DEVELOPMENT AND ANALYSIS OF A FOXA2 CONDITIONAL OVEREXPRESSION MOUSE MODEL

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Nomenclature

cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
CL	Corpus luteum
cOE	Conditionally overexpression
E2	Estrogen
ECM	Extracellular matrix
EEC	Endometrioid endometrial cancers
EMT	Epithelial–mesenchymal transition
ESRs	Estrogen receptors
FISH	Fluorescence in situ hybridization
FSH	Follicle-stimulating hormone
GD	Gestational day
GE	Glandular epithelium
GnRH	Gonadotropin-releasing hormone
hESCs	Human embryonic stem cells
ICM	Inner cell mass
LE	Luminal epithelium
LH	Luteinizing hormone
lncRNAs	Long non-coding RNAs
MAPK	Mitogen-activated protein kinase
MD	Paramesonephric duct

MMPs	Matrix metalloproteinases
NK	Natural killer cell
P4	Progesterone
PCR	Polymerase Chain Reaction
PDZ	Primary decidual zone
PGR	Progesterone receptor
PGs	Prostaglandins
PRKO	PGR deletion mice
PRL	Prolactin
SDZ	Secondary decidual zone
TE	Trophectoderm
TIMPs	Inhibitors of metalloproteinases
TSS	Transcription start site
uNK	Uterine natural killer cell
UTR	Untranslated region

Abstract

The uterus is essential for mammalian reproduction as it provides environment for conceptus implantation and subsequent development. Endometrial glands synthesize and secrete or transport substances critical for conceptus survival and implantation, demonstrated by the fact that female sheep and mice containing no uterine glands are infertile mainly due to impaired implantation and early pregnancy loss. FOXA2, a transcription factor, has been showed indispensable for not only the development of uterine glands in the neonate but also its differentiated function in the adult. The goal of the current study is to (1) generate a mouse model for the conditional overexpression of FOXA2, and to (2) determine the effects of FOXA2 overexpression on uterine morphogenesis and female fertility.

In this thesis, Chapter 1 will review the early pregnancy of mice and discuss in detail how the early pregnancy events including blastocyst activation, uterine receptivity, apposition, attachment, penetration, stromal cell decidualization are regulated by different factors and signaling pathways. Chapter 1 will also introduce FOXA2 including its finding, structure, functions in organ development and differentiated function, functions in carcinogenesis and the regulation of its expression. Chapter 2, the research chapter, shows that we developed a mouse model which could express FOXA2 continuously only in the cells with expression of Cre recombinase. By applying two different mouse strains with special Cre expression, we found that misexpression of FOXA2 in the neonatal mouse uterus alters or inhibits normal differentiation and genesis of endometrial glands and function of the adult uterus, leading to female infertility. It also suggests that regulatory elements may localize inside the *Foxa2* coding sequence and can be targeted

by some unknown epigenetic mechanism. This chapter has been recently published in a journal: Endocrinology.

Chapter 1 Literature Review

1.1 Early pregnancy in mice

1.1.1 Overview of the estrous cycle and pregnancy in mice

The house mouse (*Mus musculus*) has been widely used as a valuable model in biology and medicine because of their genetic and physiological similarities to humans. For the past two decades, our knowledge about reproduction in mammals was considerably enriched via the usage of transgenic mouse models. Mice have regularly recurring estrus cycles throughout the whole year. They spontaneously ovulate with or without mating. Their intervals between parturitions can be as short as 21 days. The estrous cycle in rodents can be divided into 4 stages (proestrus, estrus, metestrus, and diestrus) and repeats every 4 to 5 days. Their reproductive cycle will be prolonged if pregnancy, pseudopregnancy, or anestrus occurs (Allen 1922). The hypothalamus-pituitary-gonadal axis regulates the reproductive changes along the estrous cycle via the action of specific hormones, including gonadotropin-releasing hormone (GnRH), follicle-stimulating hormone (FSH), luteinizing hormone (LH), estrogen (E2) and progesterone (P4). GnRH secreted from the hypothalamus stimulates the secretion of FSH and LH from pituitary, which are essential for the development of antral follicles. FSH acts primarily to promote follicular growth, granulosa cell proliferation, and synthesis of estrogens. LH stimulates the production of E2 and P4 in ovaries. FSH and LH cooperate to promote the secretion of E2 and P4, both of which are critical for the reproductive

cyclicity and maintaining of secondary sexual characteristics. Figure 1 illustrates the hormone profiles that accompany ovarian changes in mice.

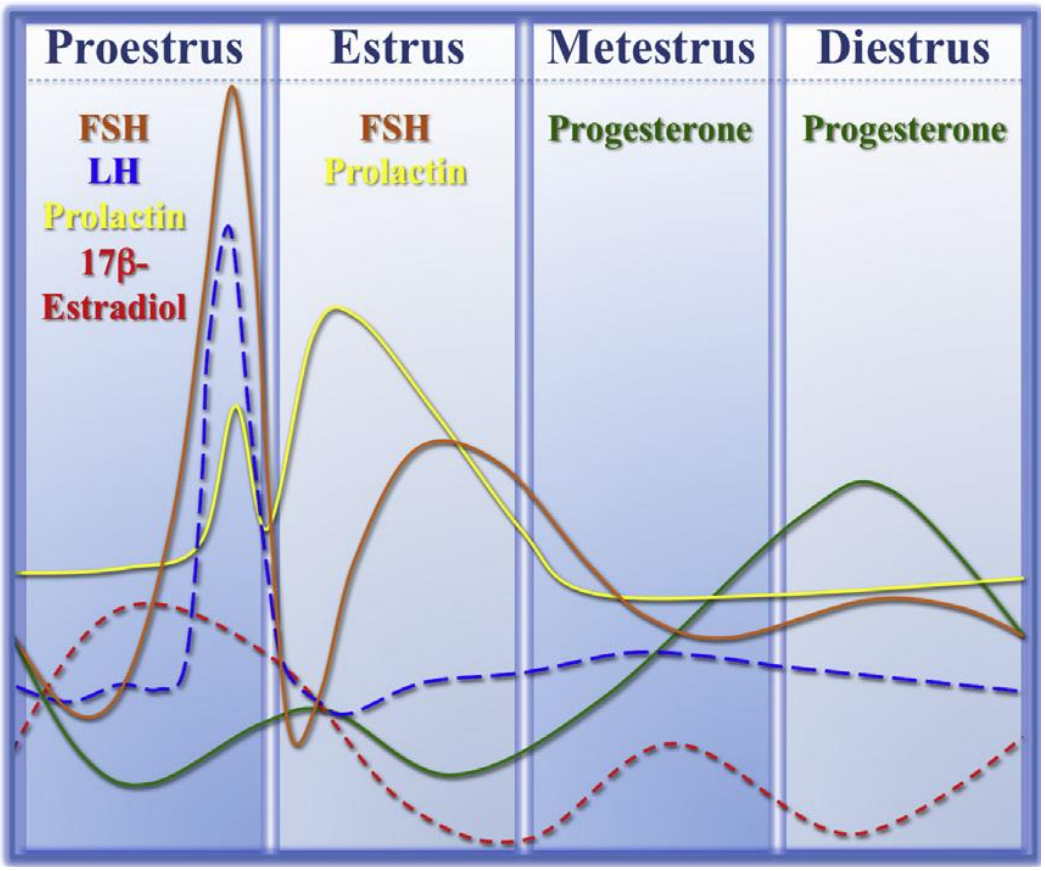

Figure 1. Diagram of the serum hormonal profile for each different stage of the cycle. FSH, orange line; LH, blue dotted lines; Prolactin (PRL), yellow line; 17β-estradiol, red dotted lines; Progesterone, green line. From (McLean, Valenzuela et al. 2012).

During proestrus, FSH stimulates the growth of antral follicles. The follicle maturation is driven first by FSH and then by LH. In the preovulatory follicles, a large amount of estrogens, mostly estradiol-17β are synthesized in theca cells and released into the bloodstream. High level of E2 affects the hypothalamus in a positive feedback manner. The rise of E2 before ovulation initiates a LH surge which stimulates the rupture of follicles. Following the peak of LH secretion in the afternoon of proestrus, mice enter estrus and ovulate in 12-14 hours (Bingel 1974). During estrus phase, the female accepts

mating with males, coinciding with biological changes in the female reproductive tract preparing for the subsequent pregnancy. After ovulation, the corpus luteum (CL) forms at the sites of ruptured follicles. The follicular theca cells and granulosa cells differentiate into luteal cells, which is called luteinization. The CL synthesize P4 instead of E2 as the principal secretory product. The estrous cycle in rodents is dominated by E2 and lacks a true luteal phase unless the mating happens. The CL is not well developed and is considered nonfunctional without mating. The nonfunctional CLs cannot support normal pregnancy because of the insufficient P4 secreted from those CLs (Neill and Knobil 2006). During the metestrus and diestrus phase, high level of P4 inhibits the secretion of LH and consequently prevents further ovulation. Metestrus is characterized by the decrease of cell proliferation in the endometrium (Wood, Fata et al. 2007). The diestrus phase is characterized by the new increase of FSH and E2 promoting the development of follicles. Small follicles are present preparing for a new estrous cycle. Examination of vaginal smears can be used to determine different estrous cycle phases in mice according to the proportion of three cells types: epithelial cells, cornified cells and leukocytes (Caligioni 2009).

The presence of a vaginal plug usually indicates that the happening of mating behavior. The secretions from the coagulating and vesicular glands forms the vaginal plug which fills the vagina and prevent the mating with other males. It usually disappears 8-24 hours after breeding. More functional CLs forms with the stimulation of mating behavior in rodents. Two daily surges of PRL from pituitary induced by mating behavior protect the CLs from degeneration. The PRL surge usually persist for 8 to 10 days (Smith, Freeman et al. 1975, Smith, McLean et al. 1976). 20α-hydroxysteroid

dehydrogenase converts progesterone into 20α-OH- progesterone which has low biological activity. PRL can inhibit expression of 20α-hydroxysteroid dehydrogenase increasing the level of progesterone (Zhong, Parmer et al. 1997). In mice, mating to a sterile male can also extend the lifespan of CLs. The vaginocervical stimulation triggers a neuroendocrine reflex similar to the neuroendocrine response of early pregnancy. PRL surges protect the CLs from degeneration, and biological changes in the female reproductive tract are similar to the ones mated with fertile males during early pregnancy, which is called pseudopregnancy. For example, changes of hormone levels along early pregnancy in pseudopregnant mice are similar to those with normal pregnancy. The uteri in pseudopregnant animals can support pregnancy to term if blastocysts are transferred into the uterine lumen during the receptive phase. Normal implantation, stromal cell decidualization and subsequent placentation occurs. Stromal cell decidualization are critical for normal pregnancy. Blastocyst implantation induces first proliferation, and then differentiation of stromal cells in the endometrium. Spindle-shaped stromal cells are turned into large, rounded cells with large round nuclei, prominent nucleoli, and abundant eosinophilic cytoplasm, which are called decidualization. Decidual cell reaction can be induced by other stimuli, such as intraluminal infusion of oil, air, and mechanical stimuli in pseudopregnant or hormonally prepared uteri, which is called artificial deciduoma. Artificial deciduoma is similar to the decidualization induced by blastocysts at some aspects like the changes of stromal cell shape and a couple molecular markers (Dey 1996). It also has difference with those induced by blastocysts like transcriptomes and total mesometrial uterine natural killer (uNK) cell density (Lundkvist and Nilsson 1982, Paria, Ma et al. 2001, Herington and Bany 2007, Kashiwagi, DiGirolamo et al. 2007).

1.1.2 Preimplantation

Synchronization between blastocyst activation and uterine receptivity are essential for normal pregnancy. Before implantation, blastocyst must be activated be certain signaling networks giving them the competency for implantation. In rodents, uterine endometrium supports blastocyst implantation only at a certain period during estrus cycle (Yoshinaga 1988, Paria, Huet-Hudson et al. 1993). A delayed-implantation model first demonstrated blastocyst activation is essential for the successful implantation (Paria, Huet-Hudson et al. 1993). Studies on this model revealed a range of the molecular signaling pathways regulating blastocyst activation or dormancy. At day 4 of pregnancy, the blastocyst hatches from its zona pellucida, and implantation is initiated by a preimplantation ovarian estrogen surge in mice. Ovariectomy before ovarian E2 surge in the morning of day 4 of pregnancy inhibits implantation resulting in metabolically dormant blastocysts (Yoshinaga and Adams 1966). Dormant and activated mouse blastocysts are two different physiological states characterized by expression of different sets of genes. (Hamatani, Daikoku et al. 2004). Expression of genes involved in the cell cycle, cell signaling, and energy metabolic pathways are mainly altered. HB-EGF/ErbB signaling (epidermal growth factor-like growth factor and EGFR subfamily of receptor tyrosine kinases) play important roles in blastocyst activation since expression of *Hegf1* (which encodes HB-EGF) and its receptors (ErbB1 and ErbB4) were both upregulated in activated blastocysts. Ovariectomy before ovarian E2 surge and dormant blastocyst in the delayed-implantation model suggests E2 triggers blastocyst activation for implantation. Further studies show estrogenic derivatives, but not estrogen itself, is responsible for the blastocyst activation likely via stimulating the synthesis of prostaglandins (PGs) (Paria,

Lim et al. 1998). E2 via its receptor (ESR1) is required for the uterine receptivity, whereas its catechol metabolite, 4-hydroxyestradiol, produced locally in the uterus regulates the blastocyst activation (Hoversland, Dey et al. 1982, Paria, Chakraborty et al. 1990, Paria, Lim et al. 1998, Reese, Das et al. 2001). Endocannabinoid signaling is another pathway that regulates the blastocyst activation. Two endogenous cannabinoid ligands, arachidonoylethanolamide (also known as anandamide) and 2-arachidonoylglycerol, produced locally in the uterus during early pregnancy. Their receptors, cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2), are both expressed in preimplantation embryos (Paria, Das et al. 1995, Paria, Deutsch et al. 1996). Further studies revealed that CB1 is the functional receptor subtype in the preimplantation embryos. During successful pregnancy, anandamide level is downregulated during implantation is required for successful pregnancy. Anandamide via its receptor CB1 modulate mitogen-activated protein kinase (MAPK) signaling and Ca2+-channel activity (Wang, Paria et al. 1999, Wang, Matsumoto et al. 2003, El-Talatini, Taylor et al. 2009). A range of WNT pathways (Wingless-Type MMTV Integration Site Family) were shown to express and act as a critical mediator of cell–cell interactions in early mouse embryos (van Amerongen and Nusse 2009). WNT signaling can be divided into canonical and non-canonical pathways based on its distinct functions (van Amerongen and Nusse 2009). Studies on female mice with oocyte specific ablation of beta-catenin (CTNNB1) suggests canonical WNT signaling pathway regulates implantation during the periimplantation stage demonstrated by the loss of their embryos during the blastocyst stage (De Vries, Evsikov et al. 2004). Overexpression of Dickkopf 1 (DKK1), an antagonist of canonical Wnt/β-catenin signaling, had no effects on the

uterine receptivity, but suppressed the acquisition of implantation competency by the blastocyst in mice (Xie, Tranguch et al. 2008). A gene expression study on the delayed implantation model revealed the activity of nuclear CTNNB1 signaling is different between the dormant and reactivated blastocysts (Xie, Tranguch et al. 2008). Taken together, these findings suggest that the canonical WNT signaling is required for blastocyst competency. Further studies suggest that canonical WNT signaling synergizes with PG signaling to confer blastocyst competency for implantation (Xie, Tranguch et al. 2008).

In placental mammals, normal implantation only occurs at the time of implantation "window" when the uterine environment is favorable for blastocyst implantation. The implantation "window" is spatiotemporally restricted during the estrus cycle (Yoshinaga 1988). This differentiated status of uteri favorable for embryo implantation is called uterine receptivity (Zhang, Lin et al. 2013). In mice, this period is limited to day 4 of pregnancy (Wang and Dey 2006). A number of signaling molecules, including cytokines, growth factors, homeobox transcription factors, lipid mediators and morphogens, together with ovarian hormones regulate uterine receptivity (Dey, Lim et al. 2004). Among them, steroid hormones including P4 and E2 act as major regulators (Dey, Lim et al. 2004). Both of ovarian P4 and E2 are essential for implantation in mice, as the mouse uterus can become receptive to blastocyst implantation only if exposed to E2 after 24 to 48 h of P4 priming (Huet-Hudson and Dey 1990). Estrogen is a critical determinant specifying the duration of the window of uterine receptivity for implantation. Treatment with a low level of E2 extends the window of uterine receptivity, whereas higher levels of E2 rapidly close the implantation window and transform the uterus into a refractory state

(Ma, Song et al. 2003). E2 and P4 function primarily via their receptors, nuclear estrogen receptors (ESR1 and ESR2) and progesterone receptors (PGR-A and PGR-B). ESR1 null uteri are hypoplastic and unable to support implantation while ESR2 null uteri are normal, and normal implantation were observed in those uteri (Lubahn, Moyer et al. 1993). Epithelial-specific deletion of ESR1 in the mouse uterus impairs female fertility likely due to lack of endometrial receptivity suggested by the aberrant expression of estrogen responsive genes and implantation failure (Winuthayanon, Hewitt et al. 2010). PGR null (PRKO) female mice are completely infertile showing impaired ovulation, uterine hyperplasia, and failure in decidualization (Lydon, DeMayo et al. 1995). Females with specific deletion of PGR-A isoform have impaired fertility likely due to the compensatory function of PGR-B isoform (Mulac-Jericevic, Mullinax et al. 2000). In the uteri with epithelium-specific ablation of PGR, P4 application is unable to inhibit E2-induced proliferation of epithelial cells, which is required for the uterine receptivity (Franco, Rubel et al. 2012). In addition, Indian hedgehog (IHH) and Leukemia inhibitory factor (LIF) are downregulated in uteri with epithelium-specific deletion of PGR comparing to the wild type, both of which are essential for implantation (Franco, Rubel et al. 2012). Collectively, these findings indicate that progesterone and its action via PGR is indispensable for uterine receptivity. Among the cytokines, LIF, a member of the interleukin-6 (IL-6) family, is required for uterine preparation for implantation as a critical mediator of E2 action in mouse uteri. LIF is secreted from the GE in response to the preovulatory E2 on GD 4 and later in the stromal cells surrounding the blastocyst during the attachment reaction, indicating that LIF regulates both the uterine receptivity and subsequent attachment reaction (Bhatt, Brunet et al. 1991, Stewart, Kaspar et al.

1992, Song, Lim et al. 2000). Females with null mutation of *Lif* are infertile due to implantation failure, but supplementation with LIF rescues this defect (Stewart, Kaspar et al. 1992). Females with Glycoprotein 130 (gp130) ablation, a common signal-transduction partner with LIF and its receptor (LIFR), also shows implantation failure (Cheng, Chen et al. 2001). Intriguingly, LIF is sufficient for both implantation and decidualization in P4-prepared ovariectomized bred mice, and E2 supplementation is unnecessary (Chen, Cheng et al. 2000).

Homeobox genes are evolutionarily conserved transcriptional regulators of embryonic morphogenesis and differentiation (Krumlauf 1994). Homeobox A10 (HOXA10) and Homeobox A11 (HOXA11), two members of the homeobox family, are required for both uterine receptivity and decidualization (Benson, Lim et al. 1996, Gendron, Paradis et al. 1997). Both *Hoxa10* and *Hoxa11* null mutant female mice are infertile due to implantation failure (Benson, Lim et al. 1996, Gendron, Paradis et al. 1997). Msh homeobox 1 and 2 (*Msx1* and *Msx2)*, two of the non-classical Hox genes, regulate uterine receptivity by maintaining uterine epithelial integrity in mice. Female mice with a double knockout of uterine *Msx1 and Msx2* are completely infertile with implantation failure due to altered luminal epithelial cell polarity and impaired stromal–epithelial communication (Daikoku, Cha et al. 2011). FK506 Binding Protein 4 (FKBP4), a P4-inducible cochaperone, regulates PR activity for normal pregnancy. The uteri of *Fkbp4* null mice showed some defects including implantation failure and no artificially induced decidual reaction (Yang, Wolf et al. 2006). *Ihh* is a downstream target gene of P4 signaling (Matsumoto, Zhao et al. 2002). *Ihh* null females are unable to support embryo implantation and fail to undergo the artificially induced decidualization (Lee, Jeong et al.

2006). The Heart and neural crest derivatives expressed transcript (HAND2), a P4-induced basic helix-loop-helix transcription factor in the stroma, regulates the uterine stromal-epithelial communication which is crucial for uterine receptivity. *Hand2* deficient uteri cannot support normal implantation likely due to the aberrant action of P4 signaling. E2-induced proliferation of epithelial cells was not suppressed by P4 in those uteri (Li, Kannan et al. 2011). Kruppel-like factor 5 (KLF5), a factor unresponsive to ovarian hormones, is indispensable for normal implantation in mice (Sun, Zhang et al. 2012). In *Klf5* deleted uteri, the attachment between blastocysts and uterine epithelium were not observed even beyond the anticipated time. Expression of Prostaglandin-endoperoxide synthase 2 (PTGS2) was altered in *Klf5* deleted uteri, which may contribute to the implantation failure (Sun, Zhang et al. 2012).

LPA3-cPLA2α-PTGS2 (Lysophosphatidic acid receptor 3-Cytosolic phospholipase A2α-Prostaglandin-endoperoxide synthase 2) signaling pathway is another determinant for uterine receptivity and on-time implantation. cPLA2α encoded by *Pla2g4a* (Phospholipase A2 Group 4A) produces arachidonic acid from membrane phospholipids as a precursor for PG synthesis. PTGSs are rate-limiting enzymes for prostaglandin synthesis from the arachidonic acid. PTGS2-derived prostacyclin (PGI2) is the primary PG that is produced at the implantation site, which is required for normal implantation. Expression of peroxisome proliferator-activated receptor δ (PPARδ) is regulated by PGI2. PGI2-PPARδ signaling pathway is a critical regulator of blastocyst implantation (Lim, Gupta et al. 1999). LPA3 is a lipid-derived G-protein-coupled receptor (GPCR) agonist regulating a variety of physiological and pathological processes. LPA3 increases the expression of *Ptgs2* mRNA (Ye, Hama et al. 2005). Deletion of

Pla2g4a and *Lpar3* in mice result in similar fertility impairment, delayed implantation. Implantation occurs beyond the normal window of implantation showing embryo crowding, conjoined placenta, retarded fetoplacental development and increased resorptions resulting in smaller litter size comparing to the control (Song, Lim et al. 2002, Ye, Hama et al. 2005). *Ptgs1* deletion has no apparent effects on female fertility while implantation defects were observed in *Ptgs2*−/− females although genetic background–dependent compensation by *Ptgs1* can partially rescue implantation (Lim, Paria et al. 1997, Dey, Lim et al. 2004).

1.1.3 Blastocyst implantation

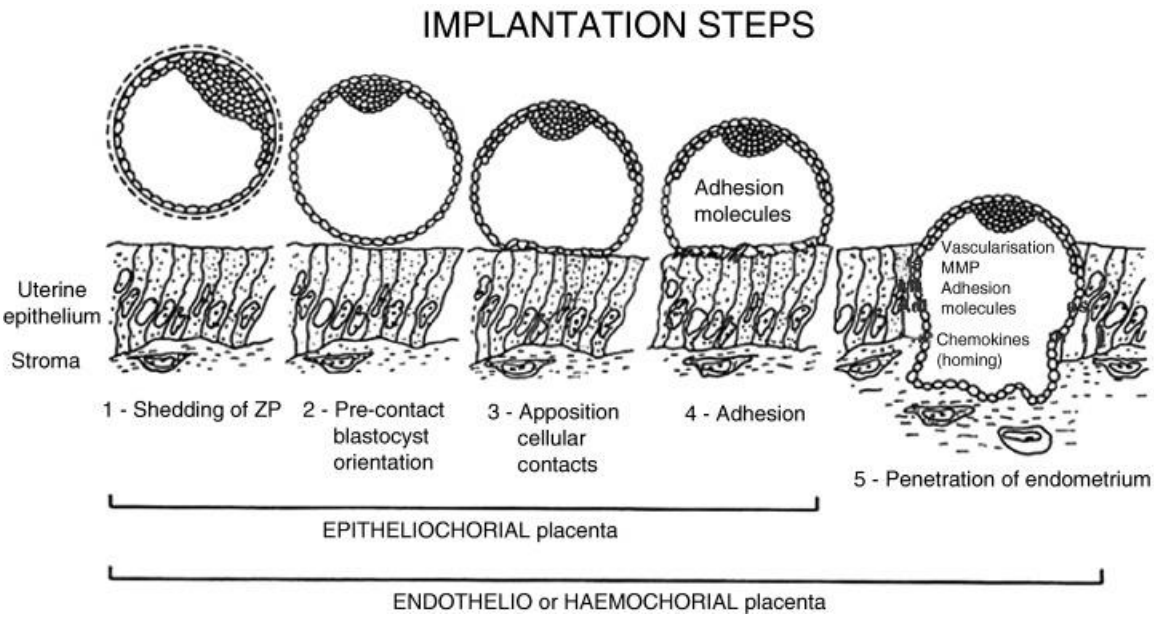

Figure 2. Implantation steps: hatching, orientation, apposition, attachment (adhesion), penetration. Adapted from (Chaouat, Ledee-Bataille et al. 2003).

The uterine endometrium contains three major types of cells, glandular epithelium, luminal epithelium and stromal cells, which are all involved in the embryo implantation. A variety of physical and physiological interactions between the uterus and

the blastocyst occur during embryo implantation (Lim and Dey 2009). The process of implantation is showed in Figure 2 (Carson, Bagchi et al. 2000, Chaouat, Ledee-Bataille et al. 2003).

Apposition is the initial contact of trophectoderm (TE) and apical surface of the LE. In rodents, apposition is facilitated by the uterine luminal closure resulting from the uterine fluid reabsorption. During the preimplantation period, uterine luminal fluid serves as a medium for the moving of embryos the female reproductive tract. The uterine fluid is reabsorbed on GD 4 results in the luminal closure and blastocyst apposition. Direct contact of the blastocyst and the uterine epithelium is established at that time (Enders and Nelson 1973). In rodents, ovarian E2 stimulates fluid secretion, while P4 promotes fluid absorption facilitating the attachment reaction. Studies on pseudopregnant females showed that their uterine lumen is also closed on GD 4 though they have no blastocysts in their uteri, suggesting the presence of blastocysts is unnecessary for the luminal closure. The luminal closure does not occur in FK506 binding protein-4 (FKBP52) deletion uteri suggesting that P4 signaling regulates uterine closure since FKBP52 is a co-chaperone of PGR and required for appropriate PGR function in the uterus (Tranguch, Cheung-Flynn et al. 2005). Interaction between the cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-activated Cl- channel, and the epithelial Na+ channel (ENaC) is believed to affect the amount of uterine and luminal closure (Salleh, Baines et al. 2005). ENaC is primarily localized to the apical membrane of uterine epithelium, while CFTR is predominantly expressed in the stromal cells (Ruan, Guo et al. 2012). Estrogen induces CFTR expression, but represses ENaC, promoting fluid accumulation in the uterine lumen, whereas progesterone acts oppositely in regulating these two genes resulting in

fluid reabsorption (Nobuzane, Tashiro et al. 2008). Invading embryos secret trypsin, a serine protease which can activate ENaC leading to fluid reabsorption (Kleyman, Carattino et al. 2009). In mice, ENaC is also regulated by serum and glucocorticoid inducible kinase-1 (SGK1), a key regulator of sodium transport in mammalian epithelia. SGK1 can directly activate ENaC by channel phosphorylation, and enhance ENaC expression by inhibiting a ubiquitin ligase, which can ubiquitinate and degrade ENaC (Diakov and Korbmacher 2004, Zhou and Snyder 2005).

EGFs with their receptors, ErbBs, act as critical mediators of apposition and attachment (Das, Yano et al. 1997). HB-EGF is an early molecular marker of embryo–uterine crosstalk, which is perhaps the first molecular marker of attachment reaction known yet. *Hbegf1* is expressed in the mouse luminal epithelium (LE) at the site of blastocyst apposition several hours before attachment, and its expression persists through the early attachment phase. During implantation, HB-EGF functions in two forms, soluble and transmembrane, which influences embryos as a paracrine and/or juxtacrine factor by interacting with ErbB1 and/or ErbB4 expressed on the blastocyst cell surface (Paria, Das et al. 1993, Raab, Kover et al. 1996, Wang, Mayernik et al. 2000). Of note, HB-EGF is not expressed at the site of blastocyst in the delayed implantation models, but is rapidly induced the E2 injection which terminates delayed implantation and triggers normal implantation (Hamatani, Daikoku et al. 2004). Uterine specific ablation of *Hbegf1* results in subfertility with delayed implantation and reduced litter size, but not infertility, which is like due to the compensation by amphiregulin (AREG) (Xie, Wang et al. 2007).

Adhesion molecules are involved in blastocyst apposition and attachment during implantation. A variety of glycoproteins and carbohydrate ligands and their receptors are

expressed in LE and TE cells around the time of implantation, including Mucin 1 (MUC1), selectins, galectins, integrins and cadherins. MUC1, a glycoprotein, is expressed in the mouse uterine epithelium, preventing direct interaction between the embryo and the LE of the uterus before implantation (Surveyor, Gendler et al. 1995). Expression of MUC1 is strongly stimulated by E2 and repressed by P4, which is consistent with the observation that MUC1 on the surface of uterine LE is downregulated on GD 4 just before the attachment reaction (Surveyor, Gendler et al. 1995). Collectively, these findings demonstrate that removal of MUC1 from the implantation sites is requied for the successful blastocyst attachment. Selectins are calcium-dependent carbohydrate binding molecules, which are expressed in TE and LE of both rodents and humans.

Selectin signaling is believed to regulate implantation in humans, but not in mice (Robinson, Frenette et al. 1999, Domino, Zhang et al. 2001, Genbacev, Prakobphol et al. 2003). Galectins are calcium-independent animal lectins (Leffler, Carlsson et al. 2002). Mouse blastocysts express galectin 1, 3, and 5 at hatching stage and both galectins 1 and 3 are present on the surface of implanting blastocyst TE but not inner cell mass (ICM) (Colnot, Fowlis et al. 1998). Galectins regulate trophoblast invasion in mice by interacting with laminin (Sutherland 2003). However, in mice with deletions of either or both of galectins 1 and 3, fertility is not affected (Colnot, Fowlis et al. 1998). The members of the integrin family serve as receptors for various extracellular matrix (ECM) ligands modulating cell-cell adhesion and signal transduction events (Giancotti and Ruoslahti 1999). Each integrin is comprised of two subunits, α and β. Integrin αvβ3 is expressed in the mouse uterine LE and the blastocyst during implantation. Injection of neutralizing antibody against the αv or β3 subunit into the mouse uterine cavity

diminishes the rate of implantation, suggesting they are involved in mediating the TE-LE interaction (Aplin, Spanswick et al. 1996, Illera, Cullinan et al. 2000). The cadherin 1 (CDH1), a calcium-dependent transmembrane cell-cell adhesion molecule, is one of the components forming the epithelial adherens junctions (Ozawa, Engel et al. 1990). It is highly expressed in the uterine LE before implantation (Thie, Fuchs et al. 1996). Females with uterine-specific ablation of CDH1 are infertile due to implantation failure and defective decidualization, indicating that CDH1 plays a role in uterine preparation for implantation (Reardon, King et al. 2012).

For many species, blastocyst implantation coincides with a reduction in the number of cellular and extracellular matrix layers. Loss of extracellular matrix and uterine epithelial cells at the implantation sites facilitates the interaction between the blastocyst and the residual epithelial basement membrane, which is called penetration (Blankenship and Given 1992). Matrix metalloproteinases (MMPs), a family of at least 17 zinc-dependent endopeptidases, are critical regulators of cellular invasion (Salamonsen 1999). In humans, the regulation of MMP activity at the maternal-fetal interface is critical for successful implantation and placentation. Trophoblast cells constitutively produce and secret MMPs which is required for the penetration during implantation (Gellersen, Reimann et al. 2010). Recent evidence shows that invading trophoblast cells express a range of MMP subtypes. However, on the maternal side, stromal cells and natural killer (NK) cells within the endometrium also express most MMP subtypes (Curry and Osteen 2003). For example, trophoblast express MMP2 and MMP9 during early pregnancy regulating trophoblast invasion (Brooks, Stromblad et al. 1996, Anacker, Segerer et al. 2011). MMP activity is also regulated by tissue inhibitors of

metalloproteinases (TIMPs) exert their affect either directly by binding to MMPs or indirectly by regulating the expression of select MMP. TIMP2 promotes the activation of MMP2 by forming a two-protein complex with MMP2 (Nishida, Miyamori et al. 2008). A decrease in TIMP-2 expression leads to a reduction in MMP-2 acitivity and trophoblast invasion (Onogi, Naruse et al. 2011).

Stromal cell decidualization is initiated by the attachment of the blastocyst and action of TE cells. The spindle-shaped stromal cells proliferate and differentiate into large, rounded cells with abundant eosinophilic cytoplasm, which is called decidual cells (Lim and Wang 2010). The decidualization and the subsequent endometrial tissue remodeling and angiogenesis is essential for embryo development and maternal immune tolerance to the fetus before the formation of functional placenta (Wang, Matsumoto et al. 2004, Peng, Li et al. 2008). In mice, decidualization is initiated at the antimesometrial site. Decidual cells first form an avascular zone surrounding the fetus in the afternoon of day 5, which is called primary decidual zone (PDZ) (Wang, Matsumoto et al. 2004). Stromal cells next to the PDZ continue to proliferate and differentiate forming a well vascularized zone, the secondary decidual zone (SDZ) (Ansell, Barlow et al. 1974). During the next 3 days of gestation, some decidual cells proliferate and differentiate into multinucleate and giant cells, which may contain as much as 64N DNA (Dey, Lim et al. 2004). Multinucleate cells generated via endoreduplication usually have enhanced gene expression and thus increased protein synthesis which may be essential to support embryonic growth (Cha, Sun et al. 2012). The enlarged multinucleate cells eventually undergo apoptosis, making room for the subsequent placental expansion and

development (Dey, Lim et al. 2004). By the day 11 of gestation, the decidual cells are barely observed in the uterus (Dey, Lim et al. 2004).

A complex interplay of transcription factors, morphogens, cytokines, cell cycle regulators and signaling pathways regulates decidualization. The P4, acting via its receptor PGR, plays a central role in regulating decidualization (Lydon, DeMayo et al. 1995). In mice, uteri with PGR deletion are refractory to an artificial deciduogenic stimulus (Lydon, DeMayo et al. 1995). Similar phenotypes are observed in *Fkbp4* null females since FKBP4 is an important regulator of PGR action in the mouse uterus (Tranguch, Wang et al. 2007). Steroid receptor coactivators (SRC1 and SRC2, encoded by *Ncoa1* and *Ncoa2* respectively) also regulate PGR activity in mouse uteri (Spencer, Jenster et al. 1997). In the females with a null mutation of either *Ncoa1* or *Ncoa2*, P4 signaling is suppressed, and decidualization is impaired. Mice with gene deletion of both *Ncoa1* and *Ncoa2* are completely infertile with a complete failure of decidual response (Xu, Qiu et al. 1998, Mukherjee, Soyal et al. 2006, Jeong, Lee et al. 2007). Recent evidence also shows that PGR-IHH-COUPTFII axis is indispensable for both uterine implantation and decidualization. The IHH is a downstream target gene of PGR which transduces PGR signal from the epithelium to stromal cells (Takamoto, Zhao et al. 2002). Chicken ovalbumin upstream promoter transcription factor II (COUP-TFII, encoded by *Nr2f2* gene) is regulated by IHH in the uterus (Lee, Jeong et al. 2006). *Ihh* and *Nr2f2* deficient mice were generated in different labs. Both females are not able to support normal implantation and show decidual reaction upon administration of an artificial stimulus (Lee, Jeong et al. 2006, Kurihara, Lee et al. 2007). Bone morphogenetic protein 2 (BMP2) is the major downstream effector of COUP-TFII for decidualization. High

expression of BMP2 is induced upon decidualization (Ying and Zhao 2000). However, in the *Nr2f2* deficient uterus, the induced expression of BMP2 upon decidualization was greatly diminished. Injection of recombinant human BMP2 into the uterine lumen can rescue the decidualization defect (Kurihara, Lee et al. 2007). Studies suggest that WNT4 may be a downstream target of BMP2 in the uterus (Franco, Dai et al. 2011). *Wnt4* deficient uteri were unable to support embryo implantation, and failed to show decidual reaction upon administration of an artificial stimulus (Franco, Dai et al. 2011). Further studies revealed that in the absence of WNT4, forkhead transcription factor FOXO1 localizes to stromal cell nuclei leading to the abnormal apoptosis of stromal cells (Franco, Dai et al. 2011). In mice, the abdominal B-like Hox genes, *Hoxa10* and *Hoxa11*, are highly expressed in the decidual cells. Defective decidualization were observed in both *Hoxa10* null mice and *Hoxa11* null mice due to the defects of stromal cell proliferation in response to P4 (Benson, Lim et al. 1996, Gendron, Paradis et al. 1997). Death effector domain–containing protein (DEDD) associated with stabilized cyclin D3 regulate stromal cell polyploidization during decidualization. Mice with DEDD deletion display defective decidualization with reduced polyploidy (Mori, Kitazume et al. 2011). Transformation-related protein 53 (*Trp53*), which encodes P53, is a tumor suppressor gene whose mutation is strongly associated with cancer. However, recent evidence suggests it also regulates apoptosis of decidual cells. In the uterus with a conditional deletion of *Trp53* at one allele, ovulation, fertilization, and implantation are not affected; however, incidence of preterm birth is increased. Studies on postimplantation uterine decidual cells showed their differentiated status was alter with higher levels of phosphorylated Protein Kinase B (AKT) and cyclin-dependent kinase inhibitor 1 (P21), indicating terminal differentiation

and senescence-associated growth restriction of those decidual cells (Hirota, Daikoku et al. 2010).

1.2 Forkhead box A2

1.2.1 Overview

Forkhead box (FOX) proteins are a family of transcription factors named after the forkhead motif, a sequence of 80 to 100 amino acids. They regulate diverse biological processes including development of various organs, metabolic homeostasis, cell senescence and proliferation (Golson and Kaestner 2016). The forkhead domain, also known as the winged-helix domain, is well conserved across the FOX family and across species (Weigel and Jackle 1990). The FOX gene was originally identified in Drosophila melanogaster whose mutation gives the insect a fork-headed appearance (Weigel, Jurgens et al. 1989). To date, 50 FOX genes have been identified in the human genome and 44 in the mouse. All the FOX genes can be classified into 19 subfamilies based on sequence similarity within and outside of the forkhead box, named FoxA-FoxS (Jackson, Carpenter et al. 2010). In 1989, FoxA transcription factors were first identified in rat liver nuclear extracts. Therefore, they were first named hepatocyte nuclear factor 3 (HNF-3) (Costa, Grayson et al. 1989). In the same year, a gene whose mutation is responsible for a fork-headed appearance in Drosophila melanogaster was cloned, which shares a highly conserved motif with the HNF-3 proteins (Weigel, Jurgens et al. 1989). Later, hundreds of homologous proteins were discovered in species ranging from yeast to human. All of those genes were streamlined by a given new name as Fox, with a letter indicating the

subclass (Kaestner, Knochel et al. 2000). Thus, HNF-3 proteins are now named FOXA1, FOXA2 and FOXA3.

1.2.2 Biological roles in organ development and function

FOXA transcription factors share 95% identity within the forkhead (FKH) domain. They can bind a similar DNA sequence only with different affinities (Golson and Kaestner 2016). The divergent sequences outside of the conserved DNA binding domain are contributed to the different function of these proteins along with their distinct gene activation patterns. Outside the FKH domain, FOXA1 and FOXA2 are 39% identical and 51% similar, but FOXA3 is quite different from FOXA1 and FOXA2 (Figure 3). The winged-helix structure of the FKH domain is similar to the structure of linker histone H1 (Clark, Halay et al. 1993). Therefore, FOXA proteins can interact directly with histones H3 and H4 within gene enhancers even when it is compacted by linker histones. Once bound to their target sites, other transcription factors can be recruited at nearby sites. Therefore, the expression of FOXA target genes can be promoted or suppressed. For example, CCAAT-enhancer-binding proteins (C/EBPs), Neurofibromatosis type 1 (NF-1) are unable to access their cognate sites within the enhancer of albumin 1 (*Alb1*) without FOXA2 (Cirillo, Lin et al. 2002). For this reason, FOXA proteins were proposed to act as ‘pioneer’ transcription factors, opening the chromatin and making its target sites accessible to other transcription factors.

Expression levels of the genes activated by nuclear hormone receptors can be affected by FOXA proteins. The glucocorticoid receptor (GR) is expressed and its ligand distributed ubiquitously, however, as its target gene, tyrosine aminotransferase (*Tat*), will only be activated in in liver and kidney, which is due to high levels of FOXA factors in the liver.

Studies on the livers with conditional *Foxa2* deletion revealed that three fasting-induced genes (*Tat*, *Pck1*, and *Igfbp1*) are all regulated by FOXA2 in vivo. Chromatin immunoprecipitation demonstrated that GR can only bind its targets with the presence of FOXA2 in vivo (Zhang, Rubins et al. 2005). A similar relationship was identified between FOXA1 and the androgen receptor. Androgen response elements and binding sites for FOXA1 are frequently colocalized in the enhancers of androgen responsive genes (Gao, Zhang et al. 2003). ChIP-on-ChIP experiments demonstrated that this relationship between FOXA1 and androgen receptor is very common in the genome-wide level (Lupien, Eeckhoute et al. 2008). In addition, a global analysis of ESR1 binding sites showed that estrogen response elements and binding sites for FOXA1 are also frequently colocalized in the enhancers of estrogen responsive genes (Laganiere, Deblois et al. 2005). Studies in vitro revealed that at least part of the estrogen response in cultured breast cancer cell lines is regulated by the cooperation of estrogen and FOXA1(Carroll, Liu et al. 2005). Interestingly, FOXA1 occupancies in breast cancer cells has only a partial overlap with those in prostate cancer cells, suggesting that gene sets regulated by FOXAs are likely different across tissues (Lupien, Eeckhoute et al. 2008).

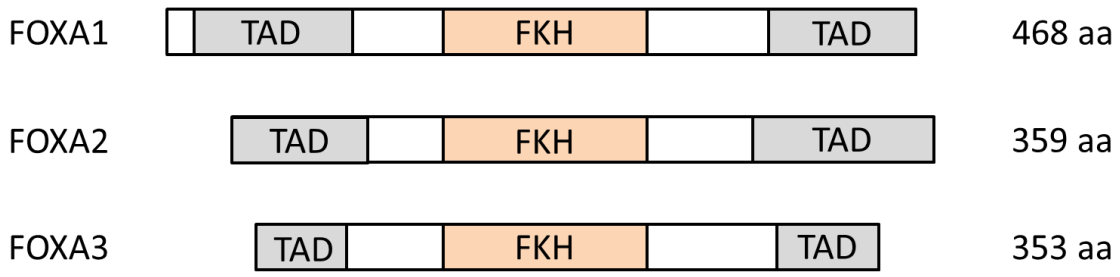

Figure 3. Schematic representation of functional domains present in mouse FOXA1–3. TAD, transactivation domain; FKH, forkhead domain/ winged helix. Adapted from (Lau, Ng et al. 2017).

FOXA proteins play essential roles in organ development and function. *Foxa2* is the only *Foxa* gene active in the early gastrula embryo. The early gastrula embryo expresses FOXA2 on day 6.5 of gestation in the primitive streak and node. FOXA2 expression is essential for the subsequent formation of the notochord (Ang, Wierda et al. 1993, Monaghan, Kaestner et al. 1993, Sasaki and Hogan 1993). *Foxa2*-deficient embryos die around day 9 of gestation due to the defects in formation of endoderm and notochord (Ang and Rossant 1994, Weinstein, Altaba et al. 1994). In contrast to FOXA2, neither FOXA1 nor FOXA3 is required during early mouse development. Both *Foxa1* and *Foxa3* null mice appear normal at birth and abnormalities are observed later in life (Kaestner, Katz et al. 1999, Shen, Scearce et al. 2001). Double deletion of *Foxa1* and *Foxa2* in mouse embryos (in the endoderm) results in severe defects on liver development. Neither liver bud development nor expression of the earliest liver marker gene alpha-fetoprotein (*Afp*) was observed in those embryos (Lee, Friedman et al. 2005). However, single ablation of either gene in foregut endoderm had little effect on liver development, indicating FOXA1 and FOXA2 may compensate for each other (Lee, Friedman et al. 2005). Like their functions during liver development, FOXA 1 and FOXA2 also regulate branching morphogenesis during the lung development. Double deletion of *Foxa1* and *Foxa2* in mouse severely blocks the branching morphogenesis in the lung, but not in the mice with ablation of either factor alone (Wan, Dingle et al. 2005). FOXA1 and FOXA2 have been showed to modulate the neuronal development. They regulate the expression of tyrosine hydroxylase, an enzyme required for the conversion of tyrosine to dopamine, a function required for normal mature dopaminergic neuron (Ferri, Lin et al. 2007, Lin, Metzakopian et al. 2009). This result was confirmed by the studies on mice with only one

Foxa2 null allele, showing age-dependent motor behavior abnormalities (Kittappa, Chang et al. 2007). Recent evidence showed that FOXA proteins also regulate organ specification, lineage differentiation, and mature function of pancreas (Lantz and Kaestner 2005, Gao, White et al. 2007). Studies on the mouse with double deletion of FOXA1 and FOXA2 showed that FOXA1/2 are critical regulators of *Pdx1* (pancreas duodenum homeobox gene) expression, a master gene of pancreas development (Gao, LeLay et al. 2008). Within the pancreas, FOXA1 and FOXA2 cooperate to control insulin secretion and to maintain the differentiated status of mature β-cells (Gao, Le Lay et al. 2010). Pancreatic β-cells with conditional deletion of *Foxa2* showed no response to the increased glucose level indicated by the lack of insulin secretion (Lantz, Vatamaniuk et al. 2004). Again, mice with single deletion of either *Foxa1* or *Foxa2* specify the pancreas normally (Lantz, Vatamaniuk et al. 2004, Vatamaniuk, Gupta et al. 2006). In addition, FOXA2 has been demonstrated to play roles in energy utilization in fat (Wolfrum, Besser et al. 2003). In summary, FOXA transcription factors have an essential biological role in organogenesis, differentiation and homeostasis.

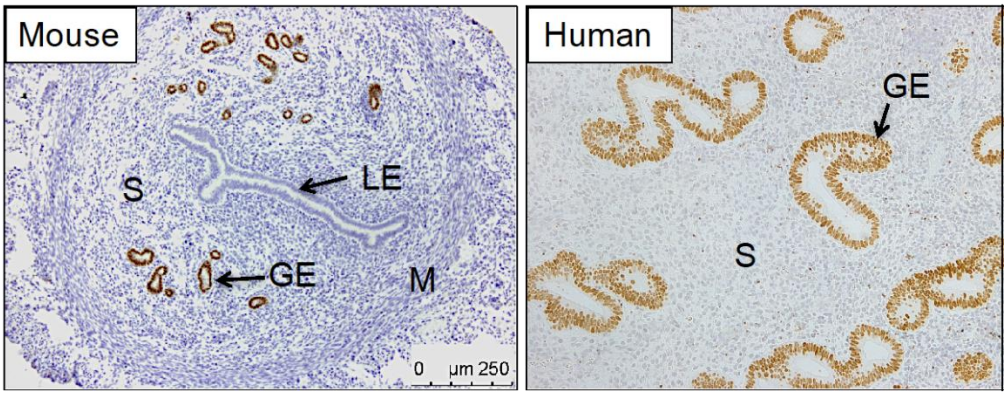

Figure 4. FOXA2 in the mouse and human uterus. FOXA2 protein in the uterus of 8-week old mice and endometrium from mid-secretory phase women. LE, luminal epithelium; GE, glandular epithelium; M, myometrium; S, stroma

FOXA2 is a critical regulator of female fertility via regulating the postnatal development and adult function of uteri. In the uterus, FOXA2 expression can be observed only in the glandular epithelium (GE), both the developing GE cells in the neonatal mouse uterus and the post-differentiated GE cells in the adult mouse and human (Figure 4) (Jeong, Kwak et al. 2010, Filant and Spencer 2013). Uterine development is initiated in early embryonic life with formation, patterning and differentiation of Müllerian or paramesonephric duct (MD). However, uterine development is not completed at birth in mammals (Spencer, Dunlap et al. 2012). At birth, the mouse uterus contains no endometrial glands with only a simple LE supported by undifferentiated mesenchyme. Started on PD 6, some LE cells differentiate into GE cells, proliferating and penetrating into the underlying stroma (Cunha, Young et al. 1989). By PD 15, the histoarchitecture of the uterus resembles that of the adult (Hu, Gray et al. 2004). Mice with floxed *Foxa2* genes was generated for conditional deletion because null mutation of *Foxa2* is embryonic lethal in mice. By crossing with the *Pgr-Cre* mouse, *Foxa2* was conditionally deleted in the uterus after birth in neonatal mice (Jeong, Kwak et al. 2010). The uteri of those mice contain no glands, and the adult mice were infertile because of defects in blastocyst implantation and stromal cell decidualization (Jeong, Kwak et al. 2010). Further, *Foxa2* was conditionally deleted using the lactotransferrin *(Ltf)-iCre* mouse model in which Cre recombinase is expressed only after puberty in the LE and GE cells of the uterus (Daikoku, Ogawa et al. 2014). The *Ltf-iCre* conditional deletion of *Foxa2* in the adult uterus results in the female infertility due to defects in blastocyst implantation stemming from a lack of LIF (Kelleher, Peng et al. 2017).

Recent studies suggest FOXA2 functions as a suppressor of carcinogenesis in several organs. Sequence analysis of 542 endometrioid endometrial cancers (EEC) shows *Foxa2* is frequently mutated in EEC, and 78.4 percent of them are loss of function mutations (Smith, Neff et al. 2016). The pattern of *Foxa2* mutations and expression in tumors points to its tumor suppressor function. A recent study found FOXA2 expression level is usually lower in Type II endometrial cancer than in Type I, which supports the idea that FOXA2 suppress the metastasis of endometrial cancer since Type II endometrial cancer is a more aggressive variant of endometrial cancers than Type I (Sung and Sohn 2014). It has been reported that FOXA2 prevented epithelial–mesenchymal transition (EMT) in both breast and lung cancers in human (Tang, Shu et al. 2011, Zhang, Yang et al. 2015). In cancer cells, a high level of FOXA2 expression usually indicates they have more features of epithelial cells. In breast cancer cells, knockdown of FOXA2 usually makes cancer cells having more mesenchymal features becoming more aggressive while overexpression of FOXA2 inhibits the EMT transition. FOXA2 also suppresses cell proliferation of both pancreas and stomach cancers *in vitro* and *in vivo* (Tang, Shu et al. 2011, Zhang, Yang et al. 2015, Zhu, Wang et al. 2015, Vorvis, Hatziapostolou et al. 2016).

1.2.3 Regulation of FOXA2 expression

FOXA proteins regulates the normal specification, differentiation and adult functions of certain organs such as liver, pancreas, intestine, lung, kidney, prostate, brain, heart and immune cells (Zhu 2016). However, the regulation of FOXA2 expression in different tissues is still poorly understood. Based on different steps during gene expression, gene regulation can be classified into epigenetic regulation, transcriptional regulation, post-transcriptional regulation and post-translational modification in eukaryotes. Epigenetic regulation is defined as the changes in a chromosome that affect gene activity and expression (Bird 2007). For example, changes of DNA methylation can affect gene expression without changing the underlying DNA sequence (Bird 2007). Transcriptional regulation is conducted by regulating the production of RNAs converted from DNA, which will then affect the production of relevant proteins. An example is transcription factors. They can be divided into two groups based on their functions, activators and repressors. They can bind the cis-regulatory elements alone or cooperating with other factors, promoting or suppressing the expression of their target genes. Activators promote the recruitment of RNA polymerase activating the gene expression, while repressors block the recruitment of RNA polymerase and the gene expression (Roeder 1996). mRNA modification makes a difference for the subsequent translation. For example, changes of the capping and a Poly(A) tail usually affect stability of an RNA after the transcription resulting in different expression of specific genes, which is called post-transcriptional regulation. microRNAs, a small non-coding RNA molecule (containing about 22 nucleotides), are critical regulators of the mRNA stability. More than 60% of protein coding genes of the human genome are likely regulated by

MicroRNAs (Friedman, Farh et al. 2009). After translation, proteins can still be modified by covalently linking with a functional group, such as a group phosphate and an acyl group. Those functional groups alter protein stability or their translocation, which is called post-transcriptional regulation. Post-transcriptional regulation include phosphorylation, glycosylation, ubiquitination, nitrosylation, methylation, acetylation, lipidation and proteolysis (Duan and Walther 2015).

At the DNA level, methylation at the CpG sites within the promoter region of the *Foxa2* gene is likely to decrease its expression in cancer cells. Studies on 25 lung cancer cell lines in vitro showed that they tend to have low FOXA2 expression, and *Foxa2* promoter hypermethylation is likely responsible for the downregulation of its expression (Basseres, D'Alo et al. 2012). Studies on melanoma cells identified a CpG island localized at −354 to −229 of the *Foxa2* promoter. Hypermethylation at this island leads to *Foxa2* silencing on melanoma cells (Yu, Jo et al. 2011). However, studies on normal cells provide opposite evidence indicating expression of *Foxa2* gene was promoted by DNA methylation within its promoter. Undifferentiated human embryonic stem cells (hESCs) expresses no FOXA2 while they displayed high levels of DNA methylation at a CpG island in the promoter region of the FoxA2 gene. hESC-derived early endoderm stage cells (CXCR4+ cells) and pancreatic islet cells displayed high levels of DNA methylation at this CpG island, but expressed relative high levels of FOXA2 (Bahar Halpern, Vana et al. 2014). One possible explanation is that in some cells, DNA methylation inhibits the binding of repressing proteins to *Foxa2* promoter activating its expression (Bahar Halpern, Vana et al. 2014).

WNT signaling pathway is likely to regulate the FOXA2 expression. FOXA2 is essential for the development of uterine glands in the neonate indicated by the lack of uterine glands in the mouse models with the ablation of *Wnt7a*, *Wnt5a*, *Ctnnb1* or *Lef1*, all of which are involved in WNT signaling pathway. In the adult prostate, consecutive activation of β-Catenin induced the re-expression of FOXA2, which is normally expressed only in the embryonic prostate (Yu, Wang et al. 2009). Similar results were observed in the uterus. LE cells, which have no FOXA2 expression in normal uteri, express FOXA2 in the uterus with consecutive activation of β-Catenin (Villacorte, Suzuki et al. 2013). Three conserved candidate TCF/LEF-binding sites were identified within *Foxa2* candidate promoter regions (−1810/+79) (Villacorte, Suzuki et al. 2013). ChIP assay showed one of these binding sites contains prominent enrichment of β-catenin (Villacorte, Suzuki et al. 2013). In mice with conditional ablation of *Foxa2*, no differences in *Wnt7a* or *Wnt5a* expression was observed in the adult uterus (Jeong, Kwak et al. 2010). However, FOXA2 was completely absent in the *Wnt7a* conditional mutant uterus (Dunlap, Filant et al. 2011). Another possible signaling pathway that regulates FOXA2 expression is Hedgehog signaling pathway. FOXA2 expression is decreased in the neural tube floor plate of *Shh*-null mice from E8.5, suggesting that SHH signaling may be required for maintenance, but not induction, of FOXA2 expression (Chiang, Litingtung et al. 1996, Kaufmann and Knochel 1996). A recent study showed that an endoderm-specific lncRNA, definitive endoderm-associated lncRNA1 (DEANR1), plays an important role in human endoderm differentiation by positively regulating expression of the endoderm factor FOXA2 (Jiang, Liu et al. 2015). In different cell types, high levels of DEANR1 expression usually indicate high levels of FOXA2 expression. In human

embryonic stem cells (hESCs), FOXA2 expression is downregulated upon the knockdown of DEANR1. Dual RNA-DNA-FISH demonstrates that DEANR1 transcripts are localized to the FOXA2 gene locus. Upon binding the *Foxa2* gene, DEANR1 stimulate FOXA2 expression by recruiting and interacting with SMAD2/3 (Jiang, Liu et al. 2015). Other factors likely regulating *Foxa2* expression includes Snail Family Transcriptional Repressor 1 (SNAIL1) in colorectal cancer cells, Sex Determining Region Y-Box 17 (SOX17) in the endoderm of Xenopus laevis and eomesodermin (EOMES) in the human definitive endoderm (Sinner, Rankin et al. 2004, Teo, Arnold et al. 2011, Jagle, Busch et al. 2017).

For the post-transcriptional regulation, two MicroRNAs (miR-124a and miR-187) were identified to inhibit *Foxa2* expression (Baroukh, Ravier et al. 2007, Li, Lu et al. 2017). The miR-124a2 precursor (pre-miR-124a2) or inhibitor (anti-miR-124a2) were transfected into murine pancreatic MIN6 β-cells. Introduction of miR-124a2 precursor decreased FOXA2 protein levels and, conversely, miR-124a2 inhibitor increased FOXA2 protein amounts determined using Western blotting. Quantification of the signals revealed the administrated amount of pre-mir-124a2 was highly correlated with the FOXA2 protein level, and an inverse correlation was observed between the administrated amount of anti-miR-124a2 and the FOXA2 protein level (Baroukh, Ravier et al. 2007). In gastric cancer cells, a luciferase assay showed that overexpression of miR-187 significantly inhibited the luciferase activity of wild-type 3-UTR of FOXA2 while inhibiting miR-187 significantly increased its luciferase activity (Li, Lu et al. 2017).

The activity of FOXA2 can also be regulated via phosphorylation, sumoylation and acetylation. In the liver, PI3-kinase–AKT pathway activated by insulin induces

Foxa2 phosphorylation. Phosphorylated FOXA2 was transported out of the nucleus suppressing its activity. T156 is where the phosphorylation occurs which is absent in other FOXA members (Wolfrum, Besser et al. 2003). In hepatocellular carcinoma tumors, (IκB Kinase α) IKKα, an important downstream kinase of Tumor necrosis factor alpha (TNFα), interacts with and phosphorylates FOXA2. The phosphorylation occurs at S107/S111, which also suppresses FOXA2 activity. Expression of Protein numb homolog (NUMB) is regulated by FOXA2, and low FOXA2 activity leads to low level of NUMB expression. Low level of NUMB further activates the NOTCH pathway which regulate cell proliferation and tumorigenesis (Liu, Lee et al. 2012). In INS-1E insulinoma cells, sumoylation of FOXA2 was identified, which can increase its transcriptional activity. Sumoylation is the attachment of a small ubiquitin related modifier-1 (SUMO-1) to a protein. that increased the transcriptional activity of FOXA2. Sumoylation has been showed occurs at a single site (K6). A mutation of K6 to arginine decrease the level of FOXA2 protein, but the level of *Foxa2* mRNA are not changed in INS-1E insulinoma cells, mapping the sumoylation site to the amino acid lysine 6 (K6) (Belaguli, Zhang et al. 2012). In the HepG2 cells, Sirtuin 1 (SIRT1) was found to interact with and deacetylate FOXA2. Deacetylation of FOXA2 by SIRT1 decreases its protein stability and levels (van Gent, Di Sanza et al. 2014). A similar regulation was observed in the β-Cell. SIRT1 deacetylates FOXA2, increasing *Pdx1* level which is required for pancreas development and β-cell formation (Wang, Xu et al. 2013).

1.3 Rational for thesis

In all studied mammals, the endometrium contains glands. Their secretions are essential for female fertility demonstrated in sheep and mouse models. Uteri with no glands are unable to support normal implantation, and recurrent early pregnancy loss are observed in those models. However, the underlying mechanism regulating the development of uterine glands are not fully understood.

In mice, the development of uterine glands is a postnatal event, and the GE originates from the LE. FOXA2 is the only FOXA members expressed in the uterus, and is expressed uniquely in the GE. Uteri with *Foxa2* conditional ablation after birth do not develop any uterine glands suggesting FOXA2 is a critical regulator of the development of uterine glands (Jeong, Kwak et al. 2010). However, whether FOXA2 play roles in the differentiation from the uterine LE to GE remains unknown.

Recently, a conditional overexpression system was designed using the Cre/loxP system, and successfully overexpressed COUP-TFI specifically in the mouse uterus (Wu, Lee et al. 2010). By employing this approach, we can generate a mouse model for the conditional overexpression of FOXA2. A new iCre knock-in mouse model was generated, in which iCre expresses primarily in the epithelium of the uterus (Daikoku, Ogawa et al. 2014). This model can be used to overexpress FOXA2 specifically in the epithelium of the uterus. Overexpression of FOXA2 in the uterine epithelium, especially in the LE, will provide us valuable insights into its roles in differentiation from the uterine LE to GE.

Chapter 2 Generation of Mouse for Conditional Expression of Forkhead Box A2 (FOXA2)

This work has been published in:

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Abstract

Forkhead box A2 (FOXA2) is a pioneer transcription factor involved in organ development, function and cancer. In the uterus, FOXA2 is essential for pregnancy and expressed specifically in the glands of the endometrium loss of FOXA2 function occurs during development of endometrial cancer in humans. The present study describes the development of a mouse model for conditional expression of mouse FOXA2. Using a system consisting of a minigene located at the Rosa26 locus, a CAG-S-mFOXA2 allele was generated in embryonic stem cells and subsequently in mice; before activation, the minigene is silent due to a floxed stop cassette inserted between the promoter and the transgene. To validate functionality, mice with the CAG-S-mFOXA2 allele were crossed with progesterone receptor (Pgr)-Cre mice and lactotransferrin (Ltf)-iCre mice that express Cre in the immature and adult uterus, respectively. In immature Pgr-Cre-CAG-S-mFoxa2 mice, FOXA2 protein was expressed in the luminal epithelium (LE), glandular epithelium (GE), stroma and inner layer of the myometrium. Interestingly, FOXA2 protein was not observed in most of the luminal epithelium of uteri from adult Pgr-Cre-CAG-S-mFoxa2 mice, although FOXA2 was maintained in the stroma, glandular epithelium and myometrium. The adult Pgr-Cre-CAG-S-mFoxa2 females were completely infertile. In contrast, Ltf-iCre-CAG-S-mFoxa2 mice were fertile with no

detectable histological differences in the uterus. The adult uterus of Pgr-Cre-CAG-S-mFoxa2 mice was smaller, contained few endometrial glands, and displayed areas of partially stratified luminal and glandular epithelium. This novel transgenic mouse line is a valuable resource to understand and explore FOXA2 function.

Introduction

Forkhead box (FOX) proteins are a family of transcription factors that play important roles in regulating the expression of genes involved in cell growth, proliferation, differentiation, and longevity (Friedman and Kaestner 2006, Kaestner 2010). FOX proteins have pioneering transcription activity by being able to bind and open condensed chromatin during cell differentiation processes. FOXA2 has important roles in genesis, differentiation and function of many endoderm-derived organs, such as the al liver, lung, and gut (Friedman and Kaestner 2006, Kaestner 2010, Spencer, Dunlap et al. 2012), and differentiation and function of uterine glands in the uterus (Jeong, Kwak et al. 2010, Filant and Spencer 2013, Filant, Lydon et al. 2014). After birth, *Foxa2* is expressed exclusively in glandular epithelium cells of the neonatal mouse uterus (Jeong, Kwak et al. 2010, Filant and Spencer 2013). In the human uterus, FOXA2 protein is also expressed in the glandular epithelium (Villacorte, Suzuki et al. 2013). Homozygous *Foxa2* null embryos die shortly after gastrulation (Ang and Rossant 1994, Weinstein, Ruiz i Altaba et al. 1994), as it is required for the development of several endoderm-derived organs (Kaestner, Hiemisch et al. 1994, Kaestner 2010). To understand the role of FOXA2 in the uterus, *Foxa2* was conditionally deleted using the progesterone receptor (Pgr)-Cre mouse model in which Cre recombinase is expressed only after birth in most cells of the uterus (Soyal, Mukherjee et al. 2005). Conditional deletion of *Foxa2* in the neonate inhibited uterine gland genesis, resulting in aglandular adult mice that were infertile due to defects in blastocyst implantation and stromal cell decidualization stemming from a lack of LIF and other glandular epithelium secretions (Jeong, Kwak et al. 2010, Filant and Spencer

2013). To determine the role of FOXA2 in the adult uterus, *Foxa2* was conditionally deleted using the lactotransferrin (Ltf)-iCre mouse model in which Cre recombinase is expressed only after puberty in the luminal epithelium and glandular epithelium cells of the uterus (Daikoku, Ogawa et al. 2014). The Ltf-iCre conditional deletion of Foxa2 in the adult uterus rendered the mice infertile due defects in blastocyst implantation stemming from a lack of LIF (Kelleher, Peng et al. 2017).

In addition to roles in organ development and function, FOXA2 also has a role in disease and cancer of many organs including the liver and pancreas (Zhao and Li 2015, Vorvis, Hatziapostolou et al. 2016). In women, FOXA2 is down-regulated in ectopic as compared to eutopic endometrium in patients with endometriosis and also implicated in the molecular pathophysiology of endometriosis and endometrial adenocarcinoma (Luong, Painter et al. 2013, Villacorte, Suzuki et al. 2013, Yang, Kang et al. 2015, Yao, Shen et al. 2015). FOXA2 is a proposed tumor suppressor gene in endometrioid endometrial carcinomas (Smith, Neff et al. 2016) and regulates tumor metastasis (Wang, Zhu et al. 2014, Zhu, Wang et al. 2015). Further, FOXA2 is somatically mutated in uterine carcinomas particularly by frameshift and nonsense mutations (Le Gallo, Rudd et al. 2018). Thus, FOXA2 is a pathogenic driver gene in some of the most clinically aggressive forms of uterine cancer. The objective here was to develop a mouse model for conditional expression of FOXA2 in vivo using an established approach (Wu, Lee et al. 2010) and validate the usefulness of the mouse model by determining the consequence of FOXA2 overexpression on development and function of the mouse uterus.

Materials and Methods

Generation of Foxa2 conditional expression mouse

An established approach for generation of conditional expression mice was utilized as described by Wu and coworkers (Wu, Lee et al. 2010). The Shuttle Vector RfNLIII was generously provided by Ming-Jer Tsai (Baylor College of Medicine, Houston, Texas). To generate an embryonic stem cell targeting construct (Supplemental Fig. 1), the mouse *Foxa2* cDNA was amplified by PCR and inserted into the targeting construct downstream of a Lox-Stop-Lox (LSL) cassette. The gene targeting in AB2.2 ES cells and production of chimeras from those ES cells was performed by the Genetically Engineered Mouse Core at Baylor College of Medicine. Chimeras were bred with C57BL/6 mice, and founder *mFoxa2*^{LSL} mice (Gt(ROSA)26Sor$^{tm1(CAG-Foxa2)TES}$) screened by PCR genotyping. Primers for the gene replacement allele are 5'-GGA GCG GGA GAA ATG GAT ATG-3' (forward) and 5'-GCT TTC TGG CGT GTG ACC-3' (reverse) with a product size 0.6 kb. PCR primers for the wild-type ROSA26 locus are 5'-GGA GCG GGA GAA ATG GAT ATG-3' (forward) and 5'-AAA GTC GCT CTG AGT TGT TAT-3' (reverse) with a product size of 0.6 kb. PCR genotyping was performed using tail DNA and 35 cycles of 95^{o}C for 1 min, 56^{o}C for 1 min, and 72^{o}C for 1 min.

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Missouri and were conducted according to NIH Guide for the Care and Use of Laboratory Animals. The *mFoxa2*^{LSL} mice were bred with *Pgr*^{Cre}

(Soyal, Mukherjee et al. 2005) and Ltf^{iCre} (Daikoku, Ogawa et al. 2014) mice to generate $Pgr^{Cre/+}mFoxa2^{LSL/+}$ and $Ltf^{iCre/+}mFoxa2^{LSL/+}$ mice that conditionally overexpress (cOE) mouse Foxa2. The Pgr^{Cre} mice were generously provided by John Lydon (Baylor College of Medicine, Houston, TX). The Ltf^{iCre} mice (stock no. 022620) were obtained from The Jackson Laboratory (Bar Harbor, ME). Heterozygous $mFoxa2^{LSL/+}$ mice were used as controls.

For the fertility trial, female mice were housed individually and continuously with CD-1 male mice of proven fertility. Fertility was assessed by monitoring litter frequency and size for 6 months. Gestational time points were obtained by the mating of 8- to 10-wk old females to CD-1 male mice of known fertility, and the day of vaginal plug observation was designated as gestational day (gestational day) 0.5. Implantation sites on gestational day 5.5 were visualized by i.v. injection of 1% Evans blue dye (Sigma-Aldrich) into the tail vein 5 min before euthanasia. Other studies involved subcutaneous injections of vehicle (sesame oil), 17β-estradiol (E2; 100 ng per mouse per day), or progesterone (P4; 1 mg per mouse per day). Sesame oil and hormones were sourced from Sigma Aldrich (St. Louis, MO).

RNA Extraction and Real-Time PCR

Total RNA was isolated from uteri using a standard TRIzol-based protocol. To eliminate genomic DNA contamination, extracted RNA was treated with DNase I and purified using an RNeasy MinElute Cleanup Kit (Qiagen). Total RNA (1 μg) from each sample was reverse transcribed in a total reaction volume of 20 μL using iScript RT Supermix (Bio-Rad). Real-time PCR was performed using a CFX384 Touch Real-Time System

with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) using Bio-Rad PrimePCR primers (Supplemental Table 1). Mus musculus *Gapdh* and *Rplp0* were used as reference genes.

Histology and Immunohistochemistry

Paraformaldehyde-fixed, paraffin-embedded mouse tissues were sectioned (5 μm), mounted on slides, deparaffinized, and rehydrated in a graded alcohol series. For immunohistochemistry, sections were subjected to antigen retrieval in boiling 10 mM citrate buffer (pH 6.0) for 10 min followed by an incubation with 1.0% (vol/vol) hydrogen peroxide in methanol for 15 min. Sections were blocked with 10% (vol/vol) normal goat serum (PCN500; Life Technologies) in PBS (pH 7.2) for 30 min and then incubated with the primary antibody in PBS with 1% BSA overnight at 4^{o}C. Antibody information is provided in Table 1. Sections were washed in PBS and incubated with 5 μg/mL biotinylated secondary antibody (Vector Laboratories, Inc.) in PBS for 1 h at 37^{o}C. Immunoreactive protein was visualized using a Vectastain ABC kit (Vector Laboratories, Inc.) with diaminobenzidine tetrahydrochloride as the chromagen. Sections were lightly counterstained with hematoxylin before dehydrating and affixing coverslips with Permount.

In Situ Hybridization

In situ hybridization was performed using the RNAscope 2.5 HD Assay-Red kit from Advanced Cell Diagnostics (Newark, CA). Briefly, 4% paraformaldehyde-fixed, paraffin-embedded mouse tissues were sectioned (5 μm), mounted on slides, and deparaffinized in

xylene and alcohol. The sections were hybridized at 40^{o}C for 2 h with a RNAscope Probe-Mm-Foxa2 that is specific for mouse *Foxa2* mRNA from Advanced Cell Diagnostics. The signal was visualized using chromogenic RNAscope 2.5 HD Detection Reagent. Sections were briefly counterstained with hematoxylin before dehydrating and affixing coverslips with Permount.

Western Blot

Total protein was isolated from frozen adult mouse uteri using sing RIPA buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 1% NP-40, 0.5% SDC, 0.1% SDS, pH 7.5) containing Halt Protease Inhibitor Cocktail (Thermofisher). Total uterine protein (30 mg) was separated on a denaturing 12% SDS- gel and transferred to a nitrocellulose membrane (Millipore). The membrane was blocked and incubated overnight at 4°C with primary antibody (Table 1). After incubation with peroxidase-conjugated secondary antibody goat anti-rabbit secondary antibody (Thermofisher), immunocomplexes were visualized by enhanced chemiluminescence (Thermofisher) and a Bio-Rad Chemidoc Imaging System.

Statistics

All quantitative data were subjected to least-squares ANOVA using the general linear model procedures of the Statistical Analysis System (SAS Institute) to determine the effects of genotype. In all analyses, error terms used in tests of significance were identified according to the expectation of the mean squares for error. Significance ($P < 0.05$) was determined by probability differences of least-squares means.

Results

Generation of the conditional expression allele for mouse Foxa2

As illustrated in Supplementary Fig. 1A, a shuttle vector was made to deliver a single copy of minigene consisting of a ubiquitous CAGGS promoter, loxP-STOP-loxP (LSL) cassette, mouse *Foxa2* coding sequence (NCBI CCDS16836.1), and a polyadenylation signal. The CAGGS promoter is a strong synthetic promoter widely used in mammalian cell lines and active in almost all tissues in vivo (Okabe, Ikawa et al. 1997). The LSL cassette was placed between the CAGGS promoter and *Foxa2* coding sequence to silence expression of the mouse *Foxa2* transgene (Tuveson, Shaw et al. 2004). In the presence of Cre recombinase, the LSL cassette is removed, which allows the CAGGS promoter to drive the expression of the mouse *Foxa2* coding sequence. The minigene was inserted into the ROSA26 locus that is widely used for constitutive, ubiquitous gene expression in mice (Zambrowicz, Imamoto et al. 1997). Embryonic stem (ES) cell lines harboring one allele of $mFoxa2^{LSL}$ at the ROSA26 locus of the mouse genome were generated by homologous recombination. Chimeras from the ES cells were bred to C57BL/6 mice to establish founder mice (Supplementary Fig. 1B). Founder mice heterozygous ($mFoxa2^{LSL/+}$) and homozygous ($mFoxa2^{LSL/LSL}$) for the Foxa2 conditional allele were viable and fertile. To verify the function of $mFoxa2^{LSL}$ locus, $Pgr^{Cre}Foxa2cOE$ ($Pgr^{Cre/+}mFoxa2^{LSL/+}$) mice were generated by crossing $mFoxa2^{LSL}$ mice with Pgr^{Cre} mice (Soyal, Mukherjee et al. 2005). The Cre excision activity in the Pgr^{Cre} mouse model is restricted to cells that express the PGR after birth, including the uterus, ovary, oviduct, pituitary gland, and mammary gland (Soyal, Mukherjee et al. 2005). Expression of *Pgr* is

initiated in the reproductive tract only after birth, and PGR is present in the uterine luminal epithelium by postnatal day 3 and in the stroma by postnatal day 6 (Kurita, Cooke et al. 2001, Franco, Jeong et al. 2008). Immunohistochemical analysis of uteri from control (*mFoxa2*$^{LSL/+}$) postnatal day 30 and adult gestational day 3.5 mice found that FOXA2 protein was only in the endometrial glands (Fig. 1A). In contrast, FOXA2 protein was present in the luminal epithelium, glandular epithelium, stroma and inner layer of the myometrium of immature postnatal day 30 *Pgr*^{Cre}*Foxa2cOE* mice. In adult *Pgr*^{Cre}*Foxa2cOE* mice, FOXA2 protein was observed in the stroma, glands and myometrium, however many of the luminal epithelium cells were not FOXA2 positive. In control (*mFoxa2*$^{LSL/+}$) mice, FOXA2 was observed only in the epithelia of oviduct and cervix and not in the ovary (Supplemental Fig. 2). In *Pgr*^{Cre}*Foxa2cOE* mice, FOXA2 was observed in the epithelia and stroma of the oviduct and cervix, but not in the ovary. In situ hybridization localized *Foxa2* mRNA in the same cells as FOXA2 protein in both control and *Pgr*^{Cre}*Foxa2cOE* adult mice (Fig. 1C). Real-time quantitative PCR analysis found that *Foxa2* mRNA levels were substantially increased ($P<0.01$) in the uteri of *Pgr*^{Cre}*Foxa2cOE* as compared to control mice on both postnatal day 30 and gestational day 3.5 (Fig. 1D). Western blot analysis found that FOXA2 protein level was substantially increased in the uteri of adult *Pgr*^{Cre}*Foxa2cOE* as compared to control uteri (Fig. 1E).

Next, *mFoxa2*^{LSL} mice were crossed with *Ltf*^{iCre} mice, which express Cre recombinase only in the luminal and glandular epithelia of the uterus after puberty (Daikoku, Ogawa et al. 2014). Before puberty in immature postnatal day 30 mice, FOXA2 was present only in the glandular epithelium of the uterus of both control (*mFoxa2*$^{LSL/+}$) and *Ltf*^{iCre}*Foxa2cOE*

(*Ltf*$^{iCre/+}$*mFoxa2*$^{LSL/+}$) mice (Fig. 1B). In the uterus of adult control mice, FOXA2 was also solely expressed in the glandular epithelium. In the *Ltf*^{iCre}*Foxa2cOE* mice, FOXA2 protein was in the glandular epithelium as well as in a few cells in the luminal epithelium (Fig. 1B). As expected, *Foxa2* mRNA levels were not different (P>0.10) in uterus of immature postnatal day 30 control and *Ltf*^{iCre}*Foxa2cOE* mice and were greater (P<0.01) in the adult uterus of *Ltf*^{iCre}*Foxa2cOE* mice (Fig. 1D). Thus, conditional expression of FOXA2 is limited primarily to the glands of the uterus using the *Ltf*^{iCre} driver model. In *Ltf*^{iCre} females, Cre expression is driven by the endogenous promoter of *Ltf*, which is an estrogen (E2)-responsive gene in the mouse uterus (Teng, Beard et al. 2002), and Cre recombinase activity in immature *Ltf*^{iCre} mice can be induced by estrogen receptor alpha (ESR1) agonists (Das, Tan et al. 1998). Immature *Ltf*^{iCre}*Foxa2cOE* mice were injected subcutaneously with either vehicle (sesame oil) or 17β-estradiol (E2; 100 ng per mouse per day) on postnatal day 20 and 21 and collected on postnatal day 22. Both *iCre* and *Foxa2* mRNA levels were clearly increased by E2 treatment (Fig. 2). FOXA2 protein was present in the glandular epithelium of *Ltf*^{iCre}*Foxa2cOE* mice receiving vehicle as well as E2 treatment (Fig. 2C). Similar to adult *Ltf*^{iCre}*Foxa2cOE* mice (Fig. 1B), a few cells in the luminal epithelium were also FOXA2 positive (Fig. 2B), which could be immune cells rather than luminal epithelium cells. Thus, the *mFoxa2*^{LSL} allele is effectively recombined and FOXA2 overexpressed in the glandular epithelium of the uterus using the *Ltf*^{iCre} model, but expression is not observed in the vast majority of luminal epithelium cells.

Effects of FOXA2 conditional expression on female fertility

Both *Pgr*^{Cre}*Foxa2cOE* and *Ltf*^{iCre}*Foxa2cOE* females displayed normal mating behavior indicated by the presence of copulatory plugs in the vagina following mating. A 6-month fertility trial was conducted with control and *Foxa2cOE* mice (Table 2). As compared to control females, the *Pgr*^{Cre}*Foxa2cOE* females were infertile, whereas the *Ltf*^{iCre}*Foxa2cOE* females displayed normal fertility. Next, mice were bred to intact fertile CD-1 males. Embryo implantation was assessed in nulliparous females on gestational day 5.5 by intravenous injection of a macromolecular blue dye that accumulates at sites of increased vascular permeability and can be used to visualize the location of embryo implantation (Deb, Reese et al. 2005). Implantation sites were easily discernible on gestational day 5.5 in the uteri of control and *Ltf*^{iCre}*Foxa2cOE* mice, but *Pgr*^{Cre}*Foxa2cOE* mice had no visible implantation sites (Fig. 3A). After the uterine lumen of mated control (n=6) and *Pgr*^{Cre}*Foxa2cOE* (n=7) females was gently flushed with saline on gestational day 3.5, an average of 6 embryos were recovered from the uterine lumen of each control female, but only 1 embryo was recovered from the *Pgr*^{Cre}*Foxa2cOE* females (data not shown).

Conditional expression of FOXA2 in the uterus elicits epithelial stratification

Uterine wet weight was lower ($P<0.01$) in *Pgr*^{Cre}*Foxa2cOE* compared to control or *Ltf*^{iCre}*Foxa2cOE* mice on gestational day 3.5 (Fig. 3B). Histological analysis revealed that the uteri of *Pgr*^{Cre}*Foxa2cOE* mice appeared to have fewer uterine glands than control mice (Fig. 4C), whereas the *Ltf*^{iCre}*Foxa2cOE* mice had normal uterine histoarchitecture. Indeed, the number of glands per transverse cross-section of the uterus was substantially reduced ($P<0.01$) in the uteri of *Pgr*^{Cre}*Foxa2cOE* as compared to control or

Ltf^{iCre}*Foxa2cOE* mice (Fig. 3D). In addition to reduced gland number, several areas of the endometrium from adult *Pgr*^{Cre}*Foxa2cOE* mice displayed areas of uncharacteristic bilaminar or stratified epithelium (Fig. 3C). Of note, the stratified regions of epithelium were not present throughout the entire uterus of nulliparous adult *Pgr*^{Cre}*Foxa2cOE* mice. The upper layer of cells in the stratified areas of luminal epithelium were FOXA2 positive in the *Pgr*^{Cre}*Foxa2cOE* uterus, which is abnormal as FOXA2 is never present in the luminal epithelium of control mice (Fig. 3C). As shown in Supplementary Fig. 2, FOXA2 is normally expressed in the upper layer of the stratified squamous type epithelium of the cervix (Kelleher, Peng et al. 2017), but not vagina (Besnard, Wert et al. 2004).

The abnormal stratified luminal and glandular epithelium in the uterus of *Pgr*^{Cre}*Foxa2cOE* mice was confirmed by immunostaining for tumor protein 63 (TP63) and keratin 14 (KRT14), two markers of epithelial stratification and basal cells (Koster, Kim et al. 2004, Koster and Roop 2007). Immunostaining revealed that KRT14 and P63 positive basal cells could be observed in discrete areas of stratified epithelia in the immature postnatal day 30 uterus from *Pgr*^{Cre}*Foxa2cOE* mice (Figs. 4A and 4B). The KRT14 and P63 positive basal cells persisted into adulthood in the uterus of *Pgr*^{Cre}*Foxa2cOE* mice. Of note, embryo implantation can be compromised in mice whose uteri contain a stratified squamous luminal epithelium or lack uterine glands (Filant, DeMayo et al. 2014).

Temporal and spatial alterations in expression of the PGR, estrogen receptor alpha (ESR1), and Ki67, a marker of cell proliferation (Scholzen and Gerdes 2000), was conducted in the uterus of early pregnant mice (Fig. 5). Overall, the patterns of PGR and

ESR1 expression were not different in the uterus of control and *Pgr*^{Cre}*Foxa2cOE* mice. Cell proliferation appeared to be increased in the uterine luminal epithelium and glandular epithelium of gestational day 3.5 *Pgr*^{Cre}*Foxa2cOE* mice, and Ki67 positive cells remained in the stratified areas of the luminal epithelium on gestational day 4.5. Stromal cell proliferation was noticeably suppressed in the uterus of *Pgr*^{Cre}*Foxa2cOE* mice on gestational days 3.5 and 4.5. Expression of CXCL15, a gland-specific gene (Schmitz, McCracken et al. 2007), declined in the glandular epithelium of both control and *Pgr*^{Cre}*Foxa2cOE* mice after gestational day 3.5, but was expressed in the areas of stratified luminal epithelium in the *Pgr*^{Cre}*Foxa2cOE* uterus (Fig. 5).

Genes regulated by estrogen and progesterone and involved in uterine receptivity for embryo implantation were measured in the early pregnant uterus (Fig. 6). Expression of a number of estrogen-responsive genes (*C3, Clca3, Cxcl15, Ltf, Muc1*) were considerably higher ($P<0.01$) in the uteri of *Pgr*^{Cre}*Foxa2cOE* as compared to control mice. With the exception of *Areg*, expression of progesterone-regulated genes (*Gata2, Hoxa10, Ihh*) was not different ($P>0.01$) in the control and *Pgr*^{Cre}*Foxa2cOE* uteri during early pregnancy. Consistent with the decreased numbers of uterine glands in *Pgr*^{Cre}*Foxa2cOE* mice, expression of glandular epithelium-specific genes (*Lif, Prss29, Spink3, Ttr*) were substantially decreased ($P<0.01$) in the uterus of *Pgr*^{Cre}*Foxa2cOE* during early pregnancy. Although CXCL15 is a glandular epithelium-specific gene in the normal mouse uterus (Schmitz, McCracken et al. 2007) (Fig. 6), levels of *Cxcl15* mRNA were substantially higher in the *Pgr*^{Cre}*Foxa2cOE* uteri throughout early pregnancy (Fig. 6), likely due to expression in the areas of stratified luminal epithelium (Figs. 4 and 5).

Effects of ovariectomy and steroid hormones in immature *Pgr^{Cre}Foxa2cOE* mice

Expression of FOXA2 was clearly observed in the luminal epithelium of uteri from immature postnatal day 30 *Pgr^{Cre}Foxa2cOE* mice, however the majority of luminal epithelium cells in adult *Pgr^{Cre}Foxa2cOE* mice do not express FOXA2 (Figs. 1 and 7A). To determine the effect of ovarian hormones on FOXA2 overexpression in the luminal epithelium, postnatal day 30 *Pgr^{Cre}Foxa2cOE* mice were ovariectomized and rested for 10 days. Beginning on postnatal day 40, ovariectomized mice treated daily with either vehicle, P4 (1 mg) or E2 (100 ng), and then necropsied on postnatal day 50. Expression of FOXA2 was maintained in the uterus of ovariectomized *Pgr^{Cre}Foxa2cOE* mice receiving either vehicle or P4 (Fig. 7B). In contrast, most of the luminal epithelium cells were FOXA2 negative in E2-treated mice; however, the stroma, glandular epithelium, and myometrial cells remained FOXA2 positive. Note that expression of the PGR and Ki67 cell proliferation marker was increased in the luminal epithelium by E2 treatment.

Discussion

In the present study, conditional overexpression of FOXA2 was achieved in the neonatal uterus using the *Pgr-Cre* model and in the adult uterus using the *Ltf-iCre* model. In the uterus, the Pgr-Cre driver produced conditional FOXA2 expression in the luminal epithelium, glandular epithelium, stroma and inner circular layer of myometrium, which phenocopies expression of the PGR (Soyal, Mukherjee et al. 2005). Indeed, FOXA2 expression is specifically restricted to the differentiating and developing glandular epithelium of the mouse uterus (Filant, Zhou et al. 2012, Filant and Spencer 2013).

Curiously, expression of FOXA2 was not present in most of the luminal epithelium cells of the adult uterus of *Pgr^{Cre}Foxa2cOE* mice despite those cells uniformly expressing the PGR. The loss of FOXA2 expression was observed by postnatal day 70. The ovariectomy experiment suggests that estrogen is involved in the loss of luminal epithelium cells that overexpress FOXA2. Although some of the luminal epithelium cells remain FOXA2 positive in the adult uterus of adult *Pgr^{Cre}Foxa2cOE* mice, those cells are clearly stratified and not normally present in the uterus. On postnatal day 30, only a few cells in the luminal epithelium of the *Pgr^{Cre}Foxa2cOE* uterus were FOXA2-negative. Thus, one plausible explanation is that the subset of FOXA2-negative luminal epithelium cells selectively proliferates in the prepubertal uterus under the influence of estrogen, which is supported by findings that FOXA2 is a tumor suppressor and controls the proliferation of endometrial epithelial cells (Villacorte, Suzuki et al. 2013). Indeed, FOXA2 is down-regulated or inactivated by mutation during the development of endometrial epithelial hyperplasia and cancer (Villacorte, Suzuki et al. 2013, Makker and Goel 2016, Le Gallo, Rudd et al. 2018). A second possibility is that an unknown mechanism silenced expression of PGR-A, which is used to drive the Cre transgene in Pgr-Cre mice (Soyal, Mukherjee et al. 2005). A third possibility is that the *mFoxa2* transgene is progressively and selectively silenced in the luminal epithelium cells of the *Pgr^{Cre}Foxa2cOE* mice as the mice age and become adults. In mice, the *Ltf* gene is estrogen-responsive and specifically expressed in the luminal and glandular epithelium of the adult uterus (Das, Tan et al. 1998), and Cre expression in the Ltf-iCre driver mouse model recombines floxed alleles in both the luminal and glandular epithelium (Daikoku, Ogawa et al. 2014). However, FOXA2 overexpression was observed only in the glandular epithelium of adult

Ltf^{iCre}*Foxa2cOE* mice. Indeed, expression of *Foxa2* mRNA or FOXA2 protein was not observed in the luminal epithelium cells of the uterus in adult *Ltf*^{iCre}*Foxa2cOE* mice. Those results support the idea that the FOXA2 transgene is silenced specifically in the luminal epithelium by some unknown epigenetic mechanism. Indeed, the only current known mechanisms regulating *FOXA2* gene expression involve miRNA targeting of the 3' UTR and control of *FOXA2* mRNA stability (Tu, Pan et al. 2016). Further analysis of the conditional FOXA2 overexpression models developed here could provide significant insights into FOXA2 regulation *in vivo*.

The development of a stratified luminal epithelium with basal cells in the uteri of *Pgr*^{Cre}*Foxa2cOE* mice was an unexpected finding, particularly since the majority of luminal epithelium cells in those adult mice were negative for FOXA2 expression. The presence of basal cells was confirmed by coexpression of TP63 and KRT14, which are basal cell markers and expressed in those cells in the cervix and vagina (Kurita and Cunha 2001, Kurita 2011). The expression of TP63 is essential for the stratified squamous differentiation because the epithelium of the Müllerian vagina in *Tp63* null mice formed a uterus-like layer of columnar epithelium (Kurita and Cunha 2001). In the present study, FOXA2 expression in the luminal epithelium of adult *Pgr*^{Cre}*Foxa2cOE* mice was confined to the nuclei of the upper, nonbasal cell layer of the stratified epithelia. This same pattern of FOXA2 expression was observed in the cervix of control mice. The biological role, if any, of FOXA2 in the cervical epithelium is not known. Epithelial stratification is observed in the uterus of conditional deletion mutants of *Wnt4* and *Fgfr2* and conditional overexpression of Notch1 created using the *Pgr*^{Cre} model (Franco, Dai et al. 2011, Filant, DeMayo et al. 2014, Rubel, Wu et al. 2016, Su, Strug et

al. 2016). The development of stratified luminal epithelium in the *Pgr^{Cre}Foxa2cOE* uterus is likely due to altered stromal-epithelial communication due to misexpression of FOXA2 in the stroma and myometrium (Filant, DeMayo et al. 2014). Stratified luminal epithelium was not observed in the uterus of adult *Ltf^{iCre}Foxa2cOE* mice that were completely fertile.

In contrast to *Ltf^{iCre}Foxa2cOE* mice, adult *Pgr^{Cre}Foxa2cOE* mice were infertile. The underlying cause of this infertility may be oviductal or cervical dysfunction, as few embryos were found in the uterine flush of gestational day 3.5 *Pgr^{Cre}Foxa2cOE* mice or in their uteri on gestational day 4.5. The ciliary epithelium appeared less folded in *Pgr^{Cre}Foxa2cOE* mice, but defects in cervix or uterine function may also be responsible for the infertility. In addition to areas of stratified luminal epithelium, the uterus of *Pgr^{Cre}Foxa2cOE* mice exhibited reduced numbers of uterine glands based on histology and reduced or absent expression of *Lif*, *Prss29*, *Spink3*, and *Ttr*, which are expressed only in the glandular epithelium of gestational day 2.5 to 4.5 uteri (Filant and Spencer 2013, Filant and Spencer 2014). The glands of the uterus begin to differentiate from the luminal epithelium and develop after postnatal day 5 in the uterus of neonatal mice (Hu, Gray et al. 2004, Cooke, Ekman et al. 2012, Filant, Zhou et al. 2012). Conditional deletion of *Foxa2* using Pgr-Cre driver mice prevents the genesis and differentiation of endometrial glands in the neonatal uterus, rendering a glandless uterus in the adult (Jeong, Kwak et al. 2010, Filant, Lydon et al. 2014). Of note, conditional expression of Notch1 in the mouse uterus using Pgr-Cre driver mice aberrantly upregulates FOXA2 expression in the luminal epithelium, and the uterus is glandless in adult PgrCre-NotchcOE mice (Su, Strug et al. 2016). In addition to an absence of glands, estrogen and

progesterone responsive genes were dysregulated in the *Pgr*^{Cre}*Foxa2cOE* uterus. Thus, misexpression of FOXA2 in the luminal epithelium and/or stroma of the developing neonatal mouse uterus alters or inhibits normal differentiation and genesis of endometrial glands and function of the adult uterus.

The present study employed homologous recombination in ES cells to generate mice in which the expression of FOXA2 can be activated in a spatial- and temporal-specific fashion. As noted by Wu et al. (Wu, Lee et al. 2010), advantages of this system include: 1) fast generation of recombination construct; 2) no expression in the absence of Cre recombination; 3) a single mouse line to express a gene of interest in any given tissue or cell type of interest with available tissue-specific Cre recombinase mouse lines; 4) temporal expression is feasible when inducible expression of Cre is employed (e.g. tamoxifen or tetracycline inducible Cre); and 5) uniform expression levels wherever activated. Results of the present study and others (Wu, Lee et al. 2010, Szwarc, Kommagani et al. 2014, Vasquez, Wu et al. 2016, Wetendorf, Wu et al. 2017) confirm the utility of this overexpression strategy for FOXA2 and other transcription factors in studies of uterine development and function. Applications of this tool can be multiple, including, but not limited to, mimicking human diseases, directing cell differentiation, reprogramming cell identity, and dissecting genetic pathways. For example, FOXA2 is essential for differentiation and development of many endoderm-derived structures in the embryo, such as the pancreas and lung (Kaestner 2010), and glands in the uterus in the neonate after birth (Jeong, Kwak et al. 2010). Besides its importance in regulating organ differentiation, FOXA2 also plays a key role in regulating cell-specific gene expression in and function of adult organs including the liver and uterus (Kaestner 2010, Kelleher,

Peng et al. 2017). Further, FOXA2 is a tumor suppressor that is involved in a number of different cancers including those of the bladder, colon, liver, lung and uterus (Katoh and Katoh 2004, Smith, Neff et al. 2016, Le Gallo, Rudd et al. 2018). Thus, the FOXA2 conditional expression allele could be useful to study FOXA2 involvement in organ-specific tumor progression and metastasis. Furthermore, in combination with genetic engineered alleles of other genes that pose as cancer risk factors, this model can be used to study effects of genetic interaction or dissect signal hierarchy between FOXA2 and other genes. Finally, the conditional overexpression allele approach could be used to create a humanized mouse model to analyze FOXA2 variants in development and tumorigenesis as well as screen drugs that directly target FOXA2, accelerating the development of new therapeutic modalities.

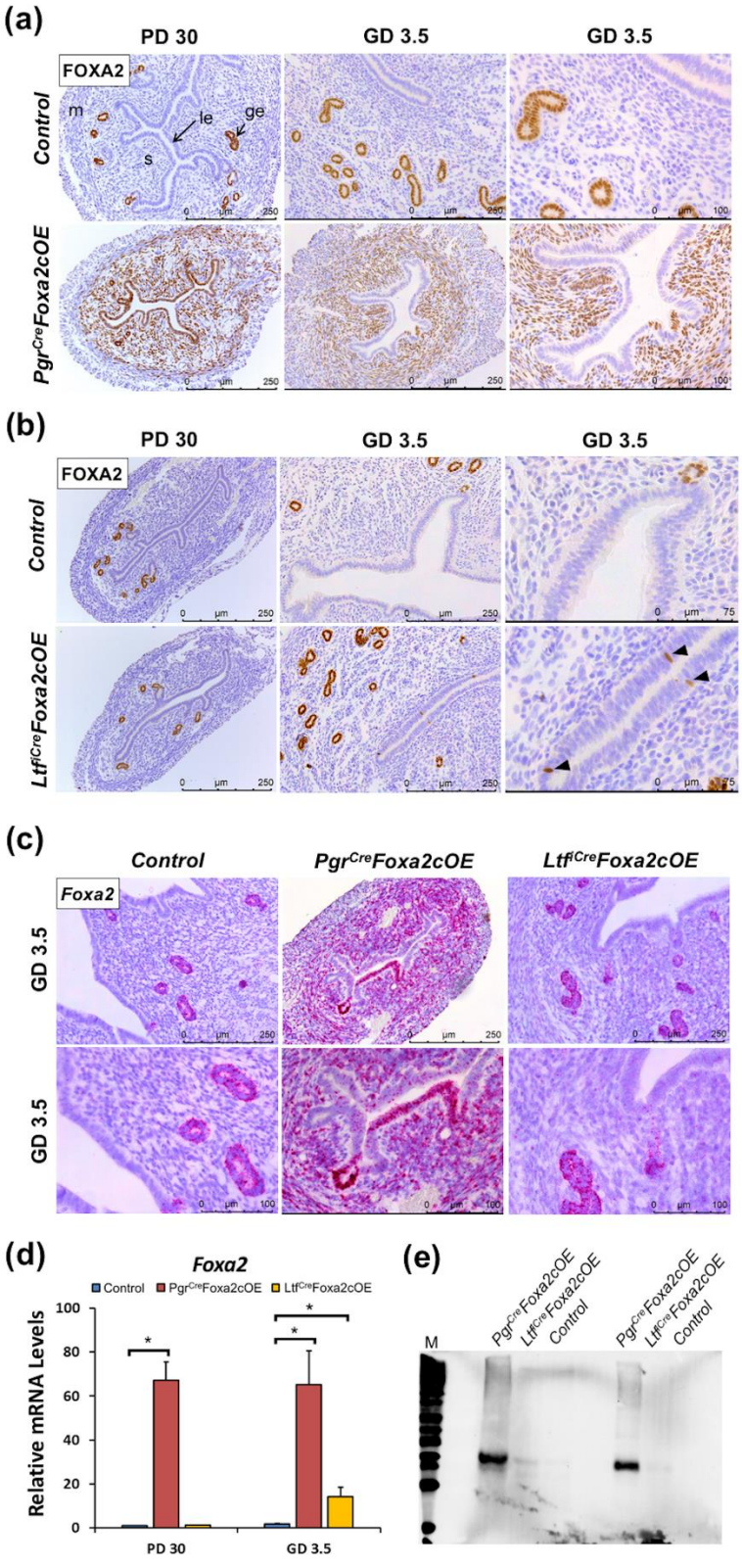
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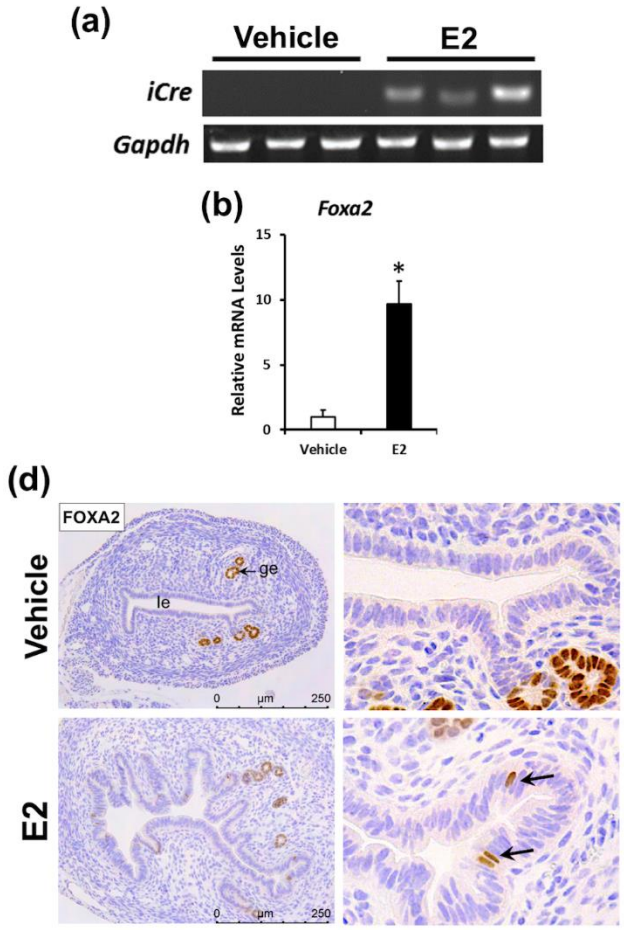
Figure 1

Figure 1. FOXA2 expression in control and Foxa2 conditional overexpression females. (a) Immunoreactive FOXA2 in cross-sections of uteri from control and *Pgr*^{Cre}*Foxa2cOE* female mice on postnatal day (PD) 30, gestational day (GD) 2.5, 3.5, and 4.5 (n = 4 mice per group). Legend: ge, glandular epithelium; le, luminal epithelium; s, stroma; m, myometrium. (b) Immunoreactive FOXA2 in cross-sections of uteri from control and *Ltf*^{iCre}*Foxa2cOE* female mice on postnatal day 30 and gestational day 3.5 (n = 4 mice per group). (c) In situ localization of *Foxa2* mRNA in cross-sections of uteri from control, *Pgr*^{Cre}*Foxa2cOE* and *Ltf*^{iCre}*Foxa2cOE* mice mice on gestational day 3.5. (d) Quantitative analysis of *Foxa2* mRNA in uteri of control, *Pgr*^{Cre}*Foxa2cOE* and *Ltf*^{iCre}*Foxa2cOE* mice on postnatal day 30 and gestational day 3.5. (n = 4 mice of each genotype per day; *P < 0.01). (e) Western blot analysis of FOXA2 in uteri from control, *Pgr*^{Cre}*Foxa2cOE* and *Ltf*^{iCre}*Foxa2cOE* mice on gestational day 3.5 (n = 2 mice per genotype).


(a)
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ge
le
0
μm
250

Figure 2

Figure 2. Effects of neonatal estrogen treatment on iCre and FOXA2 expression in *Ltf*^{iCre}*Foxa2cOE* female mice. Mice were injected (s.c.) with either vehicle (sesame oil) or estradiol-17β (E2; 100 ng per mouse per day) on postnatal days 20 and 21 (n = 3 mice per group). Uteri were collected on postnatal day 22. (a) PCR analysis of *iCre* and *Gapdh* mRNA in the uterus. (b) Quantitative analysis of *Foxa2* mRNA in uteri. (c) Immunoreactive FOXA2 in cross-sections of uteri. Legend: ge, glandular epithelium; le, luminal epithelium.

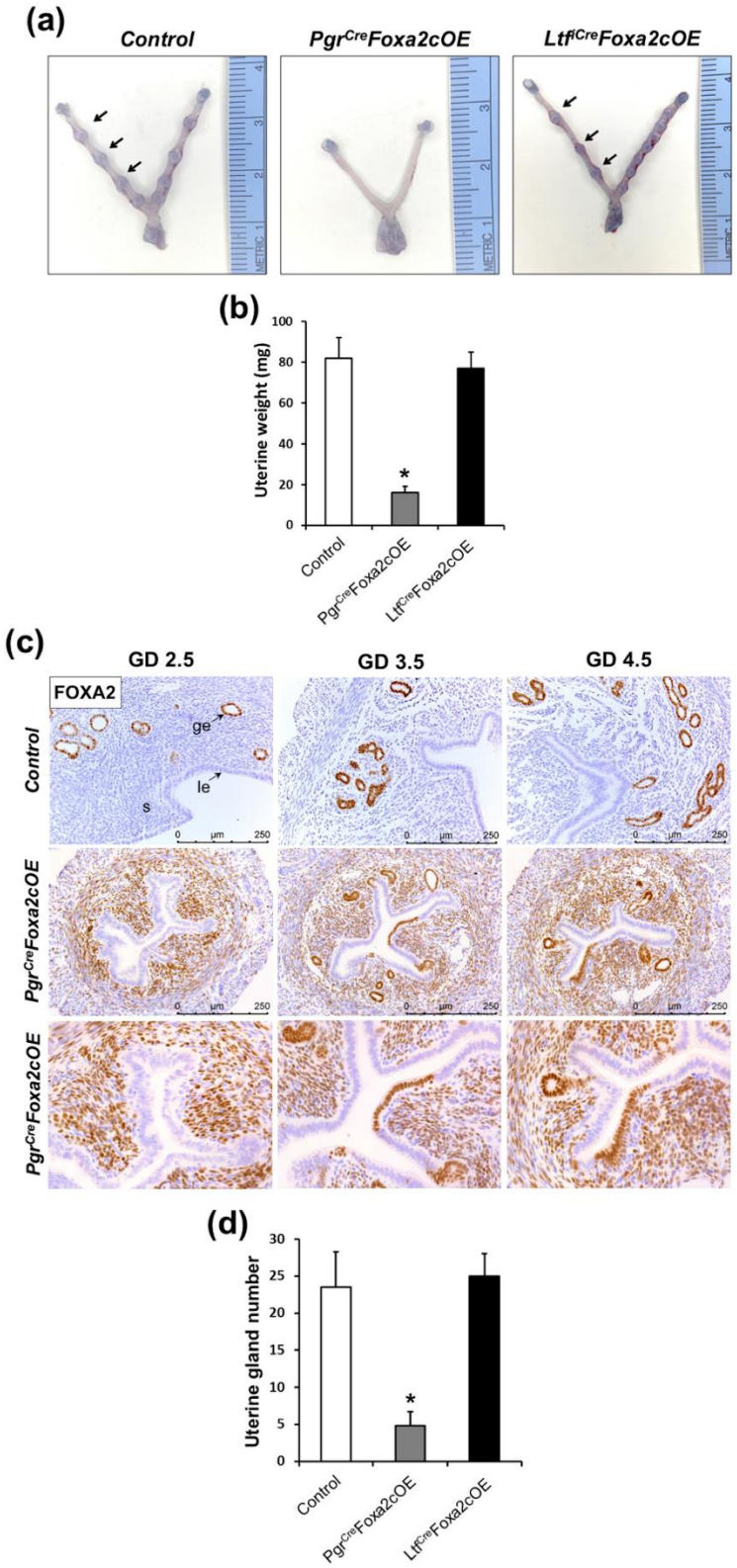

(a)
Control
PgrCreFoxa2cOE
LtfiCreFoxa2cOE
(b)
Uterine weight (mg)
100
80
60
40
20
0
*
Control
PgrCreFoxa2cOE
LtfCreFoxa2cOE
(c)
GD 2.5
GD 3.5
GD 4.5
FOXA2
ge
le
s
Control
PgrCreFoxa2cOE
PgrCreFoxa2cOE
0
μm
250
(d)
Uterine gland number
30
25
20
15
10
5
0
*
Control
PgrCreFoxa2cOE
LtfCreFoxa2cOE

Figure 3

Figure 3. Effects of FOXA2 overexpression on uterine function and histoarchitecture. (a) Embryo implantation sites (arrows) were observed in control and *Ltf*^{iCre}*Foxa2cOE* mice but not in *Pgr*^{Cre}*Foxa2cOE* mice on gestational day (GD) 5.5. (n = 5 per genotype). (b) Uterine wet weight on gestational day 3.5 was lower (P<0.01) in *Pgr*^{Cre}*Foxa2cOE* than control and *Ltf*^{iCre}*Foxa2cOE* mice. (n = 4 mice per genotype). (c) Immunoreactive FOXA2 in the uterus of adult *Pgr*^{Cre}*Foxa2cOE* mice during early pregnancy. (n = 4 mice per genotype per gestational day). Legend: ge, glandular epithelium; le, luminal epithelium; s, stroma. (d) Uterine gland number on gestational day 3.5 is substantially lower (P<0.01) in *Pgr*^{Cre}*Foxa2cOE* than control and *Ltf*^{iCre}*Foxa2cOE* mice. (n = 6 mice per genotype).

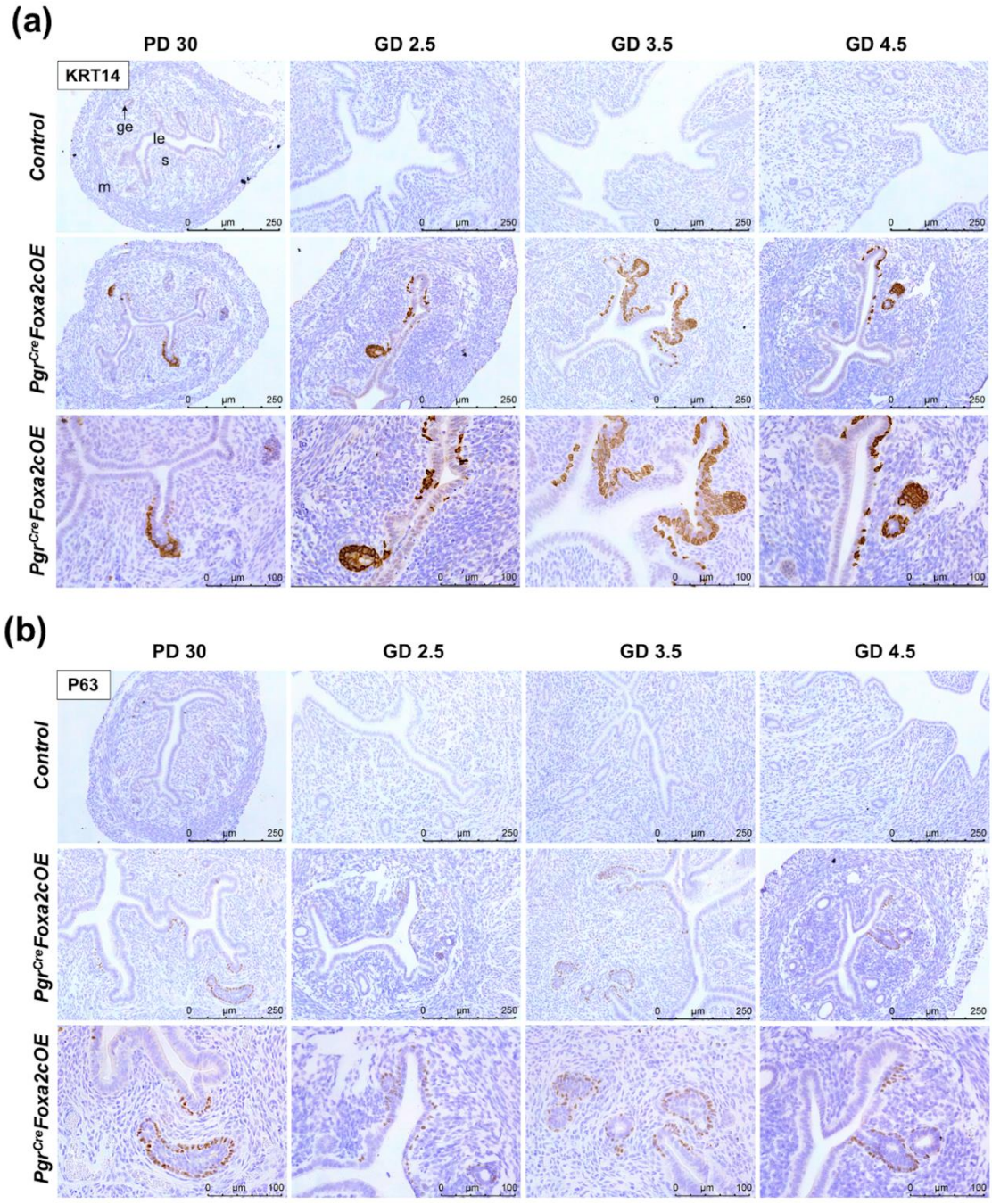

(a)
PD 30
GD 2.5
GD 3.5
GD 4.5
KRT14
Control
PgrCreFoxa2cOE
PgrCreFoxa2cOE
ge
le
s
m
(b)
PD 30
GD 2.5
GD 3.5
GD 4.5
P63
Control
PgrCreFoxa2cOE
PgrCreFoxa2cOE

Figure 4

Figure 4. Basal cells are present in areas of stratified luminal epithelium in uteri of *Pgr*^{Cre}*Foxa2cOE* mice. (a) Immunoreactive KRT14 in the uterus of control and *Pgr*^{Cre}*Foxa2cOE* mice on postnatal day (PD) 30, gestational day (GD) 2.5, 3.5 and 4.5 (n = 4 mice per genotype per day). Legend: ge, glandular epithelium; le, luminal epithelium; s, stroma; m, myometrium. (b) Immunoreactive P63 in the uterus of control and *Pgr*^{Cre}*Foxa2cOE* mice on postnatal day (PD) 30, gestational day (GD) 2.5, 3.5 and 4.5 (n = 4 mice per genotype per day). Note the presence of KRT14- and P63-positive basal cells in the luminal epithelium of the *Pgr*^{Cre}*Foxa2cOE* uterus.

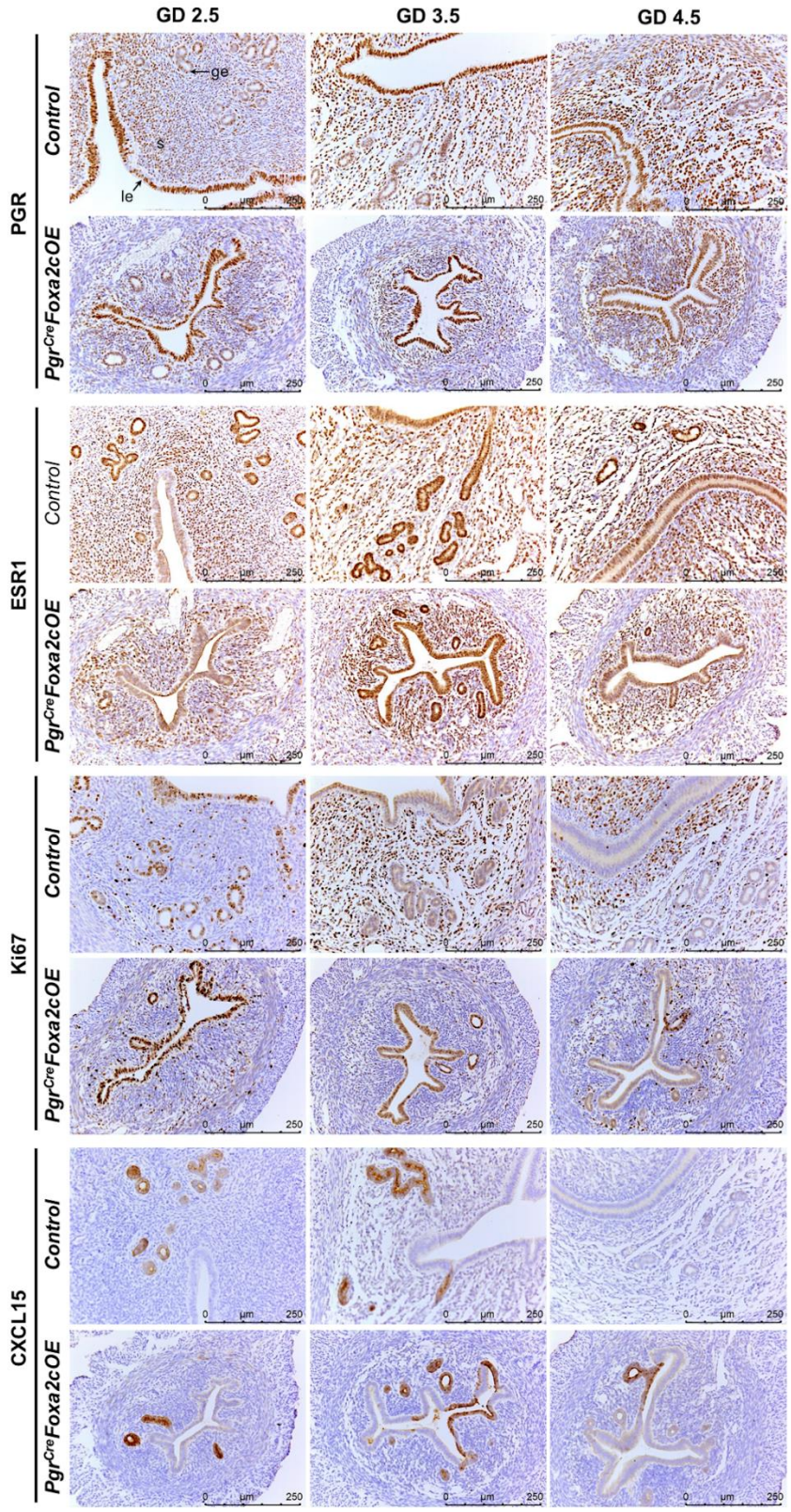

GD 2.5
GD 3.5
GD 4.5
PGR
ESR1
Ki67
CXCL15
Control
PgrCreFoxa2cOE
ge
s
le
0 µm 250

Figure 5

Figure 5. Expression of steroid receptors, cell proliferation (Ki67), and CXCL15 in adult control and *Pgr*^{Cre}*Foxa2cOE* uteri. Control and *Pgr*^{Cre}*Foxa2cOE* mice were analyzed on gestational day (GD) 2.5, 3.5 and 4.5 (n = 4 mice per genotype per day). Legend: ge, glandular epithelium; le, luminal epithelium; s, stroma.

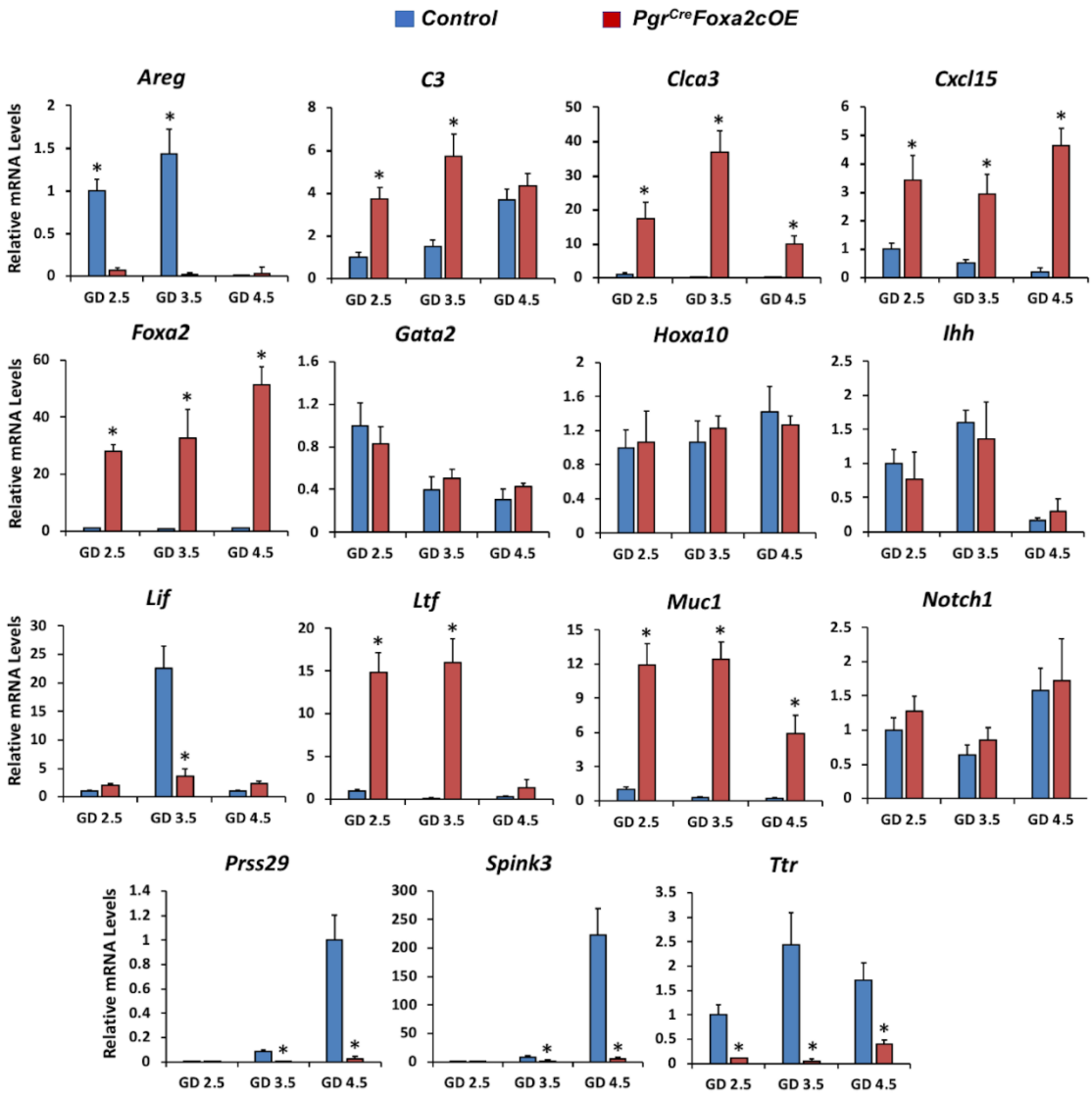

Control
Pgr^Cre Foxa2cOE
Areg
C3
Clca3
Cxcl15
Foxa2
Gata2
Hoxa10
Ihh
Lif
Ltf
Muc1
Notch1
Prss29
Spink3
Ttr
Relative mRNA Levels
GD 2.5
GD 3.5
GD 4.5

Figure 6

Figure 6. Expression of estrogen- and progesterone-responsive and gland-specific genes in the uterus during early pregnancy. Control and *Pgr*^{Cre}*Foxa2cOE* mice were analyzed on gestational day (GD) 2.5, 3.5 and 4.5 (n = 4 mice per genotype per day). (*, P<0.01).

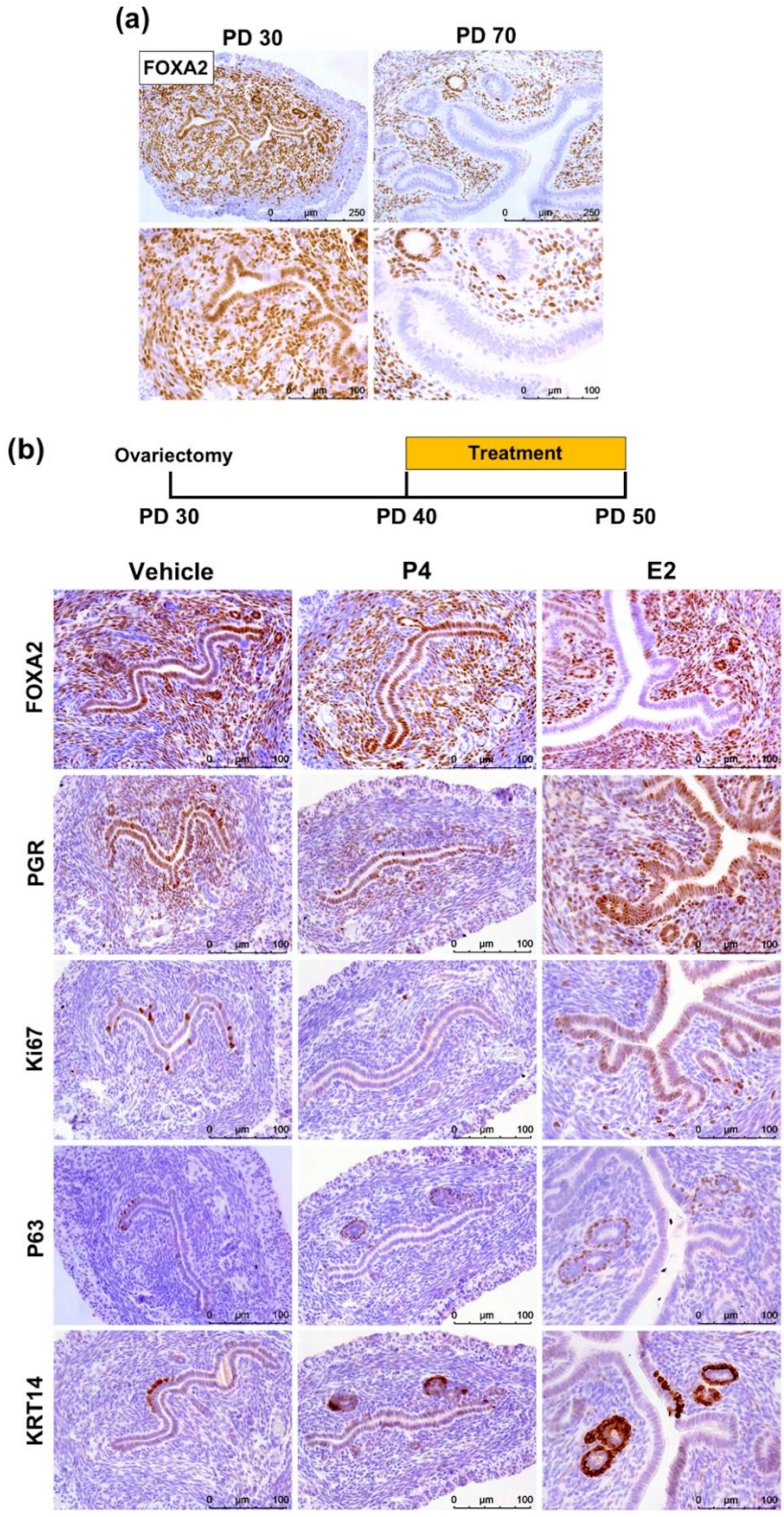
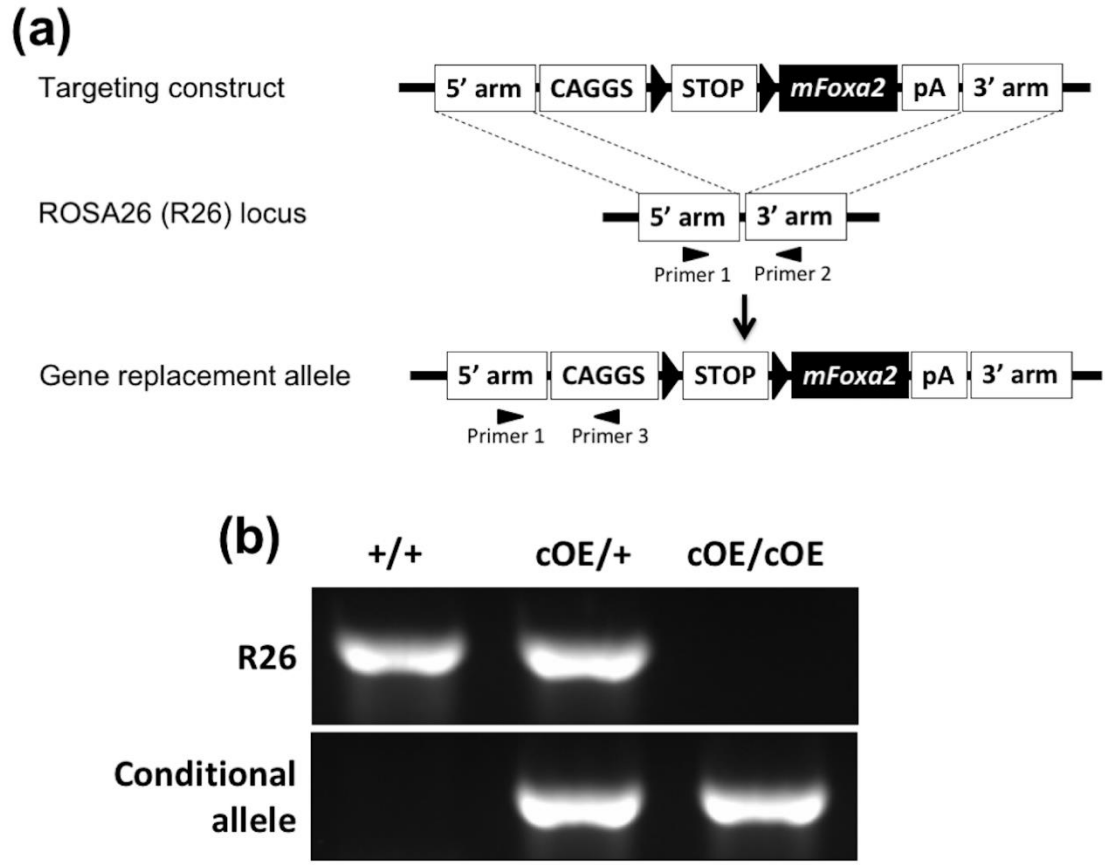

(a)
PD 30
PD 70
FOXA2
(b)
Ovariectomy
Treatment
PD 30
PD 40
PD 50
Vehicle
P4
E2
FOXA2
PGR
Ki67
P63
KRT14

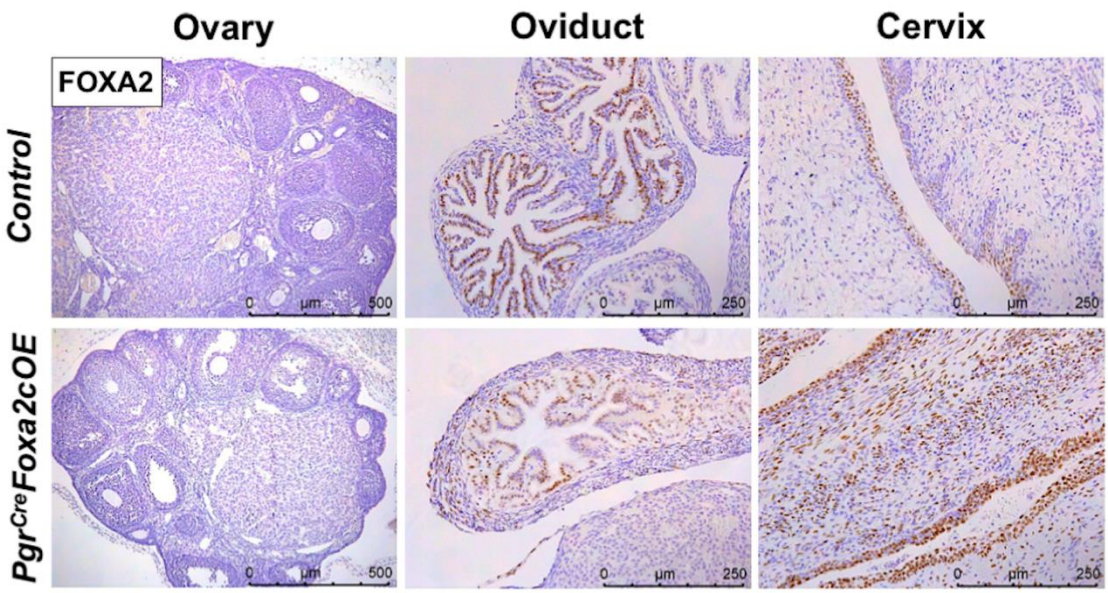
Figure 7

Figure 7. Effects of puberty and steroid hormones on FOXA2 expression in *Pgr*^{Cre}*Foxa2cOE* mice. (a) FOXA2 expression is lost from the luminal epithelium of the uterus occurs between postnatal day (PD) 30 and 70. Legend: le, luminal epithelium; s, stroma. (b) *Pgr*^{Cre}*Foxa2cOE* mice were ovariectomized on postnatal day 30, rested for 10 days, and treated daily with injections (s.c.) of vehicle (sesame oil), progesterone (P4; 1 mg), or estradiol-17β (E2; 100 ng) for 10 days (n = 4 mice per genotype per day). Immunoreactive FOXA2, PGR, Ki67, P63 and KRT14 was evaluated using immunohistochemistry.



Supplemental Figure 1

Supplemental Figure 1. Generation of the FOXA2 conditional expression system. (a) General scheme of the production process. The targeting construct contains two ROSA26 genomic sequences for gene targeting (5′- and 3′-arms) and a minigene consisting of a CAGGS promoter, a loxP-STOP-loxP cassette (STOP), and mouse *Foxa2* cDNA with a polyadenylation (pA) site. The minigene is inserted into the ROSA26 locus as indicated by *dashed lines* to generate the conditional allele. Primers for PCR genotyping on unmodified wild-type (R26) and gene replacement allele, respectively, are denoted. (b) PCR genotyping of tail DNA from WT (+/+) and conditional expressor (cOE) animals by allelic-specific primer sets indicated in panel A.


Ovary
Oviduct
Cervix
FOXA2
Control
PgrCreFoxa2cOE
0 µm 500
0 µm 250
0 µm 250
0 µm 500
0 µm 250
0 µm 250

Supplemental Figure 2. Immunoreactive FOXA2 in the ovary, oviduct and cervix of control and *Pgr*^{Cre}*Foxa2cOE* mice on gestational day 3.5.

Table 1. Antibodies used

Primary Antibody	Species	Manufacturer	Catalog No.	RRID	Dilution Ratio	Application
β-Actin	Rabbit	Cell Signaling Technology	4970	AB_2223172	1:3000	WB
FOXA2	Rabbit	LifeSpan Biosciences	LS-C 138006	AB_10946909	1:1000 (IHC)/1:2000(WB)	IHC,WB
CXCL15	Rabbit	Abcam	ab197016		1:200	IHC
ESR1	Rabbit	Santa Cruz Biotechnology	sc-542	AB_631470	1:500	IHC
Ki-67	Rabbit	Abcam	ab15580	AB_443209	1:1000	IHC
P63	Rabbit	Cell Signaling Technology	4981s	AB_2286372	1:200	IHC
PGR	Rabbit	Thermofisher	MA5-14505	AB_10980030	1:1000	IHC

Abbreviations: IHC, immunohistochemistry; WB, Western blot.

Table 2. Female fertility assessed in a 6-month breeding trial

Genotype	N	Total Litters	Total Pups	Pups per Litter
Control mFoxa2$^{LSL/+}$	6	48	432	9.0±2.4
Pgr$^{Cr/+e}$Foxa2$^{LSL/+}$	6	0	0	0
Ltf$^{iCre/+}$Foxa2 $^{LSL/+}$	6	46	404	8.8±1.8

General Discussion

A mouse model for conditional FOXA2 overexpression was generated to further investigate FOXA2 function in the uterus. This model harbors a minigene overexpressing FOXA2 conditionally at the ROSA26 locus. The inserted *Foxa2* gene is silenced originally but continuous FOXA2 expression can be induced by Cre recombinase. The *Pgr-Cre* mouse model was employed to confirm the function of the inserted *Foxa2* gene. FOXA2 was successfully overexpressed in the LE, stroma and inner layer of myometrium of $Pgr^{Cre}Foxa2cOE$ uteri on PD 30, which is consistent with PGR expression. However, in adult females (PD 60), FOXA2 was lost in the LE, while the GE, stroma and myometrium remained FOXA2-positive, and PGR expression was still observed in the LE. A 6-month breeding trial indicated that $Pgr^{Cre}Foxa2cOE$ females are completely infertile. Examination of those females revealed multiple abnormal structures present in their female reproductive tract. $Pgr^{Cre}Foxa2cOE$ uteri are smaller compared to control because, at least in part, the proliferation of stromal cells stimulated by progesterone on gestational days 3.5 and 4.5 is suppressed by the ectopic expression of FOXA2. The $Pgr^{Cre}Foxa2cOE$ uteri appeared to have fewer uterine glands than control mice. One possible explanation is that extensive FOXA2-positive LE may inhibit each other to differentiate into uterine glands at the time of endometrial adenogenesis after birth. Another possibility is that the ectopic expression of FOXA2 in the stromal cells impairs the stromal-epithelial crosstalk during the endometrial adenogenesis. The ciliary epithelium in the oviduct appeared less folded in $Pgr^{Cre}Foxa2cOE$ mice likely due to the ectopic expression of FOXA2 in their stromal cells since both $Pgr^{Cre}Foxa2cOE$ and control oviduct contains normally FOXA2-positive epithelium. These results indicate that

the inserted *Foxa2* gene functions normally, and our new mouse model is a valuable tool to study the biological roles of FOXA2 in organ development and adult functions.

FOXA2 acts as a tumor suppressor involved in a number of different cancers including those of the bladder, colon, liver, lung and uterus. Indeed, FOXA2 is down-regulated or inactivated by mutation during the development of endometrial epithelial hyperplasia and cancer. In most mammals, if not all, endometrial adenogenesis is primarily a postnatal event in which uterine GE derives from LE originally. Studies on a C3H/Balb/c chimeric mouse revealed that individual uterine glands appear to arise from one single germ cell among the LE (Lipschutz, Fukami et al. 1999). One hypothesis is that by some unknown mechanism, expression of *Foxa2* or its upstream genes are induced in some LE of immature uteri, giving them the cell fate to become the germ cells of uterine glands. Therefore, knowing how expression of FOXA2 is regulated could provide significant insights into the organ-specific tumor progression and the development of uterine glands. Analysis of the conditional FOXA2 overexpression models developed here provided some clues about the FOXA2 regulation *in vivo*. Expression of FOXA2 protein was not observed in the luminal epithelium cells of the uterus in both adult $Pgr^{Cre}Foxa2cOE$ and $Ltf^{iCre}Foxa2cOE$ mice, and even *Foxa2* mRNA is lacking in the LE of $Ltf^{iCre}Foxa2cOE$ uteri. These findings suggest that *Foxa2* transgene is silenced specifically in the luminal epithelium by some unknown epigenetic mechanism. Since the insert *Foxa2* gene only contains the FOXA2 coding sequence but not the 3' UTR and 5' UTR, we hypothesize that unknown transcription factor(s) target its coding sequence, causing the hypermethylation and silence of the inserted *Foxa2* gene. To test this hypothesis, we can use the $Pgr^{Cre}Foxa2cOE$ model. In the

Pgr^{Cre}*Foxa2cOE* adult uteri, the inserted *Foxa2* gene is silenced in the LE, but its expression is likely continued in the stromal cells because stromal cells usually have no FOXA2 expression in the control. The LE and stromal cells can be separated (De Clercq, Hennes et al. 2017). Subsequently, bisulfite sequencing can be employed to determine their methylation pattern around and within the inserted Foxa2 genes in the LE and stromal cells of the *Pgr*^{Cre}*Foxa2cOE* adult uteri. In the mouse uterus, conditional overexpression of Notch1 aberrantly upregulates FOXA2 expression in the luminal epithelium (Su, Strug et al. 2016). Therefore, by comparing the methylation pattern at the endogenous *Foxa2* gene locus in the LE cells from control and *Notch1* overexpression uteri, one could tell if the endogenous *Foxa2* gene is silenced in the LE cells due to hypermethylation. Of note, two PU.1 (Hematopoietic Transcription Factor PU.1) binding sites on the *Foxa2* coding sequence were identified, 519 and 909 bp 5' of the transcription start site (TSS), using MotifMap (motifmap.ics.uci.edu). In the uteri with *Notch1* overexpression, PU. 1 is responsible for the hypermethylation of the *Pgr* promoter and the decrease of PGR expression (Su, Strug et al. 2016). Thus, further studies are needed to determine if PU. 1 binding sites can cause the silence of *Foxa2* gene in the uterus.

Estrogen signaling is enhanced in the *Pgr*^{Cre}*Foxa2cOE* uteri indicated by the upregulation of estrogen responsive genes, *C3, Clca3, Cxcl15, Ltf* and *Muc1*. Expression of glandular epithelium-specific genes (*Lif, Prss29, Spink3, Ttr*) were substantially decreased in the uterus of *Pgr*^{Cre}*Foxa2cOE* during early pregnancy because of the decrease numbers of uterine glands and/or the defects of GE function. Interestingly, one GE-specific gene, *Cxcl15*, is upregulated in the *Pgr*^{Cre}*Foxa2cOE* uteri throughout early

pregnancy. Treated with genistein, a chemical that can bind estrogen receptors and function like estrogen, CXCL15 expression was highly upregulated in mouse oviducts (Jefferson, Padilla-Banks et al. 2012). This result indicated that *Cxcl15* is likely an estrogen responsive gene which is consistent with its expression pattern during early pregnancy. When the uterus is dominated by estrogen on GD 2.5, *Cxcl15* is highly expressed in the uterus, while on GD 4.5, when the uterus is dominated by progesterone, its expression is quite low (Chapter 2, Figure 6). The preovulatory surge of LIF is secreted in the uterine gland and induced by the nidatory estrogen on GD 4, which is essential for blastocyst implantation. However, this surge is missing in the uterus with conditional ablation of *Foxa2* while expression pattern of ESR1 is not changed in those uteri (Kelleher, Peng et al. 2017). Taken together, these findings suggest that estrogen may cooperate with FOXA2 to regulate the estrogen signaling in the female reproductive tract. Expression of *Gata2*, *Hoxa10* and *Ihh*, known as progesterone responsive genes, are not altered in $Pgr^{Cre}Foxa2cOE$ uteri (Chapter 2, Figure 6). However, expression of another two progesterone responsive gene, *Areg* and *Spink3*, are substantially suppressed in $Pgr^{Cre}Foxa2cOE$ uteri (Chapter 2, Figure 6) (Chen, Han et al. 2010). Additionally, progesterone-induced proliferation of stromal cells is inhibited in $Pgr^{Cre}Foxa2cOE$ uteri. Therefore, further studies are needed to determine whether FOXA2 plays roles in regulating progesterone signaling.

In conclusion, we established a mouse model for the FOXA2 overexpression. Studies on this model provide us some insights on the biological roles of FOXA2 in the female reproductive tract and the regulation of FOXA2 expression.

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Carroll, J. S., X. S. Liu, A. S. Brodsky, W. Li, C. A. Meyer, A. J. Szary, J. Eeckhoute, W. Shao, E. V. Hestermann, T. R. Geistlinger, E. A. Fox, P. A. Silver and M. Brown (2005). "Chromosome-wide mapping of estrogen receptor binding reveals long-range regulation requiring the forkhead protein FoxA1." Cell **122**(1): 33-43.

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