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Original Article

FoxM1 influences embryo implantation and is regulated by 17 beta-estradiol and progesterone in mouse uteri and endometrium cells

Yunpeng Xie, Dan Cui, Ying Kong

Department of Biochemistry and Molecular, Dalian Medical University, Dalian, Liaoning Provincial, China

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Abstract: To be a successful implantation, endometrial receptivity should be established. Forkhead box M1 (FoxM1) is described as a major oncogenic transcription factor in tumor initiation, promotion, and progression. FoxM1 regulates the expression of lots of targeted genes important to cell differentiation, proliferation and apoptosis; cell-cycle progression; and tumor angiogenesis, migration, invasion, and metastasis. According to these functions, we believe that FoxM1 should also play an essential role in embryo implantation. To test our hypothesis, we observed the expression and distribution of FoxM1 during the early pregnancy of mouse. Then, we used Immunohistochemistry to examine the expression of FoxM1 induced by E2 and/or P4 in the ovariectomized mouse uterus and human endometrium cells. This study further investigated whether FoxM1 was an important factor in the implantation. Our results showed that FoxM1 expressed in the mouse uterus during early pregnancy (Day 1 to 5). The expression of FoxM1 gradually increased along pregnancy process; FoxM1 expression could be increased by E2. On the contrary, FoxM1 expression could be decreased by P4 and E2 plus P4. We also detected the proliferation of human endometrium cells. We found that E2 might promote cells proliferation, while P4 and E2 plus P4 inhibited cells proliferation; Inhibiting FoxM1 could interfere the embryo implantation of mouse. Amplification or inhibiting of FoxM1 in JAR cells can increase or decrease the adhesion rate to RI95-2 and HEC-1A cells separately. Our data indicate that FoxM1 might play an important role during the process of mouse embryo implantation.

Keywords: FoxM1, E2, P4, implantation

Introduction

Embryo implantation is a reciprocal interaction between an implantation-competent blastocyst and a receptive uterus [1]. It is a crucial step for the successful establishment of mammalian pregnancy. There is a limited time period, known as the “window of implantation”, when the uterus are receptive to the implanting embryo [2, 3]. 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 [4-7]. For example, in mice, this window of implantation is tightly regulated by E2 (estrogen), with low levels being required for implantation to occur in uteri primed with P4 (progesterone); but slight E2 elevations above optimal levels render the uterus completely refractory to implantation [8]. Administration of minute doses of E2 during the per-implantation period causes pregnancy failure in mice [9]. Ovarian hyperstimulation causing increases in endogenous E2 can disrupt implantation in mice [10] and is associated with higher instances of failed implantation in humans [11]. Supra-optimal estrogen levels can prematurely close the window of uterine receptivity [8], accelerate or retard transport of blastocysts through the fallopian tubes [12], and damage the developing blastocyst itself [13]. The ratio of E2 to P4 in maternal circulation appears to be particularly important, with high E2: P4 ratios being detrimental to implantation in both mice and humans [14, 15].

Forkhead box M1 (FoxM1), as a member of Forkhead family of transcription factors, shares

homology in Winged Helix/Forkhead box DNA-binding domain [16]. It has been recognized that FoxM1 is involved in cell proliferation and apoptosis which regulates the developmental function of many organs in the body [17]. Several lines of evidence demonstrate that overexpression of FoxM1 occurs in a wide variety of human tumors frequently, including medulloblastoma [18], colorectal cancer [19], hepatocellular carcinoma [20], breast cancer [21], non-small cell lung cancer [22] and so on. Embryo implantation and cancer follow a similar progression and molecular mechanisms, such as epigenetic processes and dynamic regulation of cell migration and invasion [23]. So, given the similarity between the progress of tumor progression and embryo implantation, we presume that FoxM1 may be an indispensable factor in implantation.

To investigate whether FoxM1 could be regulated by E2 and P4, we used two human uterine epithelial cell lines as in vitro models: HEC-1A and RL95-2. HEC-1A was used as a model of non-receptive endometrium, and RL95-2 was used as a model of receptive endometrium [24-27]. The cell lines were chosen based on earlier studies which have demonstrated that RL95-2 cells have stronger adhesiveness for human JAR choriocarcinoma multicellular spheroids compared to HEC-1A cells [28-30] and are thus considered as a model of the receptive endometrium.

In this study, we examined the expression of FoxM1 in the mouse uterus during pre-implantation period. We also examined the expression of FoxM1 with ovarian steroid hormones E2 and P4 treated in the mouse uterus and human endometrium cells. We studied the effect of blocked FoxM1 on embryo implantation.

Materials and methods

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 aged 20-24 g and adult male mice aged 40-44 g were 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 superovulation and antibody injection

Each mouse was injected with pregnant mare serum gonadotropin (PMSG; 10 IU/0.1mL, Sigma) followed by hCG (10 IU/0.1mL, Sigma) 48 hours later. Then females were placed with males (one female with one male per cage). Females were checked for the presence of a vaginal plug in the next morning, which was defined as D1 if the vaginal plug came out. The mice were killed in D1-D5.

To detect whether FoxM1 plays a part in embryo implantation, 20 pregnant mice were divided into 2 groups randomly. On Day 2 of pregnancy at 10 o'clock, after 10 mice were anesthetized with pentobarbital sodium (50 mg/kg), FoxM1 polyclonal antibody (5 μl, 200 μg/mL), was injected into the right uterus horn, and IgG injected into the left uterus horn. In addition, 10 mice were anesthetized and injected with IgG into the left uterus horn, with no treatment of the right uterus horn. All mice were euthanized at 10 o'clock on Day 8 of pregnancy, and the number of embryo implanted was counted.

Immunohistochemistry

Uterine tissues were dipped in 4% (v/v) paraformaldehyde (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China) in phosphate-buffered saline (PBS; pH 7.4) for 24 h at 4°C, then dehydrated in sucrose. The frozen section of uteri which were 7 μm thick were blocked with 4% paraformaldehyde for 15 min at room temperature, washed in PBS, and endogenous peroxidase activity was blocked with 3% H_2O_2 for 15 min at room temperature, washed in PBS and incubated in 5% goat serum at 37°C for 15 min. The sections were incubated with rabbit anti-FoxM1 (1:100; Santa Cruz) and PBS (negative control) over night at 4°C. After washing three times with PBS, sections were incubated with a biotinylated secondary antibody (ZSGB-Bio Co., Ltd, Beijing, China) at 37°C for 40 min. And sections were wash with PBS, then were incubated with streptavidin-horseradish peroxidase (ZSGB-Bio Co., Ltd, Beijing, China) at 37°C for 40 min. Positive reactions were visualized with a diaminobenzidine (DAB)-peroxidase substrate (ZSGB-Bio Co., Ltd, Beijing, China) and counter-

staining with haematoxylin for 30 s. Photomicrographs were taken using OLYMPUS TH4-200 microscope.

Western blotting

Proteins from uterine tissues or cells were extracted using Lysis Buffer (KeyGen Biotech Co., Ltd., Nanjing, China) and the determination of protein concentration was tested by the BCA assay (KeyGen Biotech Co., Ltd., Nanjing, China). Equal amounts of protein extracts (30 μg) were separated by 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose filter (NC) membranes (Millipore, Bedford, MA, USA). The membranes were blocked in 5% non-fat milk in tris-buffered saline containing 0.1% Tween 20 (TBST) for 2 hours at room temperature and probed with primary antibodies FoxM1 (1:500, Santa Cruz), or β-actin (1:2000; Bioworld Technology Co., Ltd.,) overnight at 4°C. The membranes were washed with TBST three times. Then the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit antibody (1:2000; ZSGB-Bio Co., Ltd, Beijing, China) for 1 hour at room temperature. After washed with TBST four times, the membranes were detected using an enhanced chemiluminescence detection system (ECL, GE Healthcare) and visualized using Bio-Rad Laboratories. Membranes were tested with β-actin or GAPDH as a loading control.

Steroid hormonal treatments

To determine whether FoxM1 responds 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 four groups, all which were injected for 4 days: 1). Control group, injected with sesame oil; 2). E2 group, injected with E2 (100 ng/mouse; 0.1 mL); 3). P4 group, injected with P4 (2 mg/mouse; 0.1 mL); 4). 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 FoxM1 expression and distribution. The number of each group was five. The hormones were dissolved in sesame oil and injected subcutaneously.

Cell culture

RL95-2 and HEC-1A were acquired from the American Type Culture Collection (Manassas, VA, USA). RL95-2 cells were grown in DMEM/F12 (1:1) supplemented with 10% FBS, 0.005 mg/mL insulin, 100 U/mL penicillin and 100 mg/mL streptomycin. HEC-1A cells were grown in McCoy's 5A supplemented with 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin. All types of cells were maintained at 37°C, 5% CO_2 in humidified air.

Stable transfection of JAR cells

JAR cells were trypsinized and seeded into 6-well plates. When cells reached 90% confluence, overexpression and shRNA of FoxM1 were transiently transfected into cells using 0.4 mg of plasmids in the presence of 2 mL Lipofectamine reagent and plus reagent (Invitrogen) per manufacturer's instructions. The transfection was terminated 6 h later and the cells were harvested after 48 h. Stable transfected JAR cells were maintained in the media with 0.2 mg/ml of G418.

Cell adhesion assay

RL95-2 cells were grown in 96-well plates to form a confluent monolayer. Trophoblastic cells in differently treated were stained with CellTracker™ Green CMFDA (Life Technologies, USA) 1 h before the adhesion assay. The cells were gently seeded onto RL95-2 or HEC-1A cells monolayers in trophoblastic culture medium. After 1 h, unbound trophoblastic cells were removed by washing with PBS. The attached cells were detected by multimode plate reader (PerkinElmer, USA) and photographed on a fluorescent phase microscope (Olympus, Japan)

Cell proliferation assay

Cell proliferation was detected by a Cell Counting Kit-8 assay. HEC-1A and RL95-2 were suspended in medium supplemented with 15% heat-inactivated fatal bovine serum and subsequently seeded in 96-well plates and incubated for 24 h. After that, plates were divided into four groups: 1). Control group; 2). Treated with E2 group; 3). Treated with P4 group; 4). Treated

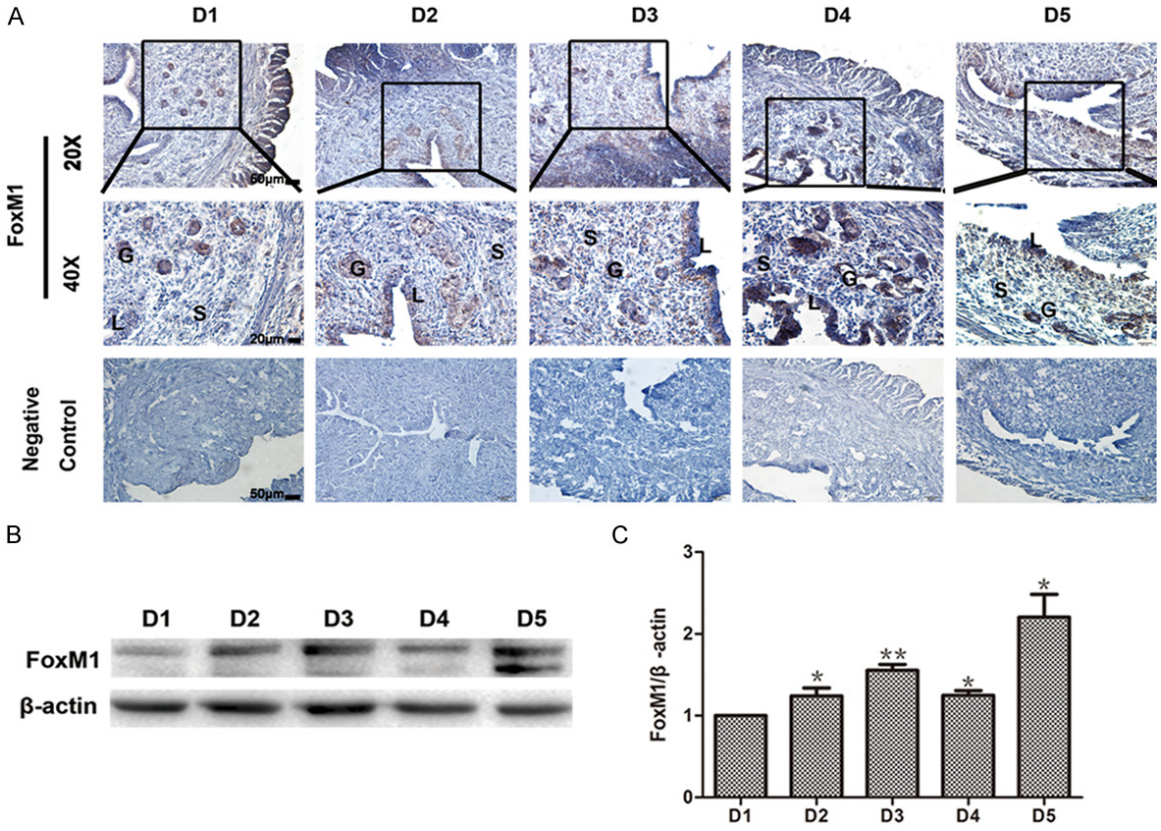

Figure 1. Expression of FoxM1 in mouse uterus during early pregnancy. A. Expression of FoxM1 detected by Immunohistochemistry of mouse endometrium, luminal epithelium (L), glandular epithelium (G), and stromal cells (S). B, C. Expression of FoxM1 detected by Wertern blotting of mouse endometrium during pre-pregnancy. β-actin blots were used as controls. Endometrial tissues were divided into 5 groups (D1-D5). (*$P < 0.05$, **$P < 0.01$).

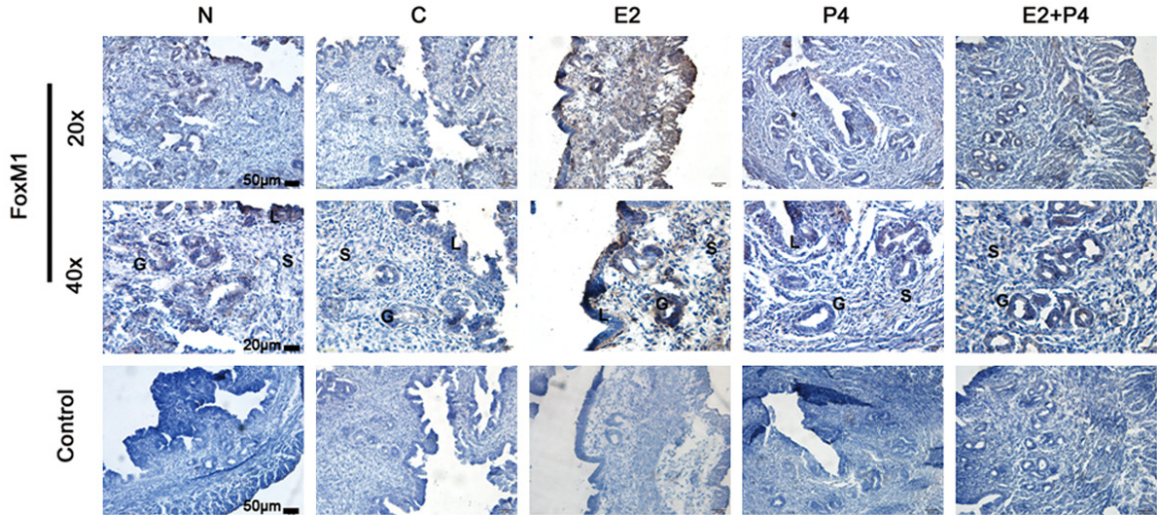

Figure 2. Effects of E2 and P4 on FoxM1 in the ovariectomized mouse uterus, luminal epithelium (L), glandular epithelium (G), and stromal cells (S).

with E2 plus P4 group. Then, plates were continued incubating 24 h, 48 h, 72 h, 96 h, 120 h. After being incubated, the cultures were added 10 μl CCK-8 solution to each well and incubated at 37°C for another 2 h. OD value of absorbance at 450 nm was measured by Thermo

Scientific Fluoroskan Ascent FL. The results were plotted as means ± SD of three independent experiments having three determination per sample for each experiment.

Statisical analysis

All of the experiments were replicated three times independently. The data were analyzed using an ANOVA followed by Fisher's Least Significant Different Test (Fisher LSD) and Independent-Samples T test with SPSS software (Version 19.0). Differences were considered significant when $P < 0.05$.

Results

Expression of FoxM1 in mouse uterus during early pregnancy

Immunohistochemistry was performed to examine the distribution of FoxM1 protein in the uteri of peri-implantation pregnant mice. FoxM1 was mainly located in the glandular epithelium and luminal epithelium on Day 1. Then FoxM1 was located in the glandular epithelium and luminal epithelium on Day 2. But FoxM1 was not detected in the stromal cells obviously on the two days. FoxM1 was negative in the luminal epithelium, but detected in the stromal and glandular epithelium cells obviously on the Day 3. The expression of FoxM1 was located in the glandular epithelium and luminal epithelium on Day 4 and Day 5 (**Figure 1A**). FoxM1 protein levels were gradually increased from Day 1 to Day 3, then low in Day 4, and reaching a peak value on Day 5 (**Figure 1B**, **1C**).

Uteri expression of FoxM1 is regulated by E2 and P4

We used immunohistochemistry to examine the expression of FoxM1 in the ovariectomized mouse uterus, the results showed that FoxM1 expression was low in ovariectomized uteri treated with tea oil and that expression was localized to the glandular epithelium. However, the expression showed a significant increase in glandular epithelium and stromal cells. A P4 injection prominently decreased in the glandular epithelium and stromal cells compared to the normal group. A combined treatment with E2 plus P4 increased the level of FoxM1 in the glandular epithelium but lower than E2 group (**Figure 2**).

E2 and P4 regulate the expression of FoxM1 in endometrial cells

Our observations of uterine expression of FoxM1 in mice suggested that FoxM1 could be regulated by ovarian E2 and P4. Therefore, we further examined the expression of FoxM1 in human endometrium cell. The expression of FoxM1 was increased by E2 in a dose-dependent manner (**Figure 3A**) and time-dependent manner (**Figure 3C**) in RL95-2 and HEC-1A cells. However, the expression of FoxM1 was decreased by P4 in a dose-dependent manner (**Figure 3B**) and time-dependent manner (**Figure 3D**) in RL95-2 and HEC-1A cells. Then we investigated the combined infection of E2 plus P4, result showed that E2 plus P4 group was significantly lower than the control group in RL95-2 and HEC-1A cells. E2 and P4 were dissolved in absolute ethyl alcohol and Con group added absolute ethyl alcohol as control (**Figure 3E**).

E2 and P4 regulate the proliferation of endometrium cells

As showed in **Figure 3F**, **3G**, E2 group significantly increased RL95-2 and HEC-1A cells proliferation compared to control group. P4 group and E2 plus P4 group hardly promoted cells proliferation in HEC-1A and RL95-2 cells.

Effects of FoxM1 antibody on embryo implantation in vivo and in vitro

To identify whether FoxM1 was necessary for the implantation, we injected FoxM1 antibody to the mouse uterus. As shown in **Figure 4A**, **4B**, injection of FoxM1 antibody interfered embryo implantation obviously: the number of implanted embryo in the horn treated with IgG (10.20 ± 0.92) was higher than that in the horn treated with FoxM1 antibody (2.20 ± 1.03, $P < 0.01$). In the other group, there was no statistical difference for the embryo implantation between the untreated horn (10.30 ± 0.95) and IgG control mice (10.20 ± 0.92).

In vitro implantation model constituting of trophoblastic cells and uterine epithelial RL95-2 cells and HEC-1A cells was utilized to analyze the role of FoxM1 in cell adhesion. The results showed that we had successfully screened out JAR-FoxM1 cells and JAR-sh FoxM1 cells (**Figure 4D**, **4E**; $P < 0.05$). The adhered trophoblastic

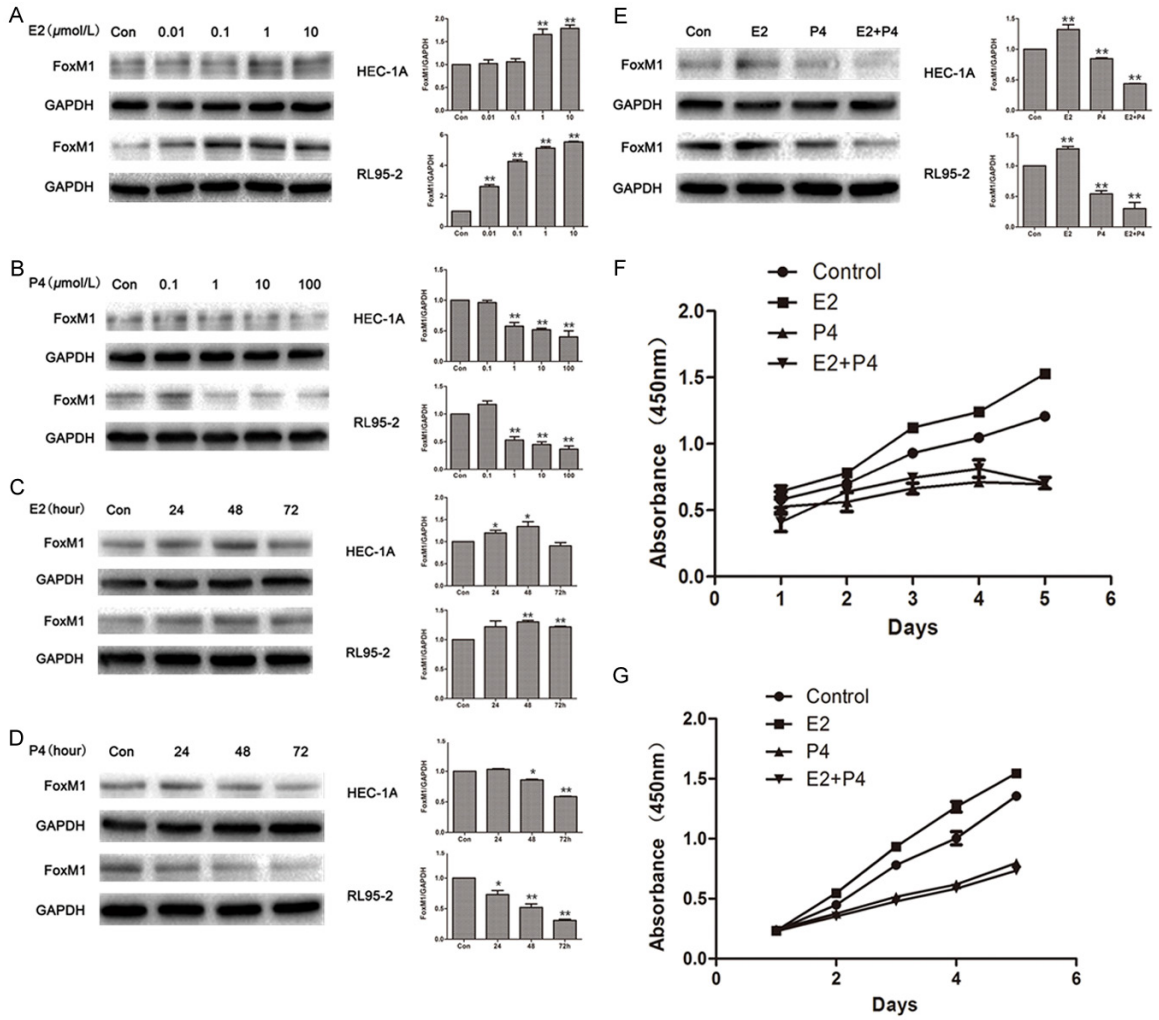

Figure 3. Effects of E2 and P4 on FoxM1 in human endometrium cells. A. The expression of FoxM1 treated by E2 with 0, 0.01, 0.1, 1, and 10 μmol/L in HEC-1A and RL95-2 cells for 48 h. B. The expression of FoxM1 treated by P4 with 0, 0.1, 1, 10 and 100 μmol/L in HEC-1A and RL95-2 cells for 48 h. C. The expression of FoxM1 treated by E2 with 0, 24, 48 and 72 h in HEC-1A and RL95-2 cells at 10 μmol/L. D. The expression of FoxM1 treated by P4 with 0, 24, 48 and 72 h in HEC-1A and RL95-2 cells at 100 μmol/L. $*P < 0.05$, $**P < 0.01$ versus control. E. The expression of FoxM1 treated by E2 10 μmol/L, P4 100 μmol/L for 48 h. F. The effect of combined treatment with E2 and P4 in the proliferation of HEC-1A cells. G. The effect of combined treatment with E2 and P4 in the proliferation of RL95-2 cells. ($*P < 0.05$, $**P < 0.01$).

cells were observed after fluorescent staining and the adhesion rate was analyzed (**Figure 4F**). It was showed that JAR-sh FoxM1 cells markedly decreased the adhesion rate of trophoblastic cells to RL95-2 cells and HEC-1A cells, compared to the control JAR cells. And JAR-FoxM1 cells increased the adhesion rate of trophoblastic cells to RL95-2 cells and HEC-1A cells, compared to the control JAR cells.

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. FoxM1, is a key regulator of both G1/S and G2/M phases of the cell cycle and mitotic spindle integrity [31]. Recent studies have strongly suggested that FoxM1 is oncogenic and plays important roles in angiogenesis, invasion and metastasis [32, 33]. Amplifications of FoxM1 gene has been reported in numerous tumors such as pancreatic carcinomas, breast cancer and hepatocellular carcinoma [34-37]. However, studies of FoxM1 in embryo implantation were few.

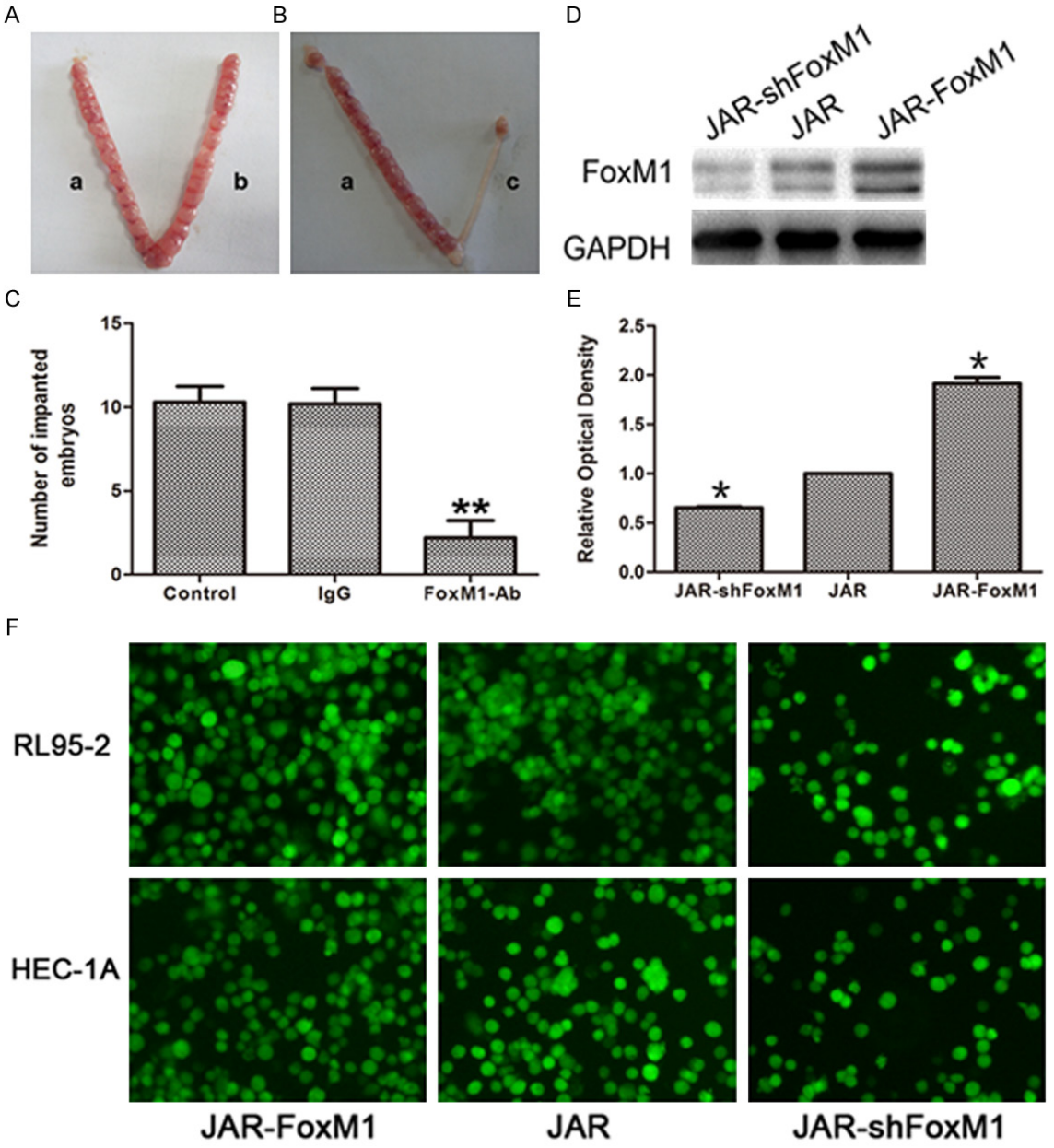

Figure 4. Effect of FoxM1 antibody on embryo implantation in vivo and in vitro. A. a, injected with IgG; b, no treatment. B. a, injected with IgG; c, injected with FoxM1 polyclonal antibody. C. The number of implanted embryos was significantly inhibited in the group injected with FoxM1 polyclonal antibody compared with IgG and no treatment groups (**$P < 0.01$). D, E. Identification of stable transfection JAR cells. F. Adhesion assay of trophoblastic cells. Cells were stained with CellTracker™ Green CMFDA 1 h before adhesion assay and photoed with fluorescence microscope.

It has been reported that embryo implantation has similar cellular processes to the invasion of cancer cells, like angiogenesis and immunosurveillance [38, 39]. The invasion of trophoblastic cells into the endometrium and formation of the placenta are the most important steps in embryo implantation. The process including endometrial epithelial, trophoblasts and stromal cells needs precise regulation of the differentiation and proliferation of trophoblast and endometrial decidualization [40]. The invasion process involves the interaction and regulation of adhesion molecules, growth factors, vasoactive factors cytokines and proteolytic [41, 42]. Matrix metalloprotrinases (MMPs), being capable of degrading all kinds of extracellular matrix

proteins, have been known to play direct roles in the process of embryonic implantation [43]. It has been shown that FoxM1 overexpression is associated with up-regulation of MMPs expression that in turn leads to degradation of the TIMPs, resulting in increased invasion and migration of tumor cells [44-46]. So it might be believed that FoxM1 regulates MMPs to impact on the invasion of trophoblasts in embryo implantation.

In our study, FoxM1 was localized to different sites on Day 1 to 5 of normal pregnancy. It is showed that the expression of FoxM1 protein was time-dependent during the embryo implantation period (**Figure 1**). The location of FoxM1 was transferred from glandular epithelium and luminal epithelium to stromal cells gradually on Days 1 to 3 (**Figure 1**). This might because that stromal cells need to proliferate for the following decidualization. Then the location of FoxM1 was back to glandular epithelium and luminal epithelium (**Figure 1**). This might be because that luminal epithelium secreted MMPs and glandular epithelium secreted growth factors, which were regulated by FoxM1. E-cadherin is a critical regulator in the development of the uterine and uterine function also is one of the best-characterized markers of epithelial-mesenchymal transition (EMT) [47, 48]. During the "window of implantation", the expression of E-cadherin decreased for a while, which is beneficial for the embryo to invade into endometrium. It is proven that Fox M1 and E-cadherin can be mutual inhibition [49, 50]. We might believe that during the Day 4, FoxM1 was inhibited by E-cadherin in the luminal epithelium, in order to embryo recognize endometrium. Then FoxM1 reemerged in the luminal epithelium in order to embryo invading endometrium.

There are several reports already revealing the effects of E2 on FoxM1 expression, such as regulation of FoxM1 by ERα in breast cancer [51], and FoxM1 co-binging with ERα in breast cancer [52]. Progesterone regulates many down-stream gene expressions through the receptor mediated way, such as Bmp2, Wnts, PAPPA and ILs, et al, which are involved in embryo implantation and development [53-56]. In this study, we investigated the regulatory effects of ovarian steroid hormones, E2 and P4, on FoxM1 expression in mouse uteri and human endometrium cells. E2 increased FoxM1 expression, however, P4 and E2 plus P4 decreased FoxM1 expression. During mouse normal pregnancy, E2 peaks around 15:30 on Day 3 until 08:30 on Day 4 of pregnancy, and P4 peaks from 03:30 of Day 4 onwards [57]. From the onset of puberty to menopause for human, the endometrium undergoes extensive remodeling in response to the ovarian steroid hormones E2 and P4 during each menstrual cycle in preparation for implanting an embryo. E2 promotes the proliferation and growth of the endometrial lining while P4 antagonizes estrogen-driven growth and promotes differentiation. In this study, E2 increased FoxM1 expression to endometrium proliferation. Then P4 plus E2 decreased FoxM1 expression to promote endometrium differentiation and decidualization.

We injected FoxM1 antibody in the mouse uterus. We found that lack of FoxM1 resulted in embryo implantation unsuccessful (**Figure 4**). These researches suggested that FoxM1 might play a significant role in the process of embryo implantation during early pregnancy.

We have concluded that the expression of FoxM1 followed a spatiotemporal pattern in the mouse endometrium during early pregnancy. The results of this investigation have demonstrated a specific expression pattern of FoxM1 in the mouse uterus and human endometrium induced by E2 and P4, also provide insights to our understanding regarding the potential roles of FoxM1 in uterine biology during implantation. High expression of FoxM1 in the mouse endometrium suggests that FoxM1 may be a novel molecule involved in the early processes of pregnancy, especially in the proliferation of endometrium and embryo invasion. However, further studies are needed to be detected the precise mechanism underlying the role of FoxM1 in the embryo implantation.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Ying Kong, Department of Biochemistry and Molecular, Dalian Medical University, Dalian, Liaoning Provincial, China. E-mail: yingkong@dlmedu.edu.cn

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