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Original Article

Regulation and expression of FOXO1 and FOXO3 in endometrial tissue during the menstrual cycle of human and mouse

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Abstract: Mammalian embryo implantation is an extremely complex process and requires endometrial receptivity. In order to establish this receptivity, sequential proliferation and differentiation during the menstrual cycle is necessary. FOXOs transcriptionally activate or inhibit downstream target genes, thereby playing an important role in proliferation, apoptosis, autophagy, metabolism, inflammation, differentiation, and stress resistance. According to these functions, we believe that FOXOs should also play an essential role in embryo implantation. In this study, we observed the expression and distribution of FoxO1 and FoxO3 during the proliferative-phase and secretory-phase in human endometrium and in Mouse Estrous Cycle firstly. Then we used Immunohistochemistry to examine the expression of FoxO1 and FoxO3 induced by E2 (Estrogen) and/or P4 (progesterone) in the ovariectomized mouse uterus and human endometrium cells. Our results showed that the expression of FOXO1 changed during the proliferative-phase and secretory-phase in human endometrium and in Mouse Estrous Cycle. Progesterone could promote FOXO1 expression, while Estrogen inhibited it expression. In this study, the expression of FoxO3 throughout did not change and was weak. Our data indicated that FOXO1 precipitated in and regulated the menstrual cycle.

Keywords: FOXO1, menstrual cycle, E2, P4

Introduction

Embryo implantation is an extremely complex process, including apposition, adhesion, penetration and trophoblast invasion. Implantation failure is one of the major reasons for infertility and remains an obstacle to the progress of assisted reproductive techniques [1]. By investigating single or limited numbers of genes, investigators have identified some of the molecules associated with receptivity, including cytokines, growth factors, adhesion molecules, and extracellular matrix components during the preparation and development of an appropriate endometrium for blastocyst adhesion and implantation [2-5].

FOX proteins are sub-divided into 19 groups with each subclass designated by a letter, from FOXA to FOXS, and each member by a number. The agreed nomenclature also distinguishes between different species: uppercase spelling, for example FOXO1, is reserved for human proteins whereas the first letter is capitalized for murine proteins (e.g. Foxo1). Capitals for the first and subclass letters are used in other chordates (e.g. FoxO1). Four members constitute this group: FOXO1 (previously known as FKHR), FOXO3 (FKHRL1), FOXO4 (AFX or Mllt7) and FOXO6 [6]. They are highly conserved through evolution and essential to various cellular processes such as regulating the expression of the genes involved in the cell cycle [7, 8], DNA damage repair [9], oxidative stress resistance [10-12], energy metabolism [13], and apoptosis [14, 15]. Fox proteins contain a sequence of 80-100 amino acids forming a motif that binds to DNA: the fork head motif. This motif is also known as the winged helix due to the butterfly-like appearance of the loops in the protein structure of the domain [16]. Hormone plays a

functional role in embryonic development and set up the receptivity of endometrium. As embryo implantation place, uterus is impacted with estrogen and progesterone.

Recent studies with baboons have shown low levels of Foxo1 mRNA in the endometrium during the late follicular phase of the menstrual cycle, increasing levels during the midluteal phase, and peak levels in a pregnant baboon's endometrium [17]. In this study, we examined the expression of FoxO1 and FoxO3 in human and mouse menstrual cycle and whether E2 and P4 could regulate FoxO1 and FoxO3 in mouse endometrium.

Materials and methods

Tissue collection

The protocol for human study was approved by the Institutional Review Board of Dalian Medical University. All human specimens used in this study were collected from patients between the ages of 30 and 45 from 2011 to 2013, with their consent; endometrial blocks were obtained from 48 hysterectomy specimens (including eight specimens each from early proliferative phase, mid-proliferative phase, late proliferative phase, early secretory phase, mid-secretory phase, late secretory phase).

Animals

Mice of Kunming species were from Lab Animal Center in Dalian Medical University of China. All experimental procedures involves in the mouse studies were approved by the Institutional Review Board in Dalian Medical University. Adult female mice weighted 20-24 g was maintained under controlled environmental conditions. The mice were housed in a temperature 22-25°C, humidity 60%, and light-controlled (12 h light: 12 h darkness) with ad libitum access to water and food.

Mouse estrous cycle determination

Briefly, the tip of a plastic pipette filled with 20 μL of PBS was gently inserted into the vagina and flushed three times. The final flush was collected and placed on a glass slide and the estrous cycle stage was determined microscopically by the types and relative numbers of cells present based on the following criteria: diestrus stage contains mostly polymorphonuclear leukocytes and few epithelial cells, proestrus stage contains mostly nucleated and cornified epithelial cells, estrus stage contains mostly cornified epithelial cells and the metestrus stage contains mostly cornified epithelial cells, PMNs and a few nucleated epithelial cells.

Steroid hormonal treatments

To determine whether FOXO1 and FOXO3 respond to E2 and/or P4, adult female mice were ovariectomized irrespective of the stage of the estrous cycle and rested for 11 days. They were given an injection of E2 (100 ng/mouse; 0.1 mL) 3 days and rested for 2 days. Then they were divided into three groups, all which were injected for 4 days: 1) E2 group, injected with E2 (100 ng/mouse; 0.1 mL); 2) P4 group, injected with P4 (2 mg/mouse; 0.1 mL); 3) E2 plus P4 group, injected with P4 (2 mg/mouse; 0.1 mL) for 3 days and injected with P4 (2 mg/mouse; 0.1 mL) plus E2 (10 ng/mouse; 0.1 mL). After 18 hours, mice were sacrificed and uteri were collected for Immunohistochemistry to study FOXO1 and FOXO3 expression and distribution. The number of each group was five. The hormones were dissolved in sesame oil and injected subcutaneously.

Immunohistochemistry

Serial sections (6 μm) were prepared from paraffin-embedded tissues. The sections were fixed at 60°C for three hours, deparaffinized in xylene and rehydrated in graded alcohol. The slides were microwaved (Defrost) for 25 min in citrate buffer in order to unmask antigen and were washed with phosphate-buffered saline (PBS) after cooling for one hour. Slides were incubated in 3% H_2O_2 for 20 min to block endogenous peroxidase activity. After washing in PBS, sections were blocked with blocking buffer supplemented with normal goat serum at 37°C for 15 min to eliminate non-specific binding of conjugated secondary antibodies before incubation overnight at 4°C with FOXO1 and FOXO3 antibody (1:100). After washing with PBS, sections were incubated with biotinylated secondary antibody for 40 min at 37°C. And sections were washed with PBS, and then were incubated with streptavidin-horseradish peroxidase 40 min at 37°C. Positive reactions were

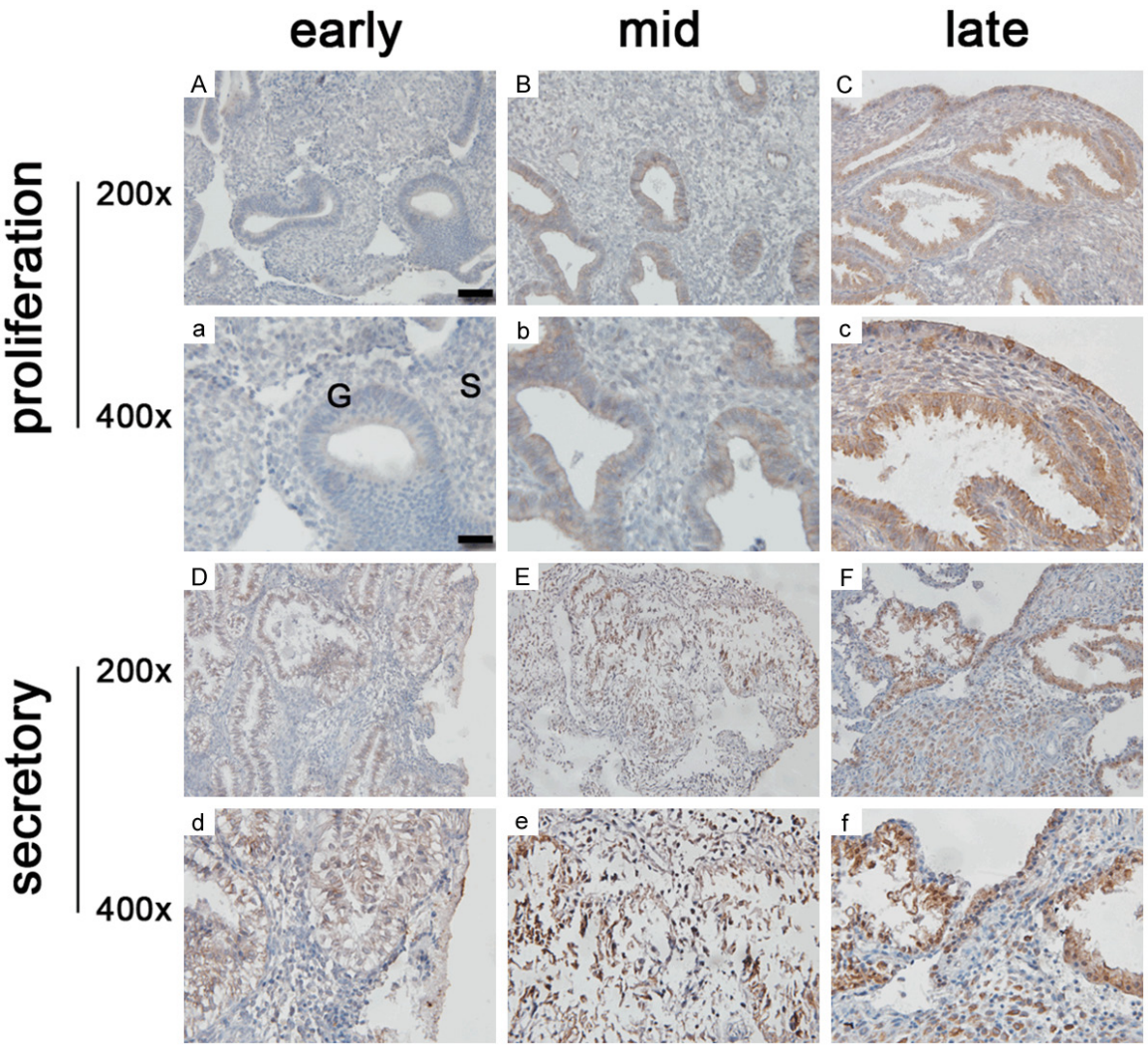

Figure 1. Expression of FOXO1 in endometrial tissue during the menstrual cycle of human. Paraffin-embedded endometrial tissues at various points of the menstrual cycle were analyzed by immunohistochemistry. FOXO1 expression in (A, a) early proliferation, (B, b) mid-proliferation, (C, c) late proliferation, (D, d) early secretory, (E, e) mid-secretory, (F, f) late secretory. glandular epithelium (G), and stromal cells (S). (A-F) 200×, Bar =50 μm; (a-f) 400×, Bar =20 μm.

visualized with a (DAB)-peroxidase substrate and counterstaining with haematoxylin for 30 s. Photomicrographs were taken using OLYMPUS TH4-200 microscopy.

Results

FOXO1 and FOXO3 expression in human endometrium

Immunohistochemistry was performed to examine the distribution of FOXO1 and FOXO3 protein in the human endometrial during proliferative-phase and secretory-phase. As shown in **Figure 1**, in the early proliferative phase, FOXO1 was minimally expressed (**Figure 1Aa**). In the mid-proliferative stage, FOXO1 was expressed in glandular (**Figure 1Bb**). In the late-proliferative stage, FOXO1 was expressed strongly in glandular epithelia and a few in stromal cells (**Figure 1Cc**). In early secretory stage, FOXO1 was also expressed in glandular epithelia but weaker than in the late-proliferative stage (**Figure 1Dd**). In the mid-secretory and late secretory stage, the expression of FOXO1 was in glandular epithelia and in stromal cells and expressed in cell nucleus (**Figure 1Ee**, **1Ff**). As shown in **Figure 2**, in the proliferative phase and early secretory stage, FOXO3 was barely expressed (**Figure 2Aa-Dd**). In the mid-secretory secretory stage, FOXO3 was only expressed

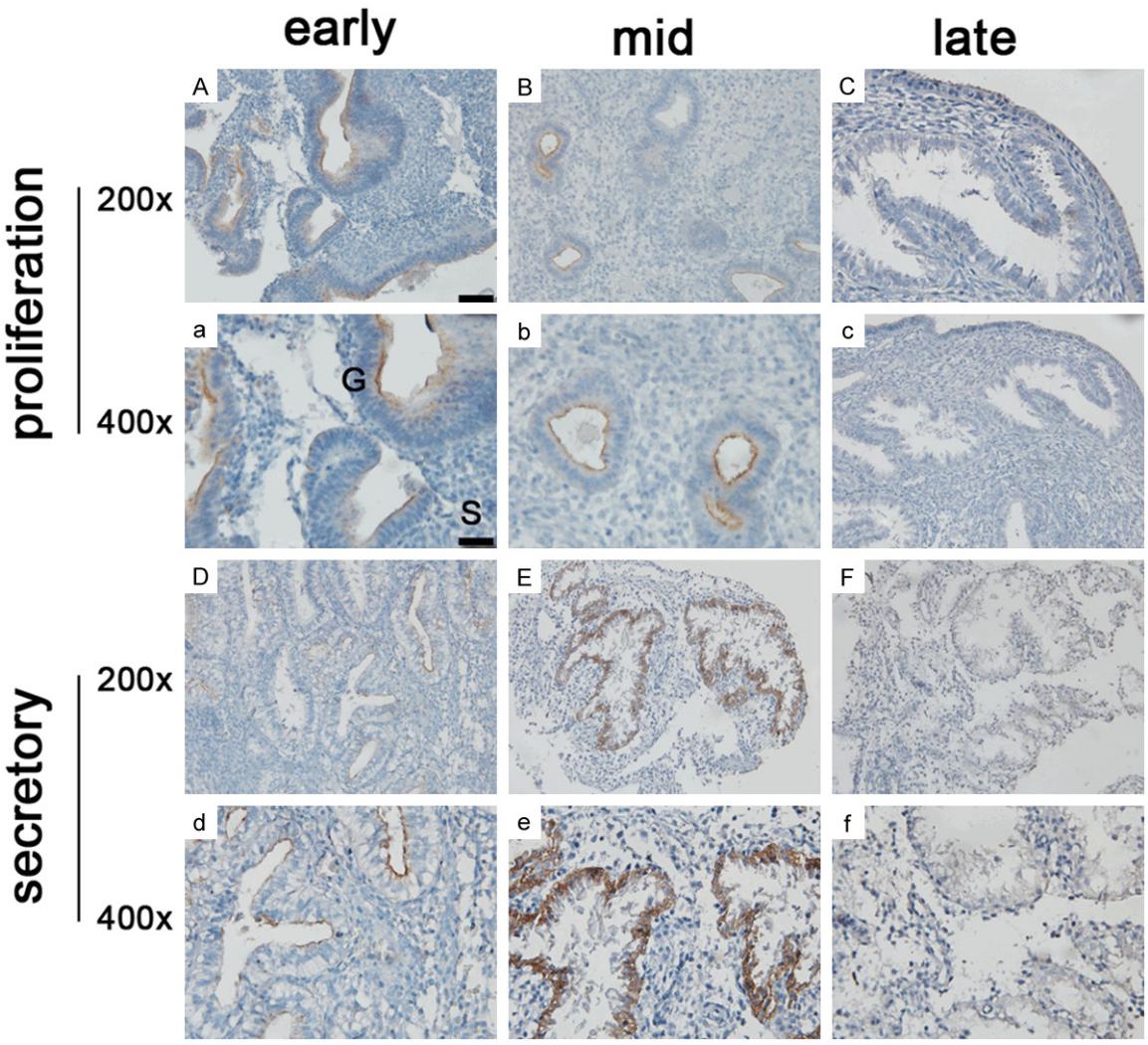

Figure 2. Expression of FOXO3 in endometrial tissue during the menstrual cycle of human. Paraffin-embedded endometrial tissues at various points of the menstrual cycle were analyzed by immunohistochemistry. FOXO3 expression in (A, a) early proliferation, (B, b) mid-proliferation, (C, c) late proliferation, (D, d) early secretory, (E, e) mid-secretory, (F, f) late secretory. glandular epithelium (G), and stromal cells (S). (A-F) 200×, Bar =50 μm; (a-f) 400×, Bar =20 μm.

in glandular epithelia (**Figure 2Ee**). And in late secretory stage, the expression of FOXO3 was not detected (**Figure 2Ff**).

FOXO1 and FOXO3 expression in mouse estrous cycle

Immunohistochemistry was performed to examine the distribution of FoxO1 and FoxO3 protein *in* Mouse Estrous Cycle. As shown in **Figure 3**, the expression of FoxO1 was weak in diestrus stage (**Figure 3Aa**). The FoxO1 was expressed in in glandular epithelia and stromal cells in proestrus stage and estrus stage (**Figure 3Bb**, **3Cc**). Then there was no expression of FoxO1 in metestrus stage (**Figure 3Dd**). As shown in **Figure 4**, the expression of FoxO3 was hardly in the whole mouse estrous cycle.

The expression of FoxO1 and FoxO3 treated by E2 and P4

We used Immunohistochemistry to examine the expression of FoxO1 and FoxO3 in the ovariectomized mouse uterus, the results showed that FoxO1 expression was low in ovariectomized uteri treated with E2 (**Figure 5Aa**, **Bb**). However, the expression of FoxO1 showed a significant increase in glandular epithelium and stromal cells in ovariectomized uteri treated

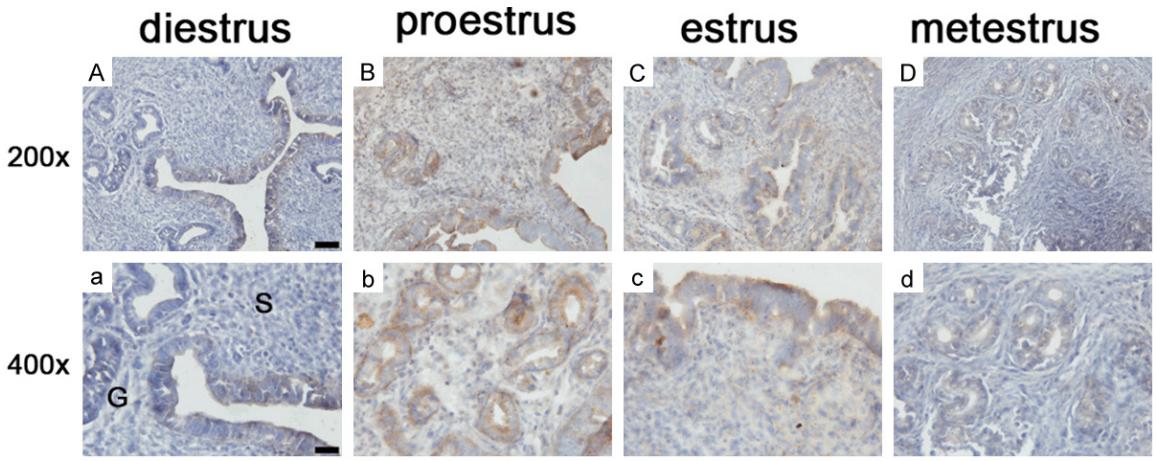

Figure 3. Expression of FOXO1 in endometrial tissue during the menstrual cycle of mouse. Paraffin-embedded endometrial tissues at various points of the menstrual cycle were analyzed by immunohistochemistry. FOXO1 expression in (A, a) diestrus, (B, b) proestrus, (C, c) estrus, (D, d) metestrus. glandular epithelium (G), and stromal cells (S). (A-D) 200×, Bar =50 μm; (a-d) 400×, Bar =20 μm.

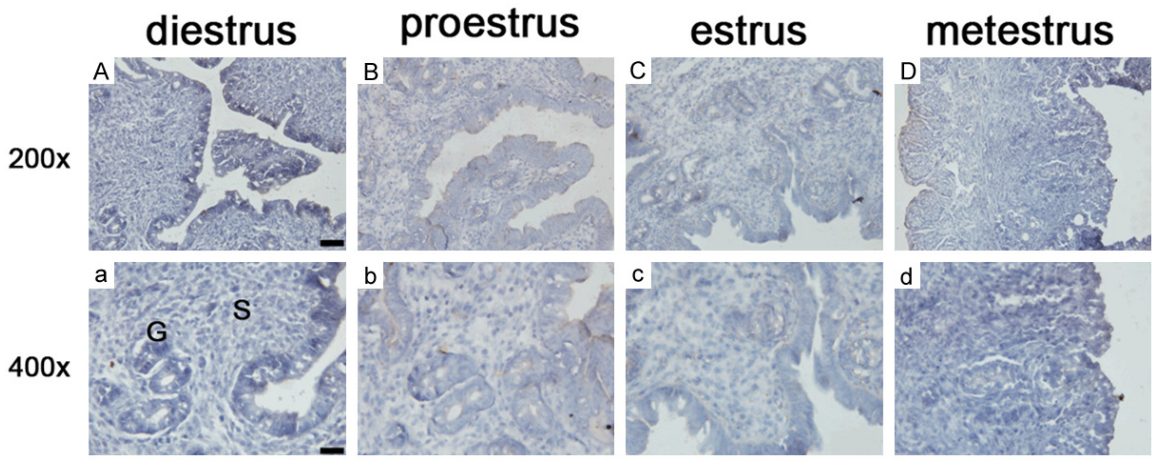

Figure 4. Expression of FOXO3 in endometrial tissue during the menstrual cycle of mouse. Paraffin-embedded endometrial tissues at various points of the menstrual cycle were analyzed by immunohistochemistry. FOXO3 expression in (A, a) diestrus, (B, b) proestrus, (C, c) estrus, (D, d) metestrus. glandular epithelium (G), and stromal cells (S). (A-D) 200×, Bar =50 μm; (a-d) 400×, Bar =20 μm.

with P4 (**Figure 5Cc**). A combined treatment with E2 plus P4 decreased the level of FoxO1 than P4 group (**Figure 5Dd**). The expression of FoxO3 was a little stronger in ovariectomized uteri treated with P4 (**Figure 6Cc**) than treated with E2 (**Figure 6Bb**) and combined treatment with E2 plus P4 (**Figure 6Dd**) and control group (**Figure 6Aa**).

Discussion

The exact molecular characteristics of the embryo implantation are still not completely characterized because of the complexity of using human embryos and endometrial tissue in research. Therefore other means must be elucidated for research of receptivity of the endometrium. During the window of implantation, endometrial epithelial cells undergo ultrastructural changes and express a variety of molecules to facilitate the adhesion of the embryo to the uterine endometrium [18]. Adhesion-related molecules such as mucins and integrins increase in the window of implantation, and are known to participate in the establishment of endometrial receptivity [19, 20]. For example, the expression of integrin

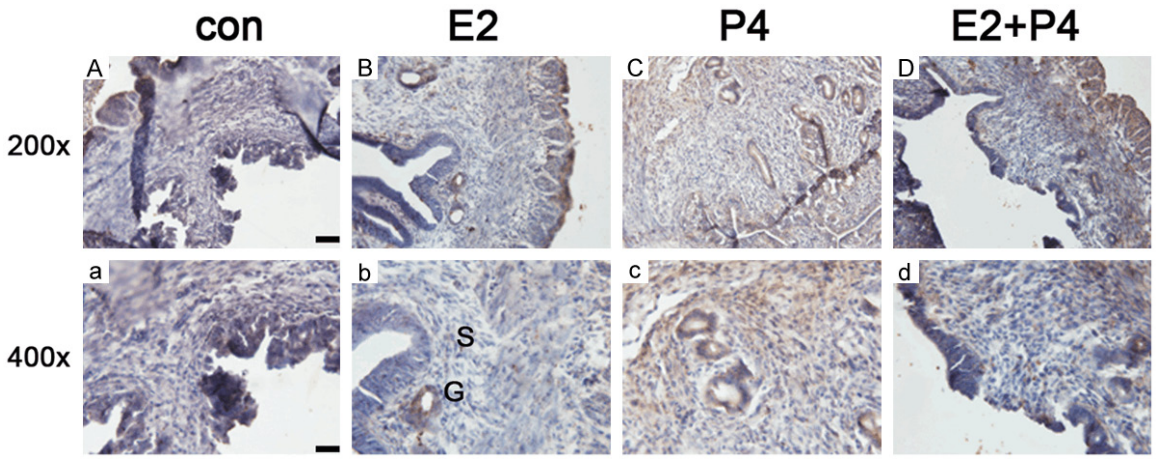

Figure 5. Effects of E2 and P4 on FoxO1 in the ovariectomized mouse uterus. A, a: The expression of FoxO1 treated by control in the ovariectomized mouse uterus; B, b: The expression of FoxO1 treated by E2 in the ovariectomized mouse uterus; C, c: The expression of FoxO1 treated by P4 in the ovariectomized mouse uterus; D, d: The expression of FoxO1 treated by E2+P4 in the ovariectomized mouse uterus. A-D: 200×, Bar =50 μm; a-d: 400×, Bar =20 μm.

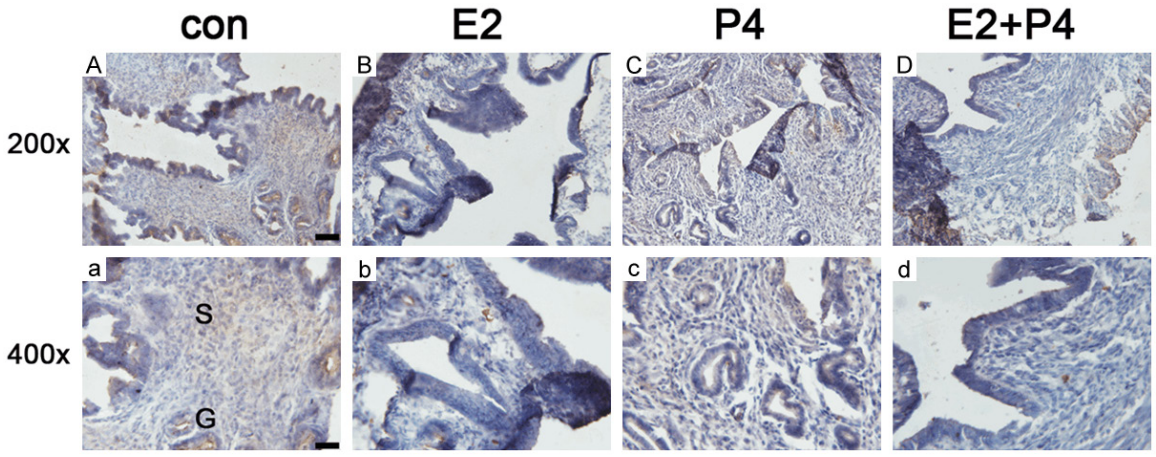

Figure 6. Effects of E2 and P4 on FoxO3 in the ovariectomized mouse uterus. A, a: The expression of FoxO3 treated by control in the ovariectomized mouse uterus; B, b: The expression of FoxO3 treated by E2 in the ovariectomized mouse uterus; C, c: The expression of FoxO3 treated by P4 in the ovariectomized mouse uterus; D, d: The expression of FoxO1 treated by E2+P4 in the ovariectomized mouse uterus. A-D: 200×, Bar =50 μm; a-d: 400×, Bar =20 μm.

avb3 is significantly increased throughout the mid-secretory and late-secretory phases [21] and has been recognized as a marker of the implantation window [22, 23].

Over the last decade, studies have demonstrated that FOXOs play critical roles in a wide variety of cellular processes. FOXOs transcriptionally activate or inhibit downstream target genes, thereby playing an important role in proliferation, apoptosis, autophagy, metabolism, inflammation, differentiation, and stress resistance. FOXO proteins have both a nuclear localization sequence and a nuclear export sequence within the C-terminal DNA binding domain. Kinases and interactions with other proteins modulate the effectiveness of these nuclear localization sequences and nuclear export sequences, which forms the basis of FOXO shuttling in and out of the nuclear compartment. The cytoplasmic sequestration of FOXO proteins is mediated by a combination of binding partners and changes in the properties of FOXO. The chaperone protein 14-3-3 binds to FOXO factors in the nucleus and allows their active export [24]. It also blocks the nuclear

localization signal to prevent FOXO re-entry into the nucleus [25].

In this study, we found that FOXO1 expression was significantly increased in the mid- and late secretory phases of the human endometrial cycle (**Figure 1**) and in proestrus stage and estrus stage of mouse estrous cycle (**Figure 3**). Altogether, these results suggest that FOXO1 may be necessary for the establishment of endometrial receptivity and its expression may serve as a potential marker in the evaluation of endometrial receptivity. As the endometrium switches from the proliferative to the secretory phase, progesterone is increased consistently, and reaches its peak in the mid-secretory phase. Increased progesterone facilitates embryo implantation in humans [26, 27]. Progesterone is an upstream regulatory factor for many molecules related with implantation [28], such as osteopontin, integrin, insulin-like growth factor, vascular endothelial growth factor and prostaglandin E2 in the human endometrial epithelium [29-32]. By immunohistochemistry staining, we found that the highest expression of CD82 occurred by P4 treated (**Figure 5**). These results *in vivo* suggest that the progesterone facilitates embryo implantation by up-regulating FOXO1.

In conclusion, FOXO1, not FOXO3 expression, up-regulated by progesterone, is likely related to endometrial receptivity, and can serve as a marker for the window of implantation.

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Disclosure of conflict of interest

None.

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