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—Original Article—

Expression and regulation of Foxa2 in the rat uterus during early pregnancy

Kazuki YAMAGAMI[1)], Nobuhiko YAMAUCHI[1)], Kaiyu KUBOTA[1)], Sho NISHIMURA[1)], Vishwajit Sur CHOWDHURY[2)], Kenichi YAMANAKA[3)], Masashi TAKAHASHI[4)], Shoji TABATA[1)] and Masa-aki HATTORI[1)]

[1)]*Department of Animal and Marine Bioresource Sciences, Graduate School Kyushu University, Fukuoka 812-8581, Japan*
[2)]*Division for Arts and Science, Faculty of Arts and Science, Kyushu University, Fukuoka 819-0395, Japan*
[3)]*Laboratory of Animal Resource Development, Faculty of Agriculture, Saga University, Saga 840-8502, Japan*
[4)]*Laboratory of Animal Breeding and Reproduction, Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan*

Abstract. The forkhead box a (Foxa) protein family has been found to play important roles in mammals. Recently, the expression of Foxa2 was reported in the mouse uterus, and it was reported to be involved in regulation of implantation. However, the regulation of Foxa2 expression in the uterus is still poorly understood. Therefore, the present study was conducted to investigate the expressional profiles of *Foxa2* in the rat uterus during the estrus cycle and pregnancy. Furthermore, the effect of steroid hormones and Hedgehog protein on the expression of *Foxa2* was analyzed *in vivo* and *in vitro*. In this study, the level of expression of *Foxa2* was low in the rat uterus during the different stages of the estrus cycle. However, the expression increased transiently during early pregnancy at 3.5 days post coitus (dpc) and decreased at 5.5 dpc. In ovariectomized rats, P4 treatment had no effect on the expression of *Foxa2* compared with the expression in control animals. Moreover, the expression of *Foxa2* in cultured epithelial cells was not increased by P4 treatment *in vitro*. However, *Foxa2* expression was significantly decreased in the rat uterus after 24 h of E2 treatment. Treatment of cells with a recombinant Hedgehog protein significantly increased the expression of *Foxa2*. These results suggest that the expression of *Foxa2* may transiently increase just before the implantation and it may be regulated by E2 and Hedgehog protein.
Key words: Foxa2, Ihh, Rat, Steroidal regulation, Uterus

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In mammalian reproduction, implantation of the embryo into the maternal uterus is a crucial step and requires spatiotemporally regulated suitable surroundings prepared by many complicated factors and processes involved with materno-fetal interaction. Successful implantation requires dramatic uterine tissue remodeling, which is strictly regulated by two sex steroid hormones, progesterone (P4) and 17β-estradiol (E2). P4 and E2 stimulate endometrial epithelial and stromal cells collectively to induce many cytokines and hormones for proliferation and differentiation for embryo attachment and acceptance. As a result, the endometrium acquires embryonic receptivity, which is even called the "window of implantation". Although the mechanism of its establishment is still poorly understood, it was recently suggested that a transcription factor, Forkhead box a 2 (Foxa2), participates in this process.

The Foxa protein family was first identified in liver nuclear extracts as transcription factors essential for regulation of the hepatocyte-specific expression of several target genes [1]. The Foxa protein contains a 110-amino acid motif that is conserved from yeast to humans [2]. The Foxa family has been found to have important roles in multiple stages of mammalian life, including early development, organogenesis, metabolism and homeostasis. The Foxa family includes *Foxa1*, *Foxa2* and *Foxa3* genes. In the early embryo, *Foxa2* is expressed in the node, notochord and floor plate, whereas *Foxa1* is detected only in the notochord and floor plate. Targeted disruption of *Foxa2* leads to an embryonic lethal phenotype in mice, with impaired formation of the node, notochord and foregut endoderm [3]. Recently, it was reported that the *Foxa2* gene and protein were localized in the mouse uterine glands [4]. Furthermore, *Foxa2* expression gradually increased until its peak at day 2.5 of pseudopregnancy (the first day a vaginal plug was observed was designated day 0.5) and then sharply decreased throughout the remaining period of pseudopregnancy. Interestingly, in *Foxa2* conditional ablation mice, implantation sites were significantly decreased compared with in normal pregnant mice [5]. Additionally, the number of uterine glands and the expression of leukemia inhibitory factor (*Lif*) were decreased in the *Foxa2* conditional ablation mice [5]. In mice, the highest levels of *Lif* were found prior to implantation in the glandular epithelium following the E2 surge in the morning of day 3.5 of pregnancy [6]. Since *Lif* null mice are infertile, it is considered that LIF is one of the essential cytokines for successful implantation [7]. These observations suggest

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Correspondence: N Yamauchi (e-mail: ny0531@agr.kyushu-u.ac.jp)

that Foxa2 plays an important role for implantation by inducing *Lif* expression in the mouse uterine gland.

In previous studies, the expression of *Foxa2* in the uterus was reported in the mouse [4], human [8] and bovine [9]. Although it was reported that *Foxa2* played an important role for implantation in mice [5], the regulatory mechanism of *Foxa2* expression remained unexplained. In the floor plate, initiation of Foxa2 expression was induced by Sonic hedgehog (*Shh*), a member of the Hedgehog (Hh) family, from the notochord [10]. Although *Shh* was not detected in the rat endometrium, Indian hedgehog (*Ihh*), a member of the Hh family, was detected in the luminal and glandular epithelium by *in situ* hybridization [11]. Since *Ihh* conditional ablation mice are infertile, it is considered that *Ihh* is one of the essential cytokines for successful implantation [12]. The deduced amino acid sequences of SHH and IHH have a high degree of similarity, particularly in the Hh signaling ligand peptide region [11]. *Ihh* peaked at day 2.5–3.5 of pseudopregnancy and decreased thereafter [13]. Takamoto *et al.* [13] also reported that the expressional profile of *Ihh* was similar to that of *Foxa2*, which transiently increased just before the implantation period. Thus, *Foxa2* may be promoted by *Ihh* in the rat uterus. However, the regulation of *Foxa2* expression by the Hedgehog protein has not yet been examined.

In the present study, we therefore investigated the expressional profiles of *Foxa2* during different stages of the estrus cycle as well as during pregnancy in the rat uterus using quantitative real-time PCR and immunohistochemistry. Then the effect of steroid hormones on the expression of *Foxa2* in the rat uterus was analyzed using ovariectomized (OVX) rats, ICI 182,780 (ICI; a pure estrogen receptor antagonist) treated rats and delayed implantation rats *in vivo*. Finally, the regulation *Foxa2* expression by the Hedgehog protein was analyzed using a recombinant Hedgehog protein in a cell culture system of endometrial epithelial cells.

Materials and Methods

Animals

This research was conducted using Wistar rats raised in our laboratory (Laboratory of Reproductive Physiology and Biotechnology, Department of Animal and Marine Bioresource Sciences, Graduate School of Agriculture, Kyushu University). The rats were housed under temperature- and light-controlled conditions (lights on at 0800 h, off at 2000 h) with free access to food and water. The stages of the estrus cycles in each rat were determined by the vaginal smear method. Adult female rats were mated with males, and the day on which spermatozoa were found in the vaginal smear was designated 0.5 days post coitus (dpc). All experiments were conducted according to the Guidelines for the Care and Use of the Laboratory Animals (Graduate School of Agriculture, Kyushu University) and with the approval of the Kyushu University Laboratory Animal Care and Use Committee.

For studies on steroid hormonal action, 7-week-old female rats were ovariectomized and subsequently rested for 2 weeks. Progesterone (P4, 10 mg/kg body weight) and 17β-estradiol (E2, 50 μg/kg body weight) were purchased from Steroids Inc. (Wilton, NH, USA) and injected subcutaneously with 200 μl sesame oil. Controls were given an equal volume of vehicle (sesame oil) only. Rats were killed after 6 h and 24 h of treatment, and uteri were rapidly isolated.

To inhibit implantation-initiating estrogenic stimuli, the pregnant rats at 3.5 dpc were treated daily with ICI 182,780 (1.5 mg/kg body weight), a pure estrogen receptor antagonist (Sigma, St. Louis, MO, USA), by subcutaneous injection. Rats were killed at 5.5 dpc, and uteri were isolated. To induce delayed implantation, the pregnant rats at 3.5 dpc were ovariectomized at 0830–0900 h. P4 (10 mg/kg body weight) was injected daily from 3.5 dpc to 6.5 dpc to maintain a delayed implantation state. At 7.5 dpc, P4 treatment was continued for half of the rats, and the remaining half was treated with P4 and E2 (1 μg/kg) to initiate implantation. Rats were euthanized at 8.5 dpc (24 h after treatment), and uteri were isolated. The number of rats used for each experimental group was three.

RNA extraction, reverse transcription (RT), polymerase chain reaction (PCR) and real-time PCR

Total RNA was extracted from homogenized uteri using a Sepasol-RNA I Super (Nacalai Tesque, Kyoto, Japan) with RQ1 RNase-Free DNase treatment (Promega, Madison, WI, USA), according to the manufacturers' protocols. RNA quality was assessed by ethidium bromide agarose gel electrophoresis and spectrophotometric UV absorbance at 260/280 nm. One microgram of each RNA sample was reverse transcribed with a MMLV High Performance Reverse Transcriptase (Epicentre, Madison, WI, USA) using an oligo-dT primer in a 20 μl solution. Real-time PCR was performed with iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) using a Chromo4 System (Bio-Rad Laboratories). The specific primers for real-time PCR were as follows: 5'- GGCATGAACACTTACATGAGC-3' (forward) and 5'-GCGCCCACATAGGATGAC-3' (reverse) for *Foxa2* (NM_012743, product length: 98 bp) and 5'-GACCGGTTCTGTCATGTCG-3' (forward) and 5'-ACCTGGTTCATCATCACTAATCAC-3' (reverse) for *Hprt* (NM_012583, product length: 61 bp). The PCR cycle parameters were an initial denaturation step at 95 C for 10 min and then 40 cycles at 95 C for 10 sec and 60 C for 30 sec. Hprt served as an internal control and was used to normalize for differences in each sample.

In situ hybridization of Foxa2

The Foxa2 PCR products were subcloned into a pGEM-T Easy Vector. Their antisense and sense RNA probes were synthesized using PCR-amplified templates and RNA polymerase promoter sequences. Digoxigenin (DIG)-labeled antisense and sense RNA probes were prepared with T7 or SP6 RNA polymerase using a DIG RNA Labeling Mix kit (Roche, Tokyo, Japan). The same primers as mentioned for real-time PCR were used for the preparation of DIG-labeled probes. Cryosections (10 μm thick) of the uterus at 3.5 dpc were processed in O.C.T. Compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and fixed with 4% paraformaldehyde. After washing with PBS, the sections were acetylated in 0.1 M triethanolamine containing 0.25% acetic anhydride. They were then hybridized with cRNA probes in 3 × SSC containing 50% formamide, 125 μg/ml yeast RNA, 100 μg/ml salmon sperm, 10% dextran sulfate, 1 × Denhardt's solution and 0.12 M phosphate at 60 C overnight. The next day, the sections were serially washed at 42 C with post-hybridization solution, i.e., 5 × SSC and 2 × SSC containing 50% formamide. They were then washed with 2 × SSC, 0.2 × SSC and 100 mM Tris-HCl (pH 7.5)

containing 150 mM NaCl and 0.01% Tween 20, treated for 60 min with 1.5% blocking reagent (Roche) and incubated with anti-DIG antibody conjugated with alkaline phosphatase at 4 C overnight. The next day, the sections were washed twice with 100 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 0.01% Tween 20 and then washed with 100 mM Tris-HCl (pH 9.5) containing 100 mM NaCl and 50 mM $MgCl_2$. A subsequent enzyme-catalyzed color reaction with BCIP/NBT produced an insoluble blue precipitate. After color development, the sections were washed with 6 mM Tris-HCl (pH 8.0) containing 0.6 mM EDTA and observed for color reaction under a microscope.

Immunohistochemistry

Pieces of uterine tissues were embedded in O.C.T. Compound and frozen immediately in liquid nitrogen. Air-dried tissue sections of 5 μm thickness were fixed for 5 min in acetone at –20 C. Nonspecific binding was blocked using 2% (v/v) goat serum in PBS (blocking buffer) for 30 min. Sections were incubated at 4 C overnight with rabbit anti-FOXA2 antibody (1:800; #3143, Cell Signaling Technology, Beverly, MA, USA) and mouse anti-Cytokeratin antibody (1:100; C2931, Sigma-Aldrich, St. Louis, MO, USA) diluted in blocking buffer. Nuclei were stained with Hoechst 33342 (Calbiochem, La Jolla, CA, USA). After washing with PBS, they were incubated for 1 h at room temperature with the secondary Goat Anti-Mouse IgG (H+L), F(ab')2 Fragment (Alexa Fluor 488 Conjugate) antibody (1:500; Cell Signaling Technology) and Alexa Fluor 594 Goat Anti-Rabbit IgG (H+L) antibody (1:500; Invitrogen, Carlsbad, CA, USA) diluted in blocking buffer. Sections were subsequently washed in PBS and mounted with Mount-Quick Aqueous (Daido Sangyo, Tokyo, Japan). Immunostaining was detected under a fluorescence microscope (Nikon, Tokyo, Japan).

Culture of the endometrial epithelial cells, real-time PCR and immunocytochemistry

Endometrial epithelial cells were isolated from uterine horns at 1.5 dpc. The uterine lumens were filled with phosphate buffered saline (PBS) containing 0.1% collagenase (Worthington Biochemical Corporation, Lakewood, NJ, USA) and incubated at 37 C for 45 min in a shaking water bath. The dissociated cells were washed and plated onto 4-well dishes coated with BD Matrigel (BD Biosciences, San Jose, CA, USA). Phenol red-free DMEM/Ham's F-12 (Nacalai Tesque) containing 10% charcoal-stripped fetal bovine serum (Invitrogen) and penicillin-streptomycin solution (Nacalai Tesque) was used as the principal culture medium. Cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37 C.

After the cells reached confluence, P4 (1 μM) or Recombinant Mouse Sonic Hedgehog N-terminus (1 μg/ml, E. coli-derived, Cys25-Gly198, with a C-terminal 6-His tag, R&D Systems, Minneapolis, MN, USA) was added to the culture medium. After 24 h of treatment, total RNA was extracted using a Sepasol-RNA I Super, followed by RT and real-time PCR performed as described above.

Immunocytochemistry analysis was performed for characterization of the cultured cells and detection of FOXA2 in the cultured cells. Stromal cell contamination was examined by using a stromal-specific Vimentin antibody (M0725, Dako, Carpinteria, CA, USA). The antibodies against Cytokeratin and FOXA2 were described above. The cells were fixed with 4% (w/v) paraformaldehyde in PBS at 4 C for 20 min and cold methanol at –20 C for 10 min. The antibody treatment and observation were performed as described above.

Statistical analysis

Each experiment was repeated three times. The results for *Foxa2* are expressed as a ratio relative to the proestrus stage for the expression profile during the estrous cycle and early pregnancy shown in Fig. 1. For the other experiments, the results are expressed as a ratio relative to the control. Values are expressed as means ± SEM, and analyses were performed by ANOVA followed by the Student-Newman-Keuls test using StatView (Abacus Concepts, Berkeley, CA, USA). Differences were considered significant at the level of $P < 0.05$.

Results

Expression of Foxa2 mRNA in the rat uterus

We first examined the expression of *Foxa2* in the rat uterus at different stages of the estrus cycle and during early pregnancy using quantitative real-time PCR. Although the expression level of the gene showed some changing patterns in different stages of estrus cycle, the values were not significantly different. On the other hand, expression of *Foxa2* significantly increased at 3.5 dpc ($P < 0.05$) during early pregnancy. The value was almost 7 times higher than that of 1.5 dpc. The elevation in the expression was transient, and the expression decreased significantly to the basal level at 5.5 dpc ($P < 0.05$) (Fig. 1A). *Foxa2* was detected using the antisense probe in the glandular epithelium at 3.5 dpc by *in situ* hybridization (Fig. 1B). Almost no signal was detected in the luminal epithelium or stroma in the endometrium. The control slide did not show any positive signals when the sense probe was used (Fig. 1C).

Localization of FOXA2 in the rat uterus

Dual-label immunohistochemical analysis of Cytokeratin and FOXA2 was performed to investigate the localization of FOXA2 in the rat uterus. Cytokeratins are proteins of keratin-containing intermediate filaments found in the intracytoplasmic cytoskeleton of epithelial tissue and were used as a marker of epithelial cells. Interestingly, FOXA2 was detected in the glandular epithelium but not in the luminal epithelium, stroma, myometrium or vessel in the rat uterus (Fig. 2). FOXA2 was detected at all stages of the estrous cycle and during early pregnancy (data not shown). To analyze the intracellular localization of the FOXA2 in glandular epithelial cells, double staining of FOXA2 and Cytokeratin with counterstaining with Hoechst was performed (Fig. 3). Keratin-containing intermediate filaments were stained as a green (Fig. 3A), and the nucleus of the glandular epithelial cells and surrounding stromal cells were stained blue (Fig. 3B). FOXA2 in the epithelial cells was stained red (Fig. 3C), and it was located in the nucleus, as shown in merged images (Fig. 3D, E).

Effect of steroid hormones on the expression of Foxa2 mRNA

To analyze the effects of steroidal regulation on *Foxa2* expression in the uterus, we used OVX rats to avoid the influence of the endogenous hormone. After 6 h of treatment, there was no significant difference in *Foxa2* expression in the uterus among the groups treated with the

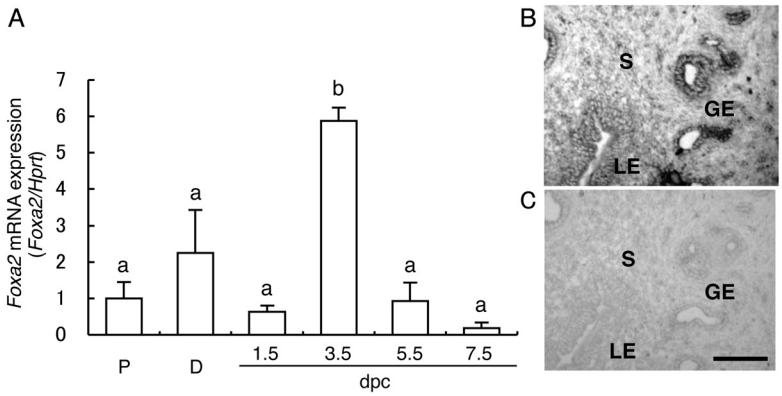

Fig. 1. Expression of *Foxa2* in the rat uterus during the estrus cycle and early pregnancy. A) Quantitative real-time PCR analysis of *Foxa2* expression. Total RNA was extracted from the rat uterus at the proestrus stage (P), diestrus stage (D) and 1.5, 3.5, 5.5 and 7.5 days post coitus (dpc). The expression of mRNA was normalized to the expression of *Hprt* measured in the same RNA preparation. The results are expressed as a ratio against the proestrus stage and as means ± SEM (n=3). Values with different superscripts in each panel are significantly different ($P < 0.05$). B, C) In situ hybridization analysis of *Foxa2* expression at 3.5 dpc. The hybridization signals were detected using an anti-digoxigenin alkaline phosphatase conjugate (B, antisense; C, sense). GE, glandular epithelium; LE, luminal epithelium; S, stroma. The scale bar represents 100 μm.

different hormones (Fig. 4A). On the other hand, after 24 h of the treatment, quantitative real-time PCR analysis revealed that treatment with E2 or the combination of P4 and E2 led to a significant reduction in *Foxa2* expression compared with the level in the in control animals ($P < 0.05$) (Fig. 4A). P4 did not affect the expression of the *Foxa2* in the uterus, at least until 24 h after treatment.

To confirm the depressive action of E2 on the expression of *Foxa2* in the uterus, pregnant rats at 3.5 dpc were treated with ICI, a pure estrogen receptor antagonist. The level of *Foxa2* expression was significantly high in the uterus of the ICI-treated rats at 5.5 dpc compared with the controls ($P < 0.05$) (Fig. 4B). A further experiment was performed using a delayed implantation rat model. The expression level of *Foxa2* was significantly decreased in the implantation-induced rats by E2 treatment compared with that of the control group treated only with daily injection of P4 ($P < 0.05$) (Fig. 4C).

Effect of Hedgehog protein on the expression of Foxa2 in vitro

In the present study, most of the cells cultured on BD Matrigel were spherical in shape, and fibroblastic cells were hardly recognized (Fig. 5Aa). The cells were positively stained with anti-Cytokeratin antibody (Fig. 5Ab) and immunoreactive vimentin-positive cells were not detected (Fig. 5Ac). No signal was detected in the negative control without the first antibody (Fig. 5Ad). To examine the effect of Hedgehog on the *Foxa2* expression in the rat uterus, cultured endometrial epithelial cells were treated with a recombinant Hedgehog protein. The expression level of *Foxa2* was not increased by the P4 treatment. However, treatment of the cells with the recombinant Hedgehog protein significantly increased the *Foxa2* expression (Fig. 5B). The expression of FOXA2 in the cells treated with the recombinant Hedgehog protein was immunocytochemically detected *in vitro*. Foxa2 was located in the nucleus of the cells (Fig. 5C).

Discussion

The expression of Foxa2 has been reported in several animal species including the mouse [4], human [8] and bovine [9]. Although an important role of Foxa2 has been suggested for implantation in mice [5], the regulation of Foxa2 expression is poorly understood in the uterus during early pregnancy. In the present study, we therefore examined the expressional profiles of *Foxa2* at different stages of the estrus cycle and during early pregnancy in rat uterus. We demonstrated that the expression of *Foxa2* transiently increased just before implantation and that it is regulated by E2 and Hedgehog protein. Thus, the results indicate that transient elevation of *Foxa2* expression may have an important role for implantation in the rat uterus.

In the rat uterus, embryo implantation occurs at 4.5 dpc following an E2 surge. Our results showed that the expression of *Foxa2* increased just before implantation and decreased thereafter, initiating of implantation. Similar results were obtained for the expression profile of *Foxa2* in the mouse uterus, which showed a transient elevation at 2.5 dpc following downregulation at 3.5 dpc [5]. Although the function has not yet been clarified sufficiently, it is suggested that Foxa2 is necessary for development of the glandular epithelium in the uterus [14]. It has also been reported that the glandular epithelium does not develop in the uterus of the *Foxa2*-deficient mouse [5]. Thus, formation of the uterine glands during implantation should be inhibited as the expression of *Foxa2* decreases during this period. It is believed that the uterine endometrial glands have an essential role in uterine function and fertility, including implantation [15]. If so, the decrease in *Foxa2* during implantation period may result in inhibition of uterine gland formation, which seems contradictory for the establishment of implantation. Although the importance of the uterine glands in implantation has been recognized, it has been reported that the glandular hyperplasia resulted in fertility defects. Jeong *et al.* [16] generated β-catenin conditional mutation

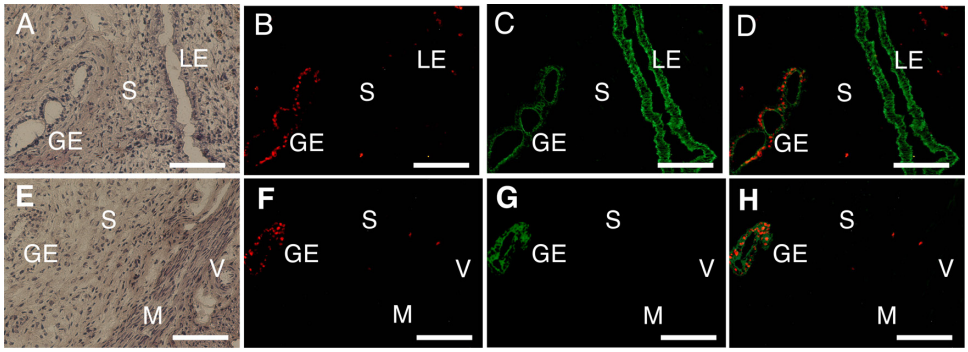

Fig. 2. Immunohistochemical detection of FOXA2 in the rat uterus (proestrus stage). After fixation, sections were stained with hematoxylin and eosin (A, E), anti-FOXA2 antibody (B, F; red) and anti-cytokeratin antibody (C, G; green). FOXA2 and cytokeratin staining merged image (D, H). GE, glandular epithelium; LE, luminal epithelium; S, stroma; M, myometrium; V, vessel. The scale bar represents 100 μm.

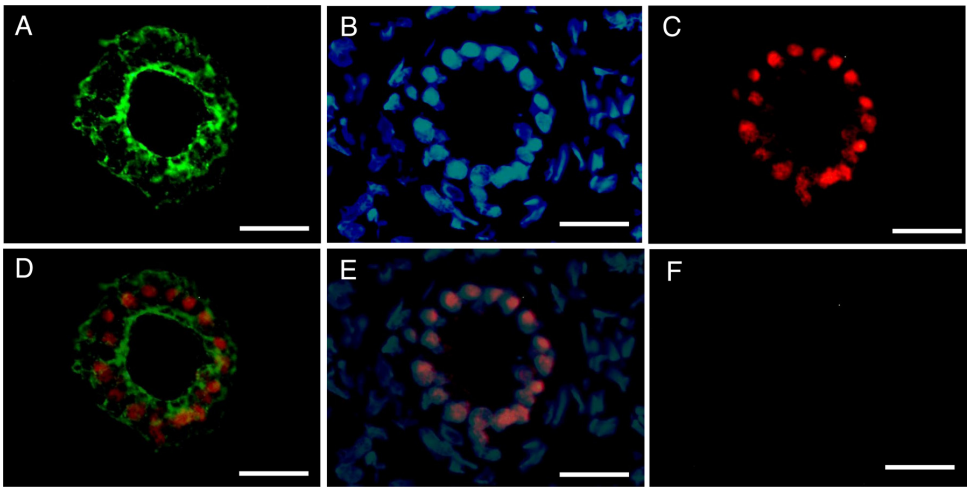

Fig. 3. Intracellular localization of FOXA2 in the glandular epithelium. Sections were stained with anti-cytokeratin antibody (A; green) and anti-FOXA2 antibody (C; red) or without a first antibody (negative control) (F). Counterstaining was performed with Hoechst, showing the location of the nuclei (B; blue). Merged images of FOXA2 and cytokeratin staining (D) and FOXA2 and Hoechst staining (E), respectively. The scale bar represents 50 μm.

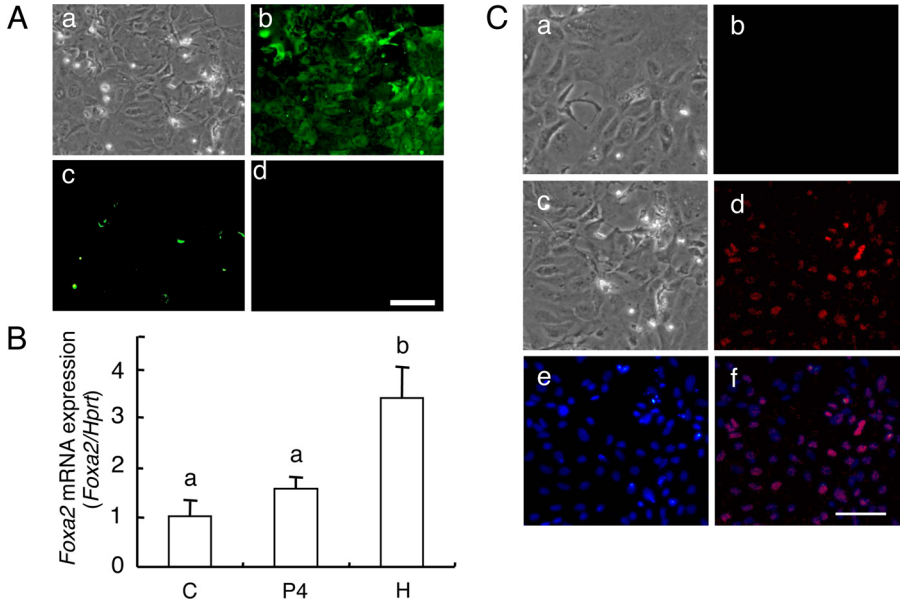

Fig. 5.

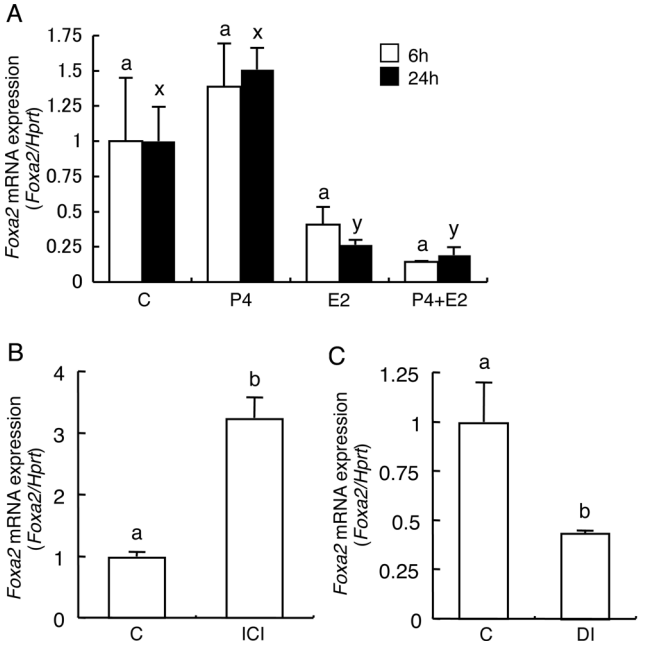

Fig. 4. Effect of steroid hormones on the expression of Foxa2 analyzed by quantitative real-time PCR. A) Ovariectomized rats were treated with progesterone (P4), 17β-estradiol (E2), a combination of P4 and E2 (P4 + E2) or vehicle (C, sesame oil). Total RNA was extracted from the rat uterus after 6 h (□) or 24 h (■) of the treatment. The results were compared individually for each time of treatment. Values with different superscripts and different times of treatment (a *vs.* b, or x *vs.* y) are significantly different ($P < 0.05$). B) The pregnant rats at 3.5 dpc were treated daily with ICI 182,780, a pure estrogen receptor antagonist, by subcutaneous injection (ICI). Sesame oil injection was used as the control (C). Total RNA was extracted from the rat uterus at 5.5 dpc (48 h after the treatment). C) The pregnant rats at 3.5 dpc were ovariectomized and P4 was injected daily from 3.5 to 7.5 dpc. The rats for initiation of implantation (DI) were treated with E2 at 7.5 dpc. E2 was not administrated to the control group (C). Total RNA was extracted from the rat uterus at 8.5 dpc (24 h after E2 treatment). The expression of mRNA was normalized to the expression of Hprt measured in the same RNA preparation. The results are expressed as a ratio against the control and as means ± SEM (n = 3). Values with different superscripts in each panel are significantly different ($P < 0.05$).

mice to investigate the role of β-catenin in uterine development. They reported that expression of the dominant stabilized β-catenin resulted in endometrial glandular hyperplasia. Interestingly, β-catenin conditionally stabilized mice had fertility defects, and the ability of the uterus to undergo a hormonally induced decidual reaction was lost [16]. Thus, it is necessary for the formation of the uterine gland to be controlled closely during pregnancy. The transient expression of *Foxa2* before implantation in the uterus may have roles in preparing a suitable environment for implantation by regulating uterine gland development. *Foxa2* may play a role through changing its expression during the implantation period or may be involved in the regulation of gene transcription, which needs to be downregulated for implantation.

Foxa2 was detected only in the glandular epithelium of the rat uterus in both mRNA and protein. Additionally, in the immunohistochemical analysis, FOXA2 was localized to the nucleus of the glandular epithelium, although it was not detected in the luminal epithelium. Franco *et al.* [17] reported that *Foxa2* can be used as a marker gene of the glandular epithelium in the mouse uterus. Considering our results along with the above report in the mouse, Foxa2 appears to be a glandular specific factor in the uterus of rodents. It is reported that Foxa2 plays an important role in epithelial budding and morphogenesis in many organs including the pancreas, liver, lung and prostate [18–20]. Since the Foxa family is conserved from yeast to humans [21], it is possible that Foxa2 may play an important role in the uterus of many mammalian species. Further analysis is needed to understand the relationship between Foxa2 and adenogenesis in the uterus for implantation.

Although *Foxa2* expression did not differ after 6 h of steroidal hormone treatments in OVX rats, its expression in E2-treated rats was significantly lower than those of the control and P4-treated groups after 24 h of treatment. This result suggested that *Foxa2* expression was reduced by E2 action. These results were similar to a report in mice, which showed repression of *Foxa2* by E2 injection in OVX mice [5]. To clarify the effect of E2 action on *Foxa2* expression during pregnancy, further experiments using an antagonist of the estrogen receptor and delayed implantation model were carried out in the present study. *Foxa2* expression in the rats treated with ICI, a selective antagonist of ERα, was significantly higher in comparison with control animals. Furthermore, since delayed implantation starts just after E2 treatment, it is assumed that *Foxa2* expression must be downregulated in accordance with the estrogenic stimuli. Indeed the expression level of *Foxa2* was lower in the implantation-induced rats treated with E2 compared with the level of the control group. The results in the OVX, ICI treatment and delayed implantation experiments clearly showed that *Foxa2* expression in the rat uterus is decreased by E2 action. The profile of *Foxa2* expression in this study is consistent with that of *Gli1* [22], which is a signal transducer of the Hedgehog pathway. *Gli1* expression transiently increases at 3.5 dpc and decreases at 5.5 dpc. Further, *Gli1* expression in the rat uterus is decreased by E2 action [22]. The similarity of *Foxa2* and

Fig. 5. Effect of Hedgehog protein on the expression of Foxa2 in cultured endometrial epithelial cells. A) Endometrial epithelial cells were isolated from uterine horns at 1.5 dpc. The morphology of the cultured cells is shown with a phase contrast image (a). After fixation, cultured cells were stained with anti-Cytokeratin antibody (b), anti-Vimentin antibody (c) or without a first antibody (negative control) (d). The scale bar represents 100 μm. B) Cultured cells were treated with progesterone (P4), treated with recombinant Mouse Sonic Hedgehog N-terminus (H) or not treated (C). Total RNA was extracted after 24 h of treatment. The expression of mRNA was normalized to the expression of Hprt measured in the same RNA preparation. The results are expressed as a ratio against the control and as means ± SEM (n = 3). Values with different superscripts are significantly different ($P < 0.05$). C) Immunocytochemical detection of FOXA2 in the cells treated with Mouse Sonic Hedgehog N-terminus. The morphology of the cultured cells is shown with a phase contrast image (a and c). After fixation, cultured cells were stained without a first antibody (negative control) (b) or with anti-FOXA2 antibody (d; red). Counterstaining was performed with Hoechst, showing the location of the nuclei (e; blue). Merged images of FOXA2 and Hoechst staining (f). The scale bar represents 100 μm.

Gli1 expression profiles during early pregnancy suggests that *Foxa2* is also one of the target genes of the Hedgehog signaling pathway. Further studies are required to elucidate the relationship between decreasing *Foxa2* expression and embryo implantation; both are induced by E2 stimulation.

The results of the present study clearly showed the restraint action of E2 for the expression of *Foxa2* in OVX rats, while P4 did not affect the expression level of *Foxa2* until at least 24 h after treatment. Although we assumed that *Foxa2* expression would be promoted by P4 treatment, the result was slightly unexpected. Two possible explanations for this condition are as follows: (1) The expression of *Foxa2* in the OVX rat uterus might be higher under the influence of castration, resulting in masking of the effect of P4 action. In the OVX rats, secretion of the endogenous steroidal hormones disappears, including E2. Therefore, it would be predicted that the downregulation of *Foxa2* expression caused by E2 might not occur in the OVX rat uterus. (2) It is possible that the processing time of P4 was not sufficient for elevation of *Foxa2* expression. In the present study, we demonstrated that the Hh protein induces expression of *Foxa2* in rat endometrial epithelial cells cultured *in vitro*. It was previously reported that Indian hedgehog (Ihh), which is a unique Hh protein expressed in the rat uterus, increased at 3.5 dpc [11]. It was also suggested that P4, which gradually increased from 1.5 dpc, indirectly promoted the expression of Ihh [23] after intermediation of the endometrial stroma cells [24]. Considering these results, if expression of *Foxa2* was induced by Ihh in the OVX rat uterus such as in our current *in vitro* study, the processing time of 24 h after P4 administration might be too short to elevate *Foxa2* expression. Further studies are required to elucidate the mechanism of *Foxa2* expression in the uterus, including the mechanisms of P4 stimulation, endometrial stromal function and Ihh action.

It was further reported that expression of Foxa2 in the floor plate of the vertebrate neural tube was induced by Sonic hedgehog (Shh), which is a member of the Hh proteins, derived from the notochord [10]. The results indicate the possibility that the Hh proteins may induce *Foxa2* expression also in the uterus. We assumed that Ihh would induce *Foxa2* expression in the uterus. To test this hypothesis, we used a recombinant Hh protein in the culture system of rat endometrial epithelial cells and performed an *in vitro* analysis. The results clearly demonstrated the effect of the Hh protein on the elevation of *Foxa2* expression in the rat endometrial epithelial cells. To demonstrate the relation between Ihh and Foxa2 expression in the uterus, an analysis of endogenous Ihh using a specific inhibitor or antibody for Ihh is necessary. It is suggested that the unique Hh protein in the uterus, Ihh, might induce *Foxa2* expression. The Ihh knockout mouse is known to be sterile based on results showing incompetence of implantation and decidualization [12]. Considering the expression of *Foxa2* located downstream of the Hh pathway, the cause of pregnancy imperfection in the Ihh knockout mouse might result from the depletion of Foxa2 function. Recently, a genome-wide investigation of FOXA2 binding target regions was performed in the murine uterus by chromatin immunoprecipitation coupled with massively parallel sequencing (ChIP-Seq) [25]. In that report, FOXA2-bound and GE-expressed genes were enriched for functional processes, including focal adhesion and WNT signaling. It is suggested that analysis of the uterine FOXA2 may provide novel insights into mechanisms governing endometrial gland development and function. Further studies are needed to understand the FOXA2-dependent network governing endometrial gland development and function for implantation.

In conclusion, the present study demonstrated the expression profile and localization of Foxa2 in the rat uterus. In addition, we also demonstrated that E2 downregulated the transient expression of *Foxa2* and suggested the possibility that Ihh might promote the elevation of *Foxa2* during early pregnancy.

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