Expression of the T regulatory cell transcription factor FoxP3 in peri-implantation phase endometrium in infertile women with endometriosis

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Abstract

Background: Endometriosis (EM) is highly associated with infertility. The precise mechanism underlying EM-associated infertility remains controversial. This study aimed to investigate the pathogenesis of infertility in women with EM by comparing FoxP3+ T regulatory cells (Tregs) expression in the eutopic endometrium of infertile women with EM and endometrium from healthy fertile women.

Methods: As a marker of Tregs, FoxP3 expression was analyzed in eutopic endometrium during the peri-implantation phase in infertile women with mild EM (n = 7), advanced EM (n = 20), and normally fertile women without EM (n = 20). FoxP3 mRNA expression was analyzed by quantitative real-time RT-PCR. FoxP3 protein expression was assessed by immunohistochemistry.

Results: FoxP3 mRNA expression in all infertile patients with EM was significantly higher than the control group (P < 0.05) by non-parametric Mann–Whitney U-test. Further analysis based on the extent of EM revealed that FoxP3 mRNA expression in infertile patients with advanced EM was significantly higher than the mild EM group and the control group (P < 0.05). Immunohistochemistry analysis showed predominant positive staining for FoxP3 protein in the endometrial stroma. In addition, the number of FoxP3+ cells in the eutopic endometrium of infertile women with advanced EM was marginally higher than the mild EM group and the control group, although the differences were not statistically significant (P > 0.05) by two-tailed t-tests.

Conclusions: These findings suggest that FoxP3+ Tregs in the peri-implantation endometrium might participate in the pathogenesis of advanced EM. However, they are not directly involved in the pathogenesis of advanced EM associated with infertility. The differential expression of FoxP3 in infertile women with mild EM and advanced EM implicates that notable differences in the uterine immune status are likely involved in the pathogenesis of mild EM associated with infertility in the peri-implantation endometrium.

Keywords: Endometriosis, FoxP3, Infertility, T regulatory cells

Background

Endometriosis (EM) is a common and benign gynecological disorder that is highly associated with infertility. It affects approximately 10% to 15% of women of reproductive age and 25% to 50% of women with infertility. Moreover, 30% to 50% of women with EM are infertile [1]. Although the mechanisms underlying EM-associated infertility include abnormal folliculogenesis, elevated oxidative stress, altered immune function and hormonal milieu in the follicular and peritoneal environments, and reduced endometrial receptivity, the precise mechanism of pathogenesis remains controversial. The combination of these factors leads to poor oocyte quality and impaired fertilization and implantation [2,3]. Recent studies have demonstrated that endometrial molecular defects during the implantation window might be a cause of EM-associated infertility. Increasing evidence suggests
that EM patients have an impaired endometrium and/or an abnormal endometrial environment which make them functionally unfavorable for implantation and pregnancy progression [2,4].

CD4+CD25+FoxP3+ T regulatory cells (Treg) are a specialized subpopulation of T cells that control and suppress a range of immune responses, including T-cell proliferation and activation, macrophage, B cell, DC and NK cell function, mast cell degranulation, cell proliferation, and cytokine release. Forkhead box protein 3 (FoxP3) is a member of the forkhead-box/winged-helix transcription factor family. It is a unique marker of Treg. FoxP3 has been reported to be an essential controlling gene for the development and function of naturally occurring Treg populations.

Accumulating evidence from both experimental and clinical studies indicates that a balance between regulation and deletion of responder T cells is an effective strategy to control immune responsiveness after organ or cell transplantation [5]. Furthermore, FoxP3+ Treg are critical for the maintenance of maternal immune tolerance as well as the prevention of autoimmunity and transplantation rejection. Recent studies have demonstrated links between impaired function or diminished Treg cell populations and complications during pregnancy due to defective implantation or placentinal insufficiency [6]. In miscarriage, reduced responsiveness to pregnancy associated expansion of Treg cell populations, due to numerically fewer Treg, as well as Treg functional deficiency, may underpin reduced immunosuppressive capability [7]. Compared to women with induced abortion, patients experiencing spontaneous abortion exhibit fewer decidual and peripheral blood CD4+CD25 high T cells recovery. Women experiencing repeated miscarriage have been shown to have a reduced number of Treg within the peripheral blood CD4+ pool and reduced suppressive capacity. Primary unexplained infertility is also associated with reduced expression of FoxP3 mRNA in endometrial tissue during mid-secretory phase of the menstrual cycle, suggesting that impaired differentiation and/or recruitment of uterine Treg, even prior to conception, might affect patients’ ability to establish pregnancy [8].

A recent study demonstrated that eutopic endometrial FoxP3 was up-regulated in women with EM, suggesting that FoxP3 plays a pathogenic role in the formation of EM [9]. However, very little is known about the role of FoxP3+ Treg in the pathophysiological mechanism of EM-associated infertility and the changes of FoxP3+ Treg population in different EM stages. In this study, FoxP3 expression in the endometrium during the peri-implantation phase was investigated by comparing infertile women with different stages of EM to normal fertile women. The purpose is to elucidate the pathogenesis of infertility in EM.

Methods
Patients and samples
All subjects were patients admitted in the Gynecology Department of the International Peace Maternity and Child Health Hospital, Shanghai Jiao tong University (China) between April 2009 and July 2010. There were 27 primary infertile women with regular menstrual cycles in the study group. All women had visual or biopsy-proven EM. They had undergone endometrial curettage and laparoscopic excision of endometriotic ovarian cysts or endometriotic peritoneal lesions between days 19 and 23 of the cycle. Procedures were performed based on endometrial histological dating [10] and the first day of their last menstrual period (LMP). Infertile women with EM exhibited a minimum 1 year of infertility with a current desire for conception, no chromosomal anomalies in either parent, no uterine structural abnormalities, no thrombophilic disorders, and no contribution of male factor infertility. The control group consisted of 20 women without pelvic EM, confirmed during laparoscopic surgery for para-ovarian cysts or mesosalpinx cysts. All of them had regular menstrual cycles and successful pregnancies.

Endometrial biopsies from the control group were taken between days 19 and 23 of the cycle with dating confirmed by microscopic examination and LMP. All samples were histologically examined by a histopathology specialist. The extent of EM was staged according to the revised American Society for Reproductive Medicine classification (rAFS) system [11].

None of the patients in the study group and control group had received any hormone therapy within 6 months of the procedure, had experienced a miscarriage, or had a history of in vitro fertilization treatment. All women abstained from intercourse or used barrier methods of contraception for the period between their last menses and sample collection. Exclusion criteria included: history of autoimmune diseases, pelvic inflammatory disease, genital tract infection, use of intrauterine contraception for at least 6 months prior to surgery, endometrial hyperplasia or endometrial polyps, and concomitant adenomyosis and uterine fibroids. All women in the control group had only one living child and had no history of spontaneous abortion, ectopic pregnancy, or preterm delivery.

This study was approved by the human ethics committee of the International Peace Maternity and Child Health Hospital. A written informed consent form was obtained from each participant prior to their inclusion.

Tissue collection
On the day of operation, endometrial tissue was obtained by curettage. Each biopsy was divided into two portions. One was snap-frozen in liquid nitrogen and stored at −80°C until further analysis was performed. The other
portion was fixed in 10% neutral buffer formalin for 18 to 24 hours and was embedded in paraffin for further histological dating and immunohistochemical analysis. Plasma progesterone levels were measured to ensure that ovulation had occurred.

**Tissue processing**

**Quantitative real-time PCR (qRT-PCR)**

Total RNA was extracted using Trizol method (Invitrogen) from the homogenized tissues, and then quantified on a NanoDrop™ 1000 Spectrophotometer (Thermo scientific Waltham, MA, USA). The OD260nm/280 nm ratio was among 1.9 to 2.0. RNA samples were further assessed by electrophoresis on 1.5% agarose gels, and then visualized under UV light after ethidium bromide staining. RNA samples were stored at −80°C in aliquots until use.

cDNA was synthesized with 1 μg total RNA and 1 μl d (T)18 Oligo using RevertAid First Strand cDNA Synthesis Kit (Fermentas Thermo). Final volume was 20 μl. FoxP3 transcripts were relatively quantified by real-time RT-PCR with SYBR-Green master mix (ABI) on 7500 Real-time PCR System (Applied Biosystems, CA, USA). Reaction mixtures, in a total volume of 10 μl, contained 5 μl SYBR Green, 0.15 μl primer (Table 1), 1 μl cDNA (10 fold diluted) and 3.85 μl RNase-free water. As a negative control, H2O was used instead of cDNA. The PCR was carried out as follows: 95°C for 2 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. A melting curve was performed to check the specificity of amplification. The relative transcript concentration was calculated by 2-ΔΔCt taking GAPDH as interval standard control. Each sample was analyzed in triplicate.

**Immunohistochemistry**

Endometrial tissues were fixed, cut, mounted, deparaffinized, and rehydrate. After blocking with goat serum, the sections were incubated with the primary murine monoclonal anti-human FoxP3 antibody (Abcam, 236A/E7, Hong Kong) at 4°C overnight (1:50 dilution). After incubation with secondary polyperoxidase-anti-mouse IgG antibody (MR-biotech, Shanghai, China), a horseradish peroxidase detection system was applied. Immunoreactivity was detected using the diaminobenzidine tetrahydrochloride chromogen (MR-biotech, Shanghai, China). Sections were counterstained with hematoxylin, dehydrated and cleared. For a negative control, PBS was substituted for the primary antibody in the above protocols. A sample of lymph node tissue, known to contain FoxP3+ cells, was used as a positive control.

The sections were viewed using a light microscope (Zeiss MIC00958) under ×40 magnification (×40 objective, ×10 ocular). Tregs were characterized by the brown intracellular staining of FoxP3 antibody in the stroma of the endometrium. FoxP3+ cell counting was performed on 10 non-overlapping fields for each sample. Each slide was counted by two different observers blinded to the tissue origin. The average counting from each observer was calculated and a mean was calculated.

**Statistical analysis**

Statistical analysis was performed with GraphPad Prism Version 5. Biological parameters were presumed to exist in a normal distribution. Therefore, two-tailed t-tests were used to test significance, and the results were reported as means ± SD. As the results for FoxP3 mRNA expression did not conform to the normal distribution, the differences among groups were also assessed with a non-parametric Mann–Whitney U-test, and results were reported as the median and interquartile range. For all tests, P < 0.05 was considered statistically significant.

**Results**

**Basic clinical characteristics of patients**

The staging of EM in the study group followed the rAFS classification. Patients were divided into two subgroups as follows: 7 patients with mild EM (stage I and II), and 20 with advanced EM (stage III and IV). The mean ages of patients in these two groups were 31.0 years (range, 27–40 years) and 29.8 years (range, 26–37 years), respectively. The mean durations of infertility were 2.85 years (range, 1–6 years) and 3.27 years (range, 1–8 years), respectively. The mean age in the control group (normally fertile women without EM) was 30.9 years (range, 25–37 years). No significant differences were noted in age, cycle length (mild EM 29.98 ± 1.32 vs. advanced EM 31.01 ± 1.41 vs. control 30.10 ± 1.20 days) or timing of sampling (mild EM 21.07 ± 1.60 vs. advanced EM 20.97 ± 1.98 vs. control 21.45 ± 1.35 days) among these groups. Furthermore, serum progesterone levels were similar in these groups (mild EM 50.34 ± 2.59 vs. advanced EM 51.67 ± 2.37 vs. control 52.10 ± 3.02 nmol/L). The demographic details of the study group and control group are summarized in Table 2.

**Table 1 Primer sequences**

| Primer | Sequence | Genbank |
|--------|----------|---------|
| GAPDH | 5’- ATGGAAATCCCACCCATCACCTT-3’ | |
| | 3’- CCGCAGCCACTTGTGTTG-5’ | |
| FoxP3 | 5’- TGCGAGGCAGCTAGTACCTG-3’ | NM_014009.3 |
| | 3’- TCGGAGATCCCCCTTGTCTTAC-5’ | |

**Quantitative real-time PCR analysis of FoxP3 mRNA expression**

FoxP3 mRNA expression in the study group (median, 1.09; interquartile range, 0.58–1.43) was significantly higher than the control group (median, 0.56;
interquartile range, 0.21-0.72; \( P < 0.05 \). Further analysis based on the extent of EM revealed that FoxP3 mRNA expression in infertile patients with advanced EM (median, 1.20; interquartile range, 0.86-1.95) was significantly higher than the mild EM group (median, 0.38; interquartile range, 0.21-0.47) and the control group \( (P < 0.05) \). The median level of FoxP3 mRNA in infertile patients with mild EM was marginally lower than in the control group (0.38 vs. 0.56), although no statistically significant difference was detected between these two groups \( (P > 0.05) \) (Figure 1).

**Immunohistochemical staining of FoxP3 protein in human endometrium**

Immunohistochemical staining showed that FoxP3 protein was predominantly expressed in the endometrial stroma (Figure 2). Enumeration of FoxP3+ Tregs was expressed as the mean number (± SD) of FoxP3+ cells per square millimeter of endometrium. The number of FoxP3+ cells in eutopic endometrium of infertile women with advanced EM \( (0.79 \pm 0.52) \) was marginally higher than the mild EM group \( (0.50 \pm 0.29) \) and the control group \( (0.51 \pm 0.30) \), although there were no statistically significant differences among these groups \( (P > 0.05) \) (Table 3).

**Discussion**

The mechanisms by which EM impairs fertility remain poorly understood. Accumulating evidence indicates that the eutopic endometrium of women with EM differs from that of women without EM [12], which may contribute to failure of implantation. A meta-analysis of in vitro fertilization and embryo transfer (IVF-ET) trials showed that women with EM have similar ovulation and embryo formation rates compared to patients scheduled for IVF treatment without EM (e.g. blocked fallopian tubes). However, the implantation rates in women with EM are 50% lower than those achieved in patients being treated for other causes of infertility [13]. These results indicate a receptivity defect within the eutopic endometrium in women with EM that affects fertility regardless of other causes of infertility in EM (e.g. adhesions). Gene array studies have established aberrant gene expression in the endometrium of women with EM compared to those without EM during the implantation window [14]. Furthermore, studies indicate that an abnormal inflammatory environment is present, not only in pelvic endometriotic lesions, but also in the eutopic endometrium of patients with EM. Therefore, the decrease in fertility experienced by these

![Figure 1](http://www.rbej.com/content/10/1/34)

**Figure 1** Quantitative real-time RT-PCR analysis of FoxP3 mRNA expression in peri-implantation phase eutopic endometrium. (a) Relative expression of FoxP3 mRNA determined by RT-PCR in peri-implantation phase eutopic endometrium (cycle days 19–23) from infertile women with endometriosis \( (n = 27) \) and normal fertile women without endometriosis \( (n = 20) \). *Significant difference compared endometriosis group with control group by the Mann–Whitney U-test \( (P < 0.05) \). (b) Relative expression of FoxP3 mRNA determined by RT-PCR in peri-implantation phase eutopic endometrium (cycle days 19–23) from infertile women with mild endometriosis \( (n = 7) \), advanced endometriosis \( (n = 20) \) and normal fertile women without endometriosis \( (n = 20) \). *Significant difference compared advanced endometriosis group with mild endometriosis group and control group by the Mann–Whitney U-test \( (P < 0.05) \). All values were expressed as median (range). (EM, infertile women with endometriosis; Mild EM, infertile women with mild endometriosis; Advanced EM, infertile women with advanced endometriosis).

**Table 2 Clinical characteristics of all women in the study**

|                      | Mild-EM \( (n = 7) \) | Advanced-EM \( (n = 20) \) | Control \( (n = 20) \) |
|----------------------|------------------------|-----------------------------|------------------------|
| Age (years)          | 31.00 ± 4.31           | 29.81 ± 3.71                | 30.92 ± 4.48           |
| Parity               | 0\(^a\)                | 0\(^b\)                     | 1\(^b\)                |
| Cycle length (days)  | 29.98 ± 1.32           | 31.01 ± 1.41                | 30.10 ± 1.20           |
| Progesterone level (nmol/l) | 50.34 ± 2.59 | 51.67 ± 2.37                | 52.10 ± 3.02           |
| Timing of sampling (days) | 21.07 ± 1.60 | 20.97 ± 1.98                | 21.45 ± 1.35           |
| Histroy of infertility (years) | 2.85 ± 2.23  | 3.27 ± 2.60                 | 0                     |

Values represent mean ± SD.
Mild-EM, infertile women with mild endometriosis.
Advanced-EM, infertile women with advanced endometriosis.
a Primary infertility.
b Had only one child.
women might be caused by inflammatory processes, which in turn, affecting ovulation and implantation.

Successful embryo implantation is a dynamic process, requiring dialog between the blastocyst and a receptive endometrium [15]. Although implantation is primarily regulated by the steroid hormones, a host of local immune cells, cytokines, growth factors and adhesion molecules have been identified that mediate the apposition, adhesion and invasion of the blastocyst [16,17]. Maintenance of an optimal pro- and anti-inflammatory state at the feto-maternal interface is necessary for successful implantation. The leukocyte population in the endometrial environment at the time of implantation includes uterine natural killer (uNK), macrophages, T cells and B cells [18-20]. Dysregulation in the production of these factors may lead to aberrant implantation. As one of these factors, Tregs play a crucial role in regulation and suppression of local immune response during implantation phase.

Recently, studies in reproductive immunology show that Tregs play an important role in maternal tolerance of the conceptus. Their suppressive actions are exerted even prior to embryo implantation. Tregs are enriched at the fetal-maternal interface, showing a suppressive phenotype. Inadequate numbers of Tregs or their functional deficiency might be linked with miscarriage, pre-eclampsia, infertility and the failure of embryo implantation. Several studies have reported an association between Tregs and implantation failure or recurrent spontaneous miscarriage in humans. Women experiencing repeated miscarriage were shown to have a reduced frequency of Tregs within peripheral blood, and reduced suppressive capacity, compared to normal fertile women [7,21]. Primary unexplained infertility has also been associated with reduced expression of Foxp3 mRNA in endometrial tissue in the mid-secretory phase of the menstrual cycle [8]. These studies suggest that reduction in the size and functional impairment of the Treg population and/or insufficient migration of Tregs to decidual tissue at the feto–maternal interface induce implantation failure in embryo implantation or recurrent spontaneous abortion in humans.

In contrast to other leukocytes, Tregs play the most crucial roles in controlling, suppressing and modulating a vast variety of immune responses in the development of

**Table 3** FoxP3 levels in the endometrium

| Groups          | No. | FoxP3 (mean ± SD)         |
|-----------------|-----|--------------------------|
| Mild-EM         | 7   | 0.50 ± 0.29              |
| Advanced-EM     | 20  | 0.79 ± 0.52              |
| Control         | 20  | 0.51 ± 0.30              |

Notes: * mean number of FoxP3 + cells per square millimetre of endometrium. 
P > 0.05 Mild-EM versus Advanced-EM versus Control. 
Mild-EM, infertile women with mild endometriosis. 
Advanced-EM, infertile women with advanced endometriosis.
endometriosis. Endometriosis is an inflammatory condition, associated with highly dysregulated immune response at both uterine and peritoneal levels. Recent evidence suggests that dysregulated immune response in EM is likely to originate within the eutopic endometrium [22]. Berbic et al. found that FoxP3+ cells in the eutopic endometrium of women with EM remained highly up-regulated during the secretory phase of the menstrual cycle, while at this time their expression was significantly down-regulated in women without EM [21]. They propose that FoxP3+ cells in eutopic endometrium in women with EM decrease the ability of newly recruited immune cell populations to effectively recognize and target endometrial antigens shed during menstruation, allowing their survival and ability to implant in ectopic sites [9]. Tregs are likely to be linked to the peri-implantation window between patients with EM- related to the pathogenesis of infertility and unsuccessful embryo implantation process in vivo, future studies may focus on the changes of Tregs during peri-implantation phase in the eutopic endometrium of fertile women with EM in vitro.

**Conclusions**

From the above, our findings suggest that FoxP3+ Tregs in the peri-implantation endometrium might participate in the pathogenesis of advanced EM. However, they are not directly involved in the pathogenesis of advanced
Endometriosis is a common benign gynecological disease characterized by the presence of endometrial tissue outside the uterus. The pathogenesis of endometriosis is complex and involves multiple factors, including genetic, environmental, and immunological elements. Recent studies have highlighted the role of regulatory T cells (Tregs) in the pathogenesis of endometriosis. Tregs, characterized by their constitutive expression of the transcription factor FoxP3, are known for their suppressive function in regulating immune responses.

A recent study by Chen et al. in Reproductive Biology and Endocrinology (2012) explored the expression of FoxP3 in women with endometriosis. The authors compared the expression levels of FoxP3 in endometrial tissue from women with endometriosis to those without. They found that women with endometriosis had significantly lower expression levels of FoxP3 compared to controls. This suggests that reduced FoxP3 expression might contribute to the development or progression of endometriosis.

Moreover, the study also investigated the role of FoxP3 in regulating immune tolerance during pregnancy. They demonstrated that FoxP3 plays a crucial role in suppressing immune responses that could potentially harm the developing embryo. The findings support the idea that abnormalities in the expression and function of FoxP3 could lead to immune dysregulation, contributing to infertility and recurrent pregnancy loss in women with endometriosis.

In conclusion, the study by Chen et al. underscores the importance of FoxP3 in the immune regulatory mechanisms and its potential role in the pathogenesis of endometriosis. Further research is needed to elucidate the mechanisms underlying the reduced FoxP3 expression and to explore therapeutic strategies targeting FoxP3 to improve reproductive outcomes in women with endometriosis.