

**Cofilin and Slingshot Localization in the Epithelium of Uterine Endometrium Changes During the Menstrual Cycle and in Endometriosis**

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## ABSTRACT

Regulation of the actin cytoskeleton is essential for epithelial cell polarity and protein trafficking within human uterine epithelium. The actin-binding protein cofilin is involved in regulation of actin dynamics by promoting actin branching and cytoskeleton reorganization. Dual immunohistochemical staining of cofilin and G-actin (represented by DNase I staining) revealed cofilin-G-actin colocalization in the apical side of luminal epithelial cells of human uterine endometrium during the proliferative phase of the menstrual cycle. Interestingly, during the secretory phase of the menstrual cycle cofilin was only present on the basolateral side. To determine whether the disease endometriosis causes a different pattern of actin remodeling, we investigated an established baboon model of induced endometriosis. The cofilin pattern in the secretory phase of baboons with endometriosis was similar to the proliferative phase in normal animals; cofilin was observed in the apical parts of luminal and glandular epithelium. A phosphatase regulating the activity of cofilin, slingshot (SSH1), revealed a similar staining pattern within these tissues. These patterns were confirmed through quantitative image analysis. Quantification of mRNA detected upregulated *SSH1* and suggested a progesterone resistance related pattern of nuclear steroid hormone receptors, but no change in membrane progesterone receptors (*mPR alpha* or *mPR beta*) was observed in endometriosis. Our data indicate that the severe dyssynchrony during menstrual cycle phases in endometriosis is connected with improper cytoskeleton rearrangements. We suggest that cofilin-mediated actin reorganization in uterine epithelial cells might be important in preparation for blastocyst implantation; dysregulation of this reorganization may lead to decreased fertility in endometriosis.

Key words: cofilin, slingshot, cytoskeleton, actin dynamics, endometriosis

## INTRODUCTION

The uterine endometrium, which lines the inner cavity of the uterus, consists of polarized luminal epithelial cells with their apical poles orientated toward the uterine cavity. This luminal epithelium contains both ciliated and microvillated columnar secretory cells <sup>1</sup>. The basal pole of both luminal and glandular epithelial cells interacts with the stroma.

Cell polarity is critical for cell-cell interactions, cell-substrate interactions, and protein trafficking <sup>2</sup>. Actin cytoskeleton remodeling plays a key role in establishment of cell polarity <sup>3</sup>. This remodeling, known as actin dynamics, occurs through the polymerization and depolymerization of actin filaments via actin-binding proteins such as cofilin <sup>4, 5</sup>. Cofilin is inactivated by phosphorylation of a single Ser-3 by LIM-kinases (LIMK) or testicular protein kinases <sup>6-8</sup>. In its phosphorylated form, cofilin is unable to bind actin <sup>9</sup>. Cofilin is activated by phosphatases such as slingshot (SSH) and chronophin (CIN) <sup>10, 11</sup>. Dephosphorylated (active) cofilin disassembles F-actin from the rear of the actin network to recycle actin monomers <sup>12</sup>. This allows for branching of actin filaments and cytoskeleton reorganization. 14-3-3 scaffolding proteins play a role in the regulation of cellular actin structures through formation of multi-protein complexes with cofilin, LIMK, and slingshot <sup>13-15</sup>.

The regulation of cell polarity is critical for normal tissue function and is often defective in disease states <sup>2</sup>. Endometriosis is an enigmatic chronic disease that affects about 10% of reproductive age women <sup>16</sup>. Endometriosis is characterized by endometrium-like tissue growing outside of the uterus, often resulting in pain, irregular bleeding, and infertility. The link between endometriosis and infertility is clinically recognized <sup>17</sup>, although the molecular mechanisms of fertility interference are less clear <sup>18</sup>. It has been shown that proteins linked with the actin

cytoskeleton (alpha-actinin, talin, and ezrin) are completely de-expressed in endometriosis tissues<sup>19</sup>.

Maintenance of a healthy endometrium is crucial for the establishment of pregnancy. Previous studies in our laboratory have shown that cofilin plays a key role in cytoskeleton remodeling during decidualization<sup>20</sup>. This study examines cofilin and its regulators in healthy uterine endometrium compared to endometriosis uterine endometrium. Our results demonstrate that cofilin and slingshot shift in epithelial cells from an apical to a basal localization during the secretory phase of the menstrual cycle. It has been recognized that a shift in apical-basal polarity may account for the increased receptivity of the endometrium<sup>21</sup>. Our results provide evidence that this shift does not occur in endometriosis, indicating that actin cytoskeleton-related polarity dysregulation may be a key factor contributing to decreased fertility in women suffering from endometriosis.

## **MATERIALS AND METHODS**

### **Materials**

Rabbit anti-cofilin antibody and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit secondary antibody were obtained from Sigma-Aldrich. Rabbit anti-slingshot antibody was from ECM Biosciences (Versailles, KY). The deoxyribonuclease I Texas Red conjugate (DNAse I) was purchased from Invitrogen (Grand Island, NY). Rabbit anti-pLIMK, rabbit anti-LIMK1, and rabbit anti-LIMK2 were acquired from Cell Signaling (Danvers, MA). Rabbit anti-14-3-3 (recognizing all subtypes of 14-3-3) was from Santa Cruz (Santa Cruz, CA). Rabbit anti-14-3-3 eta was obtained from Abcam (Cambridge, MA). Antigen unmasking solution was purchased from Vector (Burlingame, CA) and ProLong Gold antifade mounting medium with DAPI (4',6-diamidino-2-phenylindole) was purchased from Invitrogen. All other reagents were obtained from either Sigma-Aldrich or Fisher Scientific.

### **Tissue Samples**

Human endometrial tissues were obtained from the Human Female Reproductive Tissue Bank at the University of Illinois at Chicago. Samples of pre-menopausal women collected during gynecological surgical procedures, related to benign diagnoses, were categorized into the proliferative or secretory phase of the menstrual cycle according to their overall basic histological morphology; a minimum 2 samples for each phase were utilized. All studies were approved by the Institutional Review Board of the University of Illinois at Chicago.

Baboon (*Papio anubis*) endometrial tissues were obtained as additional utilization of material collected for endometriosis studies funded by the NIH U54 HD40093 grant and generously provided by Dr. A.T. Fazleabas. Cycling female baboons, ranging in age from 7 to 12 years and

weighing between 12 and 18 kg, were housed in individual cages in the Biological Research Laboratories of the University of Illinois at Chicago. Endometriosis was experimentally induced in female baboons with regular menstrual cycles by intraperitoneal inoculation of menstrual endometrium on day 2 of two consecutive menstrual cycles (where day 1 was the first day of menses). Details of the inoculation procedure have been described previously<sup>22, 23</sup>. Disease progression was monitored in each animal by consecutive laparoscopies. Endometrial tissues from animals with induced endometriosis were obtained by endometriectomy or hysterectomy on day 10 post-ovulation (PO) at 15 months post-inoculation. Control uterine tissues from the proliferative and secretory phases of the menstrual cycle were obtained from healthy baboons without disease. Tissues from at least three animals were obtained for the proliferative phase, secretory phase, and endometriosis groups. Tissues from these sets of animals were used with each antibody for immunohistochemical analysis. All animal procedures were approved by the Animal Care Committee of the University of Illinois at Chicago.

### **Immunohistochemistry**

Uterine tissues were fixed in 10% buffered formalin for 24 hours at room temperature, dehydrated in ethanol, cleared in xylene, paraffin embedded, and cut into 5µm sections. Antigen retrieval was performed using citric acid-based antigen unmasking solution. Cofilin and cofilin regulatory proteins were localized in baboon uterine tissues with rabbit polyclonal antibodies to: cofilin (1:1000), slingshot (1:100), pLIMK (1:100), LIMK1 (1:100), LIMK2 (1:100), 14-3-3 (1:50), and 14-3-3 eta (1:100). Negative controls were incubated with pre-immune serum at the same concentration as the immune serum. Incubations with the primary antibodies were conducted at 4°C overnight, followed by incubation for 1 hour with FITC-conjugated secondary

antibodies (1:500). For double staining of cofilin and G-actin, DNase I was included with a secondary FITC-conjugated antibody. Coverslips were mounted with ProLong Gold antifade mounting medium containing DAPI. Images were captured using a Zeiss LSM510 laser scanning microscope (Carl Zeiss, Thornwood, New York).

### **Quantitative real-time PCR**

Baboon tissues, at least three animals per group, were homogenized in Trizol (Invitrogen) and total RNA was isolated according to manufacturer's instructions. RNA was treated with DNase I and reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc.) according to the manufacturer's instructions. Primers for cofilin (CFL1, 100 nM): forward 5'-TCCCATGCTGCCAACTTCTA-3' and reverse 5'-CCATGAGTAGCCGTGACCAG-3', slingshot (SSH1, 200 nM): forward 5'-TTGCTGGGAGTGGACTTTTC-3' and reverse 5'-CAAGGTGGATTTTCGTGTCG-3', LIMK1 (200 nM): forward 5'-GACGAGGAGACCCAGAGGAC-3' and reverse 5'-TGTAGAGCACCCCGATGAAC-3', and LIMK2 (200 nM): forward 5'-GTCCCGGCTTACTTCACCTC-3' and reverse 5'-CCATGACAGAACTCCCCAAA-3' were designed using the Primer3 program <sup>24</sup>. PCR products were sequenced to confirm primer specificity. Previously described primers were utilized for the hormone receptors: membrane progesterin receptor (mPR) alpha <sup>25</sup>, mPR beta <sup>26</sup>, estrogen receptor (ER) alpha <sup>27</sup>, ER beta <sup>28</sup> and progesterone receptor (PR)-AB and PR-B <sup>29</sup>. Real-time quantitative PCR was carried out using the ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, California); melt curves and cycle thresholds (Ct) were determined as previously described <sup>30</sup>. Each 10 µL reaction contained 5 µL of 2X Fast SYBR Green Master Mix (Applied Biosystems), 5ng first-strand cDNA, and primers at final

concentrations indicated above, and was run for 40 cycles (3 sec at 95°C, followed by 20 sec at 60°C) after an initial denaturation at 95°C for 20 sec. Melt curves were plotted to establish the absence of confounding nonspecific amplification products, and all primer systems were tested and validated for use in the  $2^{-\Delta\Delta C_t}$  method with H3F3A (500nM) serving as the endogenous reference<sup>31</sup>.

### **Evaluation of total apical distribution intensity of immunofluorescent signal**

To quantify the immunohistochemistry results, the pixel intensities for all images were analyzed using the ImageJ program (NIH and available at <http://rsb.info.nih.gov/ij>). The pixel intensities of fluorescent signals with equal total areas were measured in 10 different locations along both the apical and the basal luminal and glandular epithelium. Pixel intensity measurements for each antigen were normalized to the staining intensity of pre-immune serum controls from the same tissue. Samples from at least 3 different animals were analyzed for each group. The apical to basal pixel intensity ratio was calculated. These values were multiplied by 0.5 because equal distribution would represent half apical and half basal localization. Results were converted into percentages to yield the total apical distribution. We considered values between 45% and 55% indicative of equal apical and basal distribution, values greater than 55% were considered apical in localization, and values less than 45% were basal in localization.

### **Statistical Analysis**

Statistical analyses on immunofluorescent images were performed with SPSS 15.0 (SPSS Inc., Chicago, IL). One-way ANOVA was used to test the null hypothesis of group differences, followed by post hoc tests using the Bonferroni correction for multiple comparisons. The results



from quantitative real-time PCR were analyzed with GraphPad Prism 4.00 (GraphPad Software Inc., La Jolla, CA). Relative mRNA expression levels were calculated using unpaired t-tests. Statistical significance is indicated by an asterisk ( $p < 0.05$ ). All results are expressed as the mean  $\pm$  SD.

## RESULTS

### **Cofilin colocalizes with G-actin and shifts in epithelial cells of human endometrium during the menstrual cycle**

The localization of cofilin during menstrual cycle phases was examined in human uterine endometrium to determine whether it may play a part in the cyclic regulation of endometrial tissues. Cofilin was seen in both the apical and basal portions of the luminal epithelium in the proliferative phase of the menstrual cycle (Figure 1a, d, and detail image i). However, cofilin shifted to the basal portion of the luminal epithelium in the secretory phase (Figure 1e, h, and detail j). Because active cofilin binds to monomeric (G) actin, dual staining of cofilin and G-actin (via staining for DNase I, a G-actin binding protein) was performed to determine their localization. The merged images clearly show their distinct colocalization in the apical portion of the luminal epithelium in the proliferative phase (shown in yellow, Figure 1i). In contrast, no sites of colocalization were noticed in either the apical or the basolateral parts of luminal epithelial cells during the secretory phase of the menstrual cycle (Figure 1j).

### **Cofilin translocates during the normal baboon menstrual cycle, but not in baboons with endometriosis**

To test the hypothesis that cell polarity could be affected in diseased tissue, we examined the localization of cofilin in uterine endometrium from a baboon model of induced endometriosis. The localization of cofilin in healthy baboon tissue was similar to its localization in human tissue (Figure 2A a, b, d, e). Cofilin was more abundant in the apical portion of the luminal epithelium in the proliferative phase (Figure 2A a, d), but this localization shifted towards the basal pole of the luminal epithelium during the secretory phase (Figure 2A b, e). Interestingly, the localization of cofilin in the secretory phase of animals with induced endometriosis (Figure 2A c, f) was very similar to the proliferative phase of the healthy animals (Figure 2A a, d). This same pattern was observed in the glandular epithelium as well (Figure 2B) where the distribution of cofilin in healthy proliferative phase animals (Figure 2B a, d) was similar to endometriosis secretory phase animals (Figure 2B c, f). Quantification of image staining intensity (on at least 3 animals per group) confirmed that there was significantly more cofilin in the apical portion of the luminal epithelium in healthy proliferative phase animals ( $78\% \pm 4\%$ ) and endometriosis secretory phase animals ( $75\% \pm 12\%$ ) compared to healthy secretory phase animals ( $58\% \pm 2\%$ ) as shown in Figure 2C.

### **Slingshot displays a staining pattern similar to cofilin**

To determine the involvement of cofilin regulators in the shift in cofilin distribution between the menstrual cycle phases, the localization of the regulators of cofilin activity were examined. The phosphatase slingshot, an activator of cofilin, had a similar localization throughout the menstrual phases as cofilin (Figure 3). Slingshot was mainly located in the apical luminal epithelium in both the healthy proliferative phase (Figure 3 a, d) and endometriosis secretory phase tissues (Figure 3A c, f), but shifted to a more basal localization in the healthy secretory phase tissues

(Figure 3A b, e). The same slingshot staining pattern was observed in the glandular epithelium (Figure 3B). Staining intensity quantification confirmed that slingshot shifted significantly from the apical portion of the luminal epithelium in the proliferative phase ( $59\% \pm 10\%$ ) to the basal portion of the luminal epithelium in secretory phase ( $39\% \pm 3\%$ ) in healthy animals (Figure 3C). However, slingshot was significantly more apically localized in the endometriosis secretory phase ( $60\% \pm 5\%$ ) compared to the secretory phase healthy animals (Figure 3C).

The localization of the deactivators of cofilin (regulatory kinases LIMK1, LIMK2 and their phosphorylated form pLIMK1/2), in healthy and endometriosis baboon tissues was similarly analyzed through immunofluorescent staining and subsequent image quantification (Figure 4). Interestingly, staining detecting the activated forms of both LIMK1 and 2 (pLIMK1/2) revealed a trend in localization opposite to cofilin and slingshot, with more apical distribution in the secretory phase of the menstrual cycle (Figure 4A). However, the observed difference did not reach statistical significance (Figure 4A). No significant change was detected for the localization of non-phosphorylated LIMK1 (Figure 4B) or LIMK2 (Figure 4C).

The distribution of 14-3-3, a scaffolding protein known to bind to cofilin and cofilin regulators, was evaluated via immunofluorescence staining with an antibody common to all subtypes of 14-3-3. Our results did not indicate a different localization pattern for 14-3-3 in the baboon tissues (Figure 4D). Likewise, no change in the localization of specific subtype 14-3-3 eta protein was detected in baboon tissues (data not shown).

**Slingshot, ER alpha, and PR-AB mRNA levels are increased, but mPR alpha and mPR beta levels are not changed in the endometrium of the baboon model of induced endometriosis**

Since changes in the protein distributions of cofilin and some of its regulators were observed between the menstrual cycle phases and in endometriosis, the mRNA expression levels for cofilin (*CFLI*), slingshot (*SSH1*), *LIMK1*, and *LIMK2* in healthy animals and animals with endometriosis were analyzed via quantitative PCR (qPCR). There was no change in *CFLI* and *LIMK1* mRNA expression in tested tissues (Figure 5a, c). However, *SSH1* mRNA increased significantly from the proliferative phase to the secretory phase in healthy animals (Figure 5b). Moreover, there was significantly more *SSH1* mRNA detected in tissues from animals with endometriosis than in both the healthy proliferative and the secretory phase animal tissues (Figure 5b). Interestingly, a similar trend was observed for *LIMK2* mRNA (Figure 5d); however, the increased levels of *LIMK2* mRNA in endometriosis animals were not statistically different from secretory phase healthy animals.

Because changes in the uterine endometrium during the menstrual cycle are due to the tissue's response to ovarian steroid hormones, we analyzed the relative mRNA expression levels of estrogen and progesterone receptors within the baboon tissues. Relative levels of estrogen receptor *ER alpha* mRNA declined in tissues from baboons in the secretory phase of the menstrual cycle as estimated by qPCR (Figure 5e). However, the tissues from baboons with induced endometriosis in the secretory phase of the menstrual cycle had relative *ER alpha* mRNA levels comparable to levels found in the proliferative phase healthy animals, and were significantly different from message levels in secretory phase healthy animals. Overall relative *ER beta* mRNA levels were on the low edge of the detection range; no distinguishable changes in *ER beta* gene expression were detected in tested baboon uterine tissues (data not shown), although we detected a positive signal in human testis and prostate tissues (data not shown). Relative levels of PR detected with primers recognizing a region common to the A and B form of

PR (*PR-AB*) detected significantly increased *PR-AB* mRNA in animals with endometriosis in comparison with healthy animals (Figure 5f). The increase in *PR-B* specific mRNA was borderline significant ( $p=0.07$ ) in animals with endometriosis in comparison with healthy animals (Figure 5g). To test the hypothesis that a novel membrane progesterin receptors might be involved in the pathophysiology of endometriosis, we measured relative mRNA levels for *mPR alpha* and *mPR beta*. Although levels for both receptors seemed increased in the secretory phase of the menstrual cycle in healthy baboons, only *mPR beta* relative expression was significantly increased (Figure 5i). However, there was no change in mRNA expression for *mPR alpha* and *mPR beta* detected in animals with endometriosis in comparison with healthy animals (Figure 5h, i).

## DISCUSSION

The uterine endometrium undergoes both morphological and functional changes during the secretory phase of the menstrual cycle that are essential to prepare for blastocyst attachment and to establish pregnancy. Since reorganization of the actin cytoskeleton is an early cellular response common to many dynamic processes, this study has examined the localization of key regulators of cytoskeleton organization in the healthy human endometrial epithelium and in a non-human model of endometriosis. The results of this study point at new cytoskeleton related targets to focus on in future studies involving women suffering from endometriosis related infertility.

A recent review summarizing current knowledge about the functions of cofilin discusses the profoundly multifaceted role that this well-established regulator of actin dynamics has in cell biology <sup>32</sup>. The authors discuss cofilin's ability to respond to a wide variety of inputs and also modulate physiological changes affecting those inputs not only through its role in actin dynamics, but also its role in apoptosis, phospholipid metabolism and gene expression; these characteristics strongly indicate that cofilin is a homeostatic regulator or “functional node” in cell biology <sup>32</sup>. So far studies related to cofilin in reproductive tissues have been limited. In rat preovulatory granulosa cells, cofilin activation is necessary for the actin cytoskeleton rearrangement which coincides with cell remodeling and progesterone production <sup>33</sup>. We have reported that manipulating actin dynamics has a negative effect on decidualization in human stromal fibroblasts, and have also observed the movement of cofilin into the nucleus of cells undergoing decidualization <sup>20</sup>. In a recent study, overexpression of cofilin in endometrial stromal cells isolated from the eutopic endometrium of patients with advanced ovarian endometriosis

was linked to malignant-like properties; silencing cofilin in these cells attenuated the malignant-like features<sup>34</sup>.

Cellular interactions with the extracellular environment are achieved through dynamic remodeling of the actin cytoskeleton<sup>35</sup>. This study indicates that the actin-binding protein cofilin and its regulator slingshot are involved in actin cytoskeleton remodeling during the menstrual cycle. This remodeling may be essential for implantation during the secretory phase because the apical cell membrane of luminal epithelial cells is the first point of contact between blastocyst and endometrium. Shifts in apical-basal polarity such as those described here may account for the increased receptivity of the endometrium as it prepares for pregnancy<sup>21</sup>. The term “plasma membrane transformation” was proposed to describe the changes in uterine epithelial cells during blastocyst attachment and implantation, further emphasizing the importance of architectural restructuring in this process<sup>21</sup>. The plasma membrane transformation of rat epithelial cells involves the dissociation of actin filaments which make up the apical terminal web<sup>36-38</sup>. Remodeling of the actin cytoskeleton during early rat pregnancy has been shown to involve the actin-binding proteins gelsolin, alpha-actinin and vinculin<sup>39</sup>.

This study has demonstrated that in healthy human endometrial epithelial cells, cofilin and slingshot change localization from the apical pole in the proliferative phase of the menstrual cycle to the basal pole in the secretory phase. Slingshot may migrate with cofilin in order to maintain an active cofilin pool, thereby promoting actin remodeling and uterine receptivity. These results also suggest that the activated phosphorylated form of LIM kinases 1 and 2 (pLIMK1/2) moves in the opposite direction, from a basal position in the proliferative phase to a more apical location in the secretory phase in healthy individuals (see proposed model in Figure

6). These kinases are able to phosphorylate and thus inactivate cofilin. The non-phosphorylated forms of LIMK1 and 2 did not display any migratory pattern.

Members of the 14-3-3 family of scaffolding proteins have been shown to interact with cofilin and cofilin regulators<sup>13, 14, 40</sup>. In this study changes in 14-3-3 (as detected with an antibody common to all subtypes) location were not observed in uterine endometrial epithelial cells; their location remained equally distributed between the apical and basal poles. The localization of 14-3-3 eta which is a specific subtype of 14-3-3 that has been shown to have increased gene expression in patients with endometriosis was also examined<sup>41</sup>; however, no change in 14-3-3 eta localization was detected in the baboon endometriosis secretory phase epithelial endometrium.

Uterine tissue growing outside of the uterine cavity, such as occurs in endometriosis, might alter or distort the signals from the uterine environment causing a different expression pattern of cytoskeleton remodeling proteins and their genes resulting in decreased fertility. Changes in *CFL1* mRNA expression were not detected in baboon endometrial tissues during the menstrual cycle, suggesting that cofilin gene expression does not depend on changes in steroid hormones during the menstrual cycle. In contrast, progesterone might be involved in regulation of cofilin's up-stream kinase *LIMK2* and phosphatase *SSH*, since their increased mRNA levels were detected in the secretory phase. Additionally, *SSH* mRNA expression was further increased in animals with endometriosis. This upregulation of slingshot may be important for implantation; slingshot-regulated cofilin activity has been shown to be mediated by Rac1<sup>14</sup>, which has been reported to be involved in implantation<sup>42</sup>.

Estrogen and progesterone are the major regulators of the cyclical changes in the uterine endometrium. Estrogen influence is dominant during the proliferative phase and promotes



endometrium cell growth. Progesterone, the main hormone of the secretory phase, is responsible for cell differentiation in preparation for blastocyst implantation which is critical for the establishment of pregnancy. Several studies indicate that endometriosis has been associated with a decreased progesterone response in the eutopic and ectopic endometrium <sup>43</sup>. Previous analysis of the eutopic endometrium in the baboon model of induced endometriosis suggests that the development of progesterone resistance is a gradual process and becomes evident 6 months after disease induction <sup>44</sup>. In this study, eutopic endometrial tissues from animals 15 months after disease induction were utilized. Quantification of mRNA from these tissues suggested a progesterone resistance related pattern of nuclear steroid hormone receptors, although further protein analysis is necessary to confirm this observation. Loss of progesterone activity may result in the persistence of ER-alpha gene expression seen in tissues from baboons with endometriosis, since down-regulating ER is one of the primary actions of progesterone. A similar trend was described on the protein level in women with endometriosis who were suspected to have defects in uterine receptivity <sup>45</sup>. In the baboon induced endometriosis model used in our study, PR mRNA levels (represented by PR-AB, detected by a primer specific for both PR-A and PR-B) were not downregulated as seen in healthy animals, but remained elevated.

This study also investigated the involvement of the novel membrane progesterone receptors mPR alpha and mPR beta <sup>46-48</sup> in endometriosis. Data from this study support the previously observed trend of their upregulated mRNA levels during the secretory phase of the healthy menstrual cycle <sup>25</sup>. However, the data from this study do not implicate their involvement in endometriosis eutopic endometrium physiology; no difference was seen in their mRNA expression in diseased tissues compared to tissues from healthy baboons.

The results of this study indicate that there is a dysregulation in endometriosis connected to improper cytoskeleton rearrangements during the menstrual cycle (please see Figure 6). This implies that luminal epithelial cells of the endometriotic endometrium are not properly prepared for blastocyst arrival, since these cells allow attachment of trophoblast cells to their apical pole during embryo implantation. A shift in apical-basal polarity may play a key role in cytoskeletal reorganization and uterine receptivity; disruption or dysregulation of this cytoskeletal remodeling may contribute to decreased fertility in women suffering from endometriosis.

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## FIGURE LEGENDS

### **Figure 1. Association of cofilin with monomeric actin in human uterine endometrium during the menstrual cycle**

Human tissues in the proliferative phase (proliferative, a, b, c, d) and secretory phase (secretory, e, f, g, h) of the menstrual cycle were used for immunostaining. Confocal microscopy of double-immunofluorescent staining for cofilin (green; a, e) and G-actin (visualized with DNase I Texas red dye; DNase I: b, f). Nuclear staining with DAPI (blue; c, g) and merged images (d, h, i, j) are shown. Detail of merged image in proliferative phase (i) and secretory phase (j). Note that cofilin localization is altered between the proliferative phase and secretory phase. Arrows indicate cofilin localization. The scale bar in a-h represents 50 $\mu$ m, and the scale bar in i-j represents 20 $\mu$ m.

### **Figure 2. Cofilin localization in healthy and induced endometriosis baboon uteri**

**A. B.** Cofilin localization in baboon uterine endometrium luminal epithelium (A) and glandular epithelium (B) detected by immunostaining. Confocal microscopy was used to visualize cofilin (green) distribution in healthy baboon tissues in the proliferative phase (a, d), the secretory phase (b, e) of the menstrual cycle and in secretory phase tissues from animals with induced endometriosis (c, f). Merged images (d, e, f) contain nuclear staining with DAPI (blue). Insets show staining with non-specific control IgG. Note that cofilin localization shifts to the basal pole in epithelial cells of the secretory phase healthy animals, but this shift is not detected in animals with endometriosis.

**C.** Cofilin distribution in luminal epithelial cells illustrated as percentage of total apical distribution quantified by ImageJ analysis as described in Material and Methods. Note the

decline in apical distribution between menstrual phases in healthy animals (\*  $p < 0.05$ ) and the absence of this shift in the secretory phase of animals with endometriosis (\*  $p < 0.05$ ).

**Figure 3. Slingshot localization in healthy and induced endometriosis baboon uteri**

**A. B.** Slingshot localization in baboon uterine endometrium luminal epithelium (A) and glandular epithelium (B) detected by immunostaining. Confocal microscopy was used to visualize slingshot (green) distribution in healthy baboon tissues in the proliferative phase (a, d) and secretory phase (b, e) of the menstrual cycle and in secretory phase tissues from animals with induced endometriosis (c, f). Merged images (d, e, f) contain nuclear staining with DAPI (blue). Insets show staining with non-specific control IgG. Note that slingshot localization in the apical pole of epithelial cells has diminished in the secretory phase healthy animals, but this change was not detected in animals with endometriosis.

**C.** Slingshot distribution in luminal epithelial cells is illustrated as percentage of total apical distribution quantified by ImageJ analysis as described in Material and Methods. Note the shift from apical to basal distribution between menstrual phases in healthy animals (\*  $p < 0.05$ ) and the absence of this shift in the secretory phase of animals with endometriosis (\*  $p < 0.05$ ).

**Figure 4. Distribution of cofilin regulators in baboon uterine luminal epithelial cells during the menstrual cycle and in endometriosis**

Tissues were stained with specific antibodies against phospho-LIMK1/2 (pLIMK1/2; A), LIMK1 (B), LIMK2 (C) and 14-3-3 (D). The distribution of antigens in luminal epithelial cells was quantified by ImageJ analysis as described in Material and Methods and is illustrated as percentage of total apical distribution. Note the shift to apical distribution of pLIMK1/2 (A) in

the secretory phase of the menstrual cycle in comparison to the proliferative phase in healthy animals; this trend is not observed in animals with endometriosis.

**Figure 5. *CFL1*, *SSH1*, *LIMK1*, *LIMK2* and steroid hormone receptor mRNA expression in baboon uterine endometrium**

Total RNA was isolated from healthy baboon uterine endometrial tissues in proliferative phase (Prolif., n=4) and secretory phase (Secr., n=5) and from baboons with induced endometriosis in the secretory phase of the menstrual cycle (Eosis, n=4). **A.** RNA samples were used for qPCR with primers specific for *CFL1* (a), *SSH1* (b), *LIMK1* (c) and *LIMK2* (d). The results are expressed as mRNA relative expression with *H3F3* mRNA serving as a reference gene. Note that *SSH1* mRNA was significantly different between the menstrual phases in healthy animals, and in the secretory phase between healthy animals and animals with endometriosis (\* p<0.05).

**B.** Baboon RNA samples were used for qPCR with primers specific for *ER alpha* (e), *PR-AB* (f), *PR-B* (g), *mPR alpha* (h), and *mPR beta* (i). Note that in animals with endometriosis, the mRNA levels for *ER alpha* and *PR-AB* were significantly up-regulated in comparison with healthy animals. (\* p<0.05).

**Figure 6. Model representing dyssynchrony of cofilin and its regulators in endometriosis**

Based on our results, we hypothesize that endometriosis-related progesterone resistance results in dysregulation of actin remodeling close to the apical pole of uterine endometrial epithelial cells as indicated by the improper localization of cofilin and slingshot. The localization of these actin regulatory proteins in endometriosis is similar to what is seen in the healthy proliferative phase. In contrast, in the healthy secretory phase epithelium there is a shift in the distribution of the

cofilin multi-protein complex which we propose results in a less rigid and more receptive apical surface that is ideal for blastocyst adhesion during implantation.

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