: new surprises in the Jak/Stat pathway. *Cell*. 2002;109(Suppl):S121-S131.
26. Liu Y, Li PK, Li C, Lin J. Inhibition of STAT3 signaling blocks the anti-apoptotic activity of IL-6 in human liver cancer cells. *J Biol Chem*. 2010;285:27429-27439.
27. Shen XH, Han YJ, Zhang DX, Cui XS, Kim NH. A link between the interleukin-6/Stat3 anti-apoptotic pathway and microRNA-21 in preimplantation mouse embryos. *Mol Reprod Dev*. 2009;76:854-862.
28. Beidler DR, Cheng YC. Camptothecin induction of a time- and concentration-dependent decrease of topoisomerase I and its implication in camptothecin activity. *Mol Pharmacol*. 1995;47:907-914.
29. Liu LF, Desai SD, Li TK, Mao Y, Sun M, Sim SP. Mechanism of action of camptothecin. *Ann N Y Acad Sci*. 2000;922:1-10.
30. Harada T, Yoshioka H, Yoshida S, et al. Increased interleukin-6 levels in peritoneal fluid of infertile patients with active endometriosis. *Am J Obstet Gynecol*. 1997;176:593-597.
31. Bergqvist A, Bruse C, Carlberg M, Carlstrom K. Interleukin 1beta, interleukin-6, and tumor necrosis factor-alpha in endometriotic tissue and in endometrium. *Fertil Steril*. 2001;75:489-495.
32. Bedaiwy MA, Falcone T, Sharma RK, et al. Prediction of endometriosis with serum and peritoneal fluid markers: a prospective controlled trial. *Hum Reprod*. 2002;17:426-431.
33. Mihalyi A, Gevaert O, Kyama CM, et al. Non-invasive diagnosis of endometriosis based on a combined analysis of six plasma biomarkers. *Hum Reprod*. 2010;25:654-664.
34. Taskin MI, Gungor AC, Adali E, Yay A, Onder GO, Inceboz U. A humanized anti-interleukin 6 receptor monoclonal antibody, tocilizumab, for the treatment of endometriosis in a rat model. *Reprod Sci*. 2016;23:662-669.
35. von Wolff M, Stieger S, Lumpp K, Bucking J, Strowitzki T, Thaler CJ. Endometrial interleukin-6 in vitro is not regulated directly by female steroid hormones, but by pro-inflammatory cytokines and hypoxia. *Mol Hum Reprod*. 2002;8:1096-1102.
36. Okamoto M, Nasu K, Abe W, et al. Enhanced miR-210 expression promotes the pathogenesis of endometriosis through activation of signal transducer and activator of transcription 3. *Hum Reprod*. 2015;30:632-641.
37. Han SJ, Jung SY, Wu SP, et al. Estrogen receptor beta modulates apoptosis complexes and the inflammasome to drive the pathogenesis of endometriosis. *Cell*. 2015;163:960-974.
38. Wing LY, Chuang PC, Wu MH, Chen HM, Tsai SJ. Expression and mitogenic effect of fibroblast growth factor-9 in human endometriotic implant is regulated by aberrant production of estrogen. *J Clin Endocrinol Metab*. 2003;88:5547-5554.
39. Zhao Y, Gong P, Chen Y, et al. Dual suppression of estrogenic and inflammatory activities for targeting of endometriosis. *Sci Transl Med*. 2015;7:271ra9.

16000897, 2017, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/aji.12690 by National Cheng Kung University, Wiley Online Library on [18/06/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

40. Classen-Linke I, Muller-Newen G, Heinrich PC, Beier HM, von Rango U. The cytokine receptor gp130 and its soluble form are under hormonal control in human endometrium and decidua. *Mol Hum Reprod*. 2004;10:495-504.
41. Bromberg JF, Wrzeszczynska MH, Devgan G, et al. Stat3 as an oncogene. *Cell*. 1999;98:295-303.
42. Niu G, Bowman T, Huang M, et al. Roles of activated Src and Stat3 signaling in melanoma tumor cell growth. *Oncogene*. 2002;21:7001-7010.
43. Kong LY, Abou-Ghazal MK, Wei J, et al. A novel inhibitor of signal transducers and activators of transcription 3 activation is efficacious against established central nervous system melanoma and inhibits regulatory T cells. *Clin Cancer Res*. 2008;14:5759-5768.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Hsiao K-Y, Chang N, Tsai J-L, Lin S-C, Tsai S-J, Wu M-H. Hypoxia-inhibited DUSP2 expression promotes IL-6/STAT3 signaling in endometriosis. *Am J Reprod Immunol*. 2017;78:e12690. https://doi.org/10.1111/aji.12690

16000897, 2017, 4, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/aji.12690 by National Cheng Kung University, Wiley Online Library on [18/06/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License