Mechanisms of uterine estrogen signaling during early pregnancy in mice: an update

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Abstract

Adherence of an embryo to the uterus represents the most critical step of the reproductive process. Implantation is a synchronized event between the blastocyst and the uterine luminal epithelium, leading to structural and functional changes for further embryonic growth and development. The milieu comprising the complex process of implantation is mediated by estrogen through diverse but interdependent signaling pathways. Mouse models have demonstrated the relevance of the expression of estrogen-modulated paracrine factors to uterine receptivity and implantation window. More importantly, some factors seem to serve as molecular links between different estrogen pathways, promoting cell growth, acting as molecular chaperones, or amplifying estrogenic effects. Abnormal expression of these factors can lead to implantation failure and infertility. This review provides an overview of several well-characterized signaling pathways that elucidates the molecular cross talk involved in the uterus during early pregnancy.

Key Words
- estrogen
- implantation
- uterus
- pregnancy
- signaling

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Introduction

Reproduction is a fundamental aspect of life. The World Health Organization (WHO) has recognized infertility, or the inability to reproduce, as a worldwide health concern with a lifetime prevalence ranging from 6.6 to 26.4% (Boivin *et al.* 2007). Although much advancement has been made using assisted reproductive technologies (ARTs) to achieve higher pregnancy rates by improving the selection of high-quality embryos, the implantation process is still very illusive.

The development of the preimplantation embryo and the differentiation of the uterus are distinct processes occurring simultaneously in early gestation and must be synchronized in order for successful implantation (Psychoyos 1973*a*, Paria *et al.* 1993). It has been shown that in a mouse, implantation occurs when the developed blastocyst attaches to the luminal epithelium of the uterine endometrium on the evening of day 4 of pregnancy (Enders & Schlafke 1969, Das *et al.* 1994). The attachment of the embryo to the epithelial lining promotes the disappearance of epithelium. This depends on a mechanism of entosis (cell-eat-cell) by the trophoblast cells followed by apoptosis at the site of implantation (Parr *et al.* 1987, Li *et al.* 2015) and subsequent stimulation of stromal cell proliferation and differentiation into secretory decidual cells. This series of events form the decidualization bed at the blastocyst site (Huet-Hudson *et al.* 1989).

These structural and functional changes occurring in the uterus promote receptivity to the invading blastocyst.

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This receptivity phase of the uterus is short-lived and primarily mediated by estrogen and progesterone (Psychoyos 1973a, Paria *et al*. 1993). The estrogenic effects in the mouse uterus are biphasic: early (phase I) responses occur within 6 h and are characterized by water inhibition, macromolecular uptake, and alteration in genes involved in vascular permeability. Late (phase II) responses occur between 18 and 30 h and are characterized by increased epithelial cell proliferation (Huet-Hudson *et al*. 1989). The presence of progesterone (P4) is inhibitory to estrogen-mediated epithelial proliferation, which can be detected on day 4 (D4) of gestation (Das & Martin 1973, Martin *et al*. 1973, Pan *et al*. 2006, Li *et al*. 2011). Ovariectomized mice on the morning of D4 before preimplantation estrogen secretion exhibit delayed implantation due to blastocyst dormancy (Yoshinaga and Adams 1966). When the uterus in ovariectomized mice is exposed to progesterone alone, it renders it a neutral or pre-receptive endometrium; however, receptivity for implantation is observed when exposed to estrogen (Paria *et al*. 1993). This demonstrates the crucial role of estrogen in the process of implantation.

The mechanisms by which estrogen transforms a progesterone-primed uterus to the receptive state, activates blastocysts, and initiates implantation are not clearly delineated. The classical estrogen signaling pathway is through nuclear estrogen receptors ERα (ESR1) and ERβ (ESR2), which act as ligand-inducible transcription factors (Tsai & O'Malley 1994, Beato *et al*. 1995). However, there is increasing evidence that gene activation and cell function modulation are initiated by estrogen through a nuclear ER-independent manner. Studies with *Erα*-null mice and also wild-type mice, in which both ERα and ERβ antagonists ICI-182,780 were used to silence ligand-dependent ER functions, have demonstrated estrogen-mediated gene expression, suggesting an alternate signaling pathway (Das *et al*. 1997, Das *et al*. 2000, Hou *et al*. 2004).

Implantation failure and infertility are associated with aberrations in molecular pathways. The knowledge attained with the development of knockout (KO) mouse models and conditional gene deletions has advanced uterine biology immensely. This is a review of the knowledge gained from previous studies on mice attempting to delineate the mechanisms of estrogen signaling. Understanding the estrogen pathways and its mediated events during early pregnancy is critical to further advancement in ART protocols that will improve treatment of this worldwide health condition.

Role of estrogen receptors during early pregnancy

Estrogen plays a pivotal role in the observed changes of the uterus during early pregnancy. In mice, during the first 2 days of gestation, pre-ovulatory estrogen stimulates proliferation of the luminal and glandular epithelial cells (phase I estrogen secretion). Once the corpora lutea is formed on day 3 of gestation, progesterone secretion stimulates stromal cell proliferation, which becomes further potentiated by preimplantation estrogen (phase II estrogen secretion) on day 4, the day of implantation (Huet-Hudson *et al*. 1989). This second wave of estrogen before implantation ceases epithelial cell proliferation and allows for differentiation to occur (Tan *et al*. 1999). During the remodeling of the uterine epithelium, the epithelial cells lose polarity through downregulation of the cell-to-cell adhesion molecule E-cadherin (Daikoku *et al*. 2011, Li *et al*. 2015). Epithelial cells also acquire inhibition of the glycoprotein mucin 1 (MUC1) and develop protrusions along the apical surface (Surveyor *et al*. 1995, DeSouza *et al*. 1998). Increased endometrial capillary permeability at the location of the blastocyst is also exhibited, lending to implantation and subsequent decidualization of stromal cells (Psychoyos 1973b, Matsumoto *et al*. 2002*a*).

The classic physiological actions of estrogen on its target organ are mediated by its binding to ER, which activates the receptor by promoting dimerization and then translocation to the nucleus to bind its responsive element in the DNA (Kumar & Chambon 1988). The distribution and expression of ER subtypes varies due to their tissue-specific physiological functions in various organ systems. ERα (ESR1), for example, is mainly present in mammary gland tissue, uterus, thecal cells of the ovary, bone, liver, adipose tissue, testes, epididymis of the male reproductive organs, and the stroma of the prostate. ERβ (ESR2) is mainly found in the epithelium of the prostate, bladder, granulosa cells of the ovary, colon adipose tissue, and the immune system (Dahlman-Wright *et al*. 2006, Heldring *et al*. 2007). Although ERα is the predominant isoform in certain tissues, both receptors have high affinity to estradiol-17β (E2) in the same estrogen response element (ERE), and they share approximately 95 and 55% homology in the DNA-binding domain and the hormone-binding domain, respectively (Kuiper *et al*. 1997, Tremblay *et al*. 1997). However, it has been demonstrated that the biological disruption of *Erα* gene causes infertility due to defects in the reproductive tract and gonads of female mice, whereas disruption of the *Erβ* gene by the insertion

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of neo-cassette into exon 3 is associated with only disruption of ovulation (Lubahn *et al*. 1993, Eddy *et al*. 1996, Krege *et al*. 1998, Couse *et al*. 2005).

The innovation of genetically induced mice has allowed for further knowledge of estrogen signaling. Studies on *Erα*- and *Erβ*-KO mice have demonstrated that *Erα* is essential for endometrial receptivity (Lubahn *et al*. 1993, Cooke *et al*. 1997, Buchanan *et al*. 1999). Similarly, studies using *Pr*-null mouse strains have demonstrated that uterine stromal cells are the mediators of progesterone inhibitory effects on estrogen-induced proliferative response of the uterine epithelium (Kurita *et al*. 1998). Simultaneously, Tan and coworkers (1999) demonstrated that there is compartmentalization of uterine *Erα*, but extremely low-to-undetectable expression of *Erβ* is associated with early peri-implantation days of gestation. During early gestation (days 1 and 2), *Erα* mRNA is primarily localized in the luminal and glandular epithelium, whereas localization is additionally seen in the stroma on days 3 and 4; however, by day 8 of gestation, *Erα* exhibits downregulation of decidual cells immediately surrounding the embryo. Collectively, these studies suggest that specific regulation of *ER* gene expression seems to define the implantation window.

Additionally, analysis of the implantation window has demonstrated that the estrogen effects on the endometrium are tightly regulated. Ma and coworkers (2003) demonstrated that lower estrogen levels tend to sustain the receptivity of the uterus; however, higher concentrations shut down this time window, although the exact mechanism is not well understood (Ma *et al*. 2003). NCOA6 is a coactivator for multiple nuclear receptors. Its absence, as demonstrated by studies using Ncoa6-KO mice, causes failure to develop due to defects noted in the placenta and other tissues (Kuang *et al*. 2002, Mahajan & Samuels 2005). Kawagoe and coworkers (2012) demonstrated that *Ncoa6* regulates estrogen sensitivity and signaling affecting the uterine receptivity status. Using a conditional KO of *Ncoa6* in mice, Kawagoe was able to demonstrate that loss of NCOA6 results in ERα accumulation in stromal cells and accumulation of steroid receptor coactivator 3 (SRC3), a potent ERα coactivator (Kawagoe *et al*. 2012). Therefore, the loss of NCOA6 leads to the inability to attenuate estrogen sensitivity via an accumulation of ERα and SRC3 at the implantation site, rendering the uterus nonreceptive with pregnancy failure.

These observations suggest a localized site of the coordinated effects of estrogen on its target tissue. As both stroma and epithelium express ERα, one would assume that estrogen-induced epithelial proliferation is controlled directly through the interaction with the specific nuclear steroid receptor. However, studies have demonstrated that estrogen-induced response in target tissue is not necessarily related to its affinity or occupancy to the receptor (Das *et al*. 1997), because an estrogen receptor antagonist, ICI-182,780, failed to inhibit uterine estrogen-responsive lactoferrin (*Ltf*) gene expression and water imbibition induced by certain estrogens in *Er*-KO mice. However, the antagonist ICI-182,780 indeed suppressed the uterine *Ltf* expression in wild-type mice induced after E2, which indicated an estrogen signaling independent of both ERα and ERβ.

Distinct estrogen signaling pathways

Specific functions of AF1 and AF-2 domains of ERα

Binding of ER at genomic sites regulates gene expression. Different physiological responses are initiated by binding of estrogen to ER, leading to receptor conformational changes that are required for transcriptional activity. Two transactivation function domains mediate transcriptional activation: activation function-1 (AF1) in the N-terminal domain and activation function-2 (AF2) in the C-terminal ligand-binding domain (LBD) (Tremblay *et al*. 1999, Kushner *et al*. 2000). Both AFs have unique differential gene activation through cell type-specific coactivators (Xu *et al*. 1998, Hsia *et al*. 2010). Previous studies demonstrated that the significance of these specific domains with regard to the functionality of ER depends on AF1 (Merot *et al*. 2004).

However, although reproduction is affected in *Erα*-null mice (Lubahn *et al*. 1993), several estrogen effects still persist, such as early responses to uterine edema and gene expression (Das *et al*. 1997, Das *et al*. 2000) and vascular injury response (Iafrati *et al*. 1997). In the uterus of this null mouse, through alternative splicing, a chimeric small ERα protein (~55 kDa), in which 64 amino acid residues belonging mainly to the B region, can be partially deleted from the N-terminal A/B regions of *Erα* (Couse *et al*. 1995). In addition, studies also reported detection of a short form of *Erα* transcript in the uterus, representing the deletion of a portion of exon 2 followed by the insertion of a frameshift and at least two stop codons at the 5′-end of exon 3 (Couse *et al*. 1995), but the significance of this remains unknown. The truncated small *Erα* variant lacks the AF1 domain, which according to Pendaries coworkers could be partially dispensable to mediate the estrogenic effects in the uterus, because the variant possesses a residual estrogen-dependent transcriptional activity with an intact AF2 region

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(Couse *et al*. 1995, Pendaries *et al*. 2002). Further studies revealed the crucial role of AF2 for estrogen-mediated endometrial epithelial proliferation using antagonists and selective ER modulators (SERMs) (Arao *et al*. 2011). However, further studies also revealed that activation of AF1 function is required for the E2-induced uterine epithelial proliferation, whereas it is partially dispensable for the induction of uterine edema by chronic estrogen stimulation (Abot *et al*. 2013). Additionally, Kurita coworkers (2005) revealed differences in the estrogen-induced proliferative responses between human and mouse epithelial cells, which seem to be species specific with regard to using AF domains within the ERα. Therefore, further investigations into these domains to evaluate the specific physiological roles of AF1 and AF2 are still needed.

Interdependent regulation by uterine epithelial and stromal cells

Deletion of *ERα* in uterine epithelial cells leads to infertility; however, this receptor loss does not prevent estrogen-induced epithelial cell proliferation (Winuthayanon *et al*. 2010). In this regard, tissue recombination studies have also shown that ERα action in stromal cells mediates the estrogenic proliferation events in the epithelium in a paracrine manner (Cooke *et al*. 1997, Cunha *et al*. 2004). In addition, Pawar and coworkers (2015) also showed that epithelial ERα controls uterine decidualization via a paracrine mechanism of epithelial–stromal cross talk during the early implantation. Similarly, downregulation of the progesterone receptor in the uterine epithelium is depended on stromal ERα (Kurita *et al*. 2000). The theory of interdependency between the endometrial epithelium and the stroma proposes an intercellular cross talk through different signaling pathways (Fig. 1), which can mimic the effects of the traditional ligand–receptor pathway.

Leukemia inhibitory factor signaling

Leukemia inhibitory factor (LIF) is a well-characterized paracrine factor produced by the glandular epithelium under estrogen stimulation that regulates implantation (Stewart *et al*. 1992). It executes its biological function by activating its own receptor (*LIFR*) followed by the recruitment of glycoprotein 130 (GP130) (Taga & Kishimoto 1997). Yang and coworkers (1995) demonstrated the expression patterns of *Lifr* and *Gp130* in the luminal epithelium on day 4 of pregnancy in mice. LIF acts on the luminal epithelium to activate Janus kinase (JAK), a nonreceptor tyrosine kinase, which mediates the phosphorylation and activation of signal transducer and activator of transcription 3 (STAT3) (Heinrich *et al*. 1998, Tomida *et al*. 1999). *Lif*-null mice demonstrate normal ER and PR expression, but absence in the expression of EGF-like growth factors such as heparin-binding epidermal growth factor (*Hbegf*), amphiregulin (*Areg*), and epiregulin (*Ereg*) near the blastocyst on day 4 of gestation (Song *et al*. 2000). Although the exact function of EGFs is unknown, the EGF receptors are expressed on stromal cells

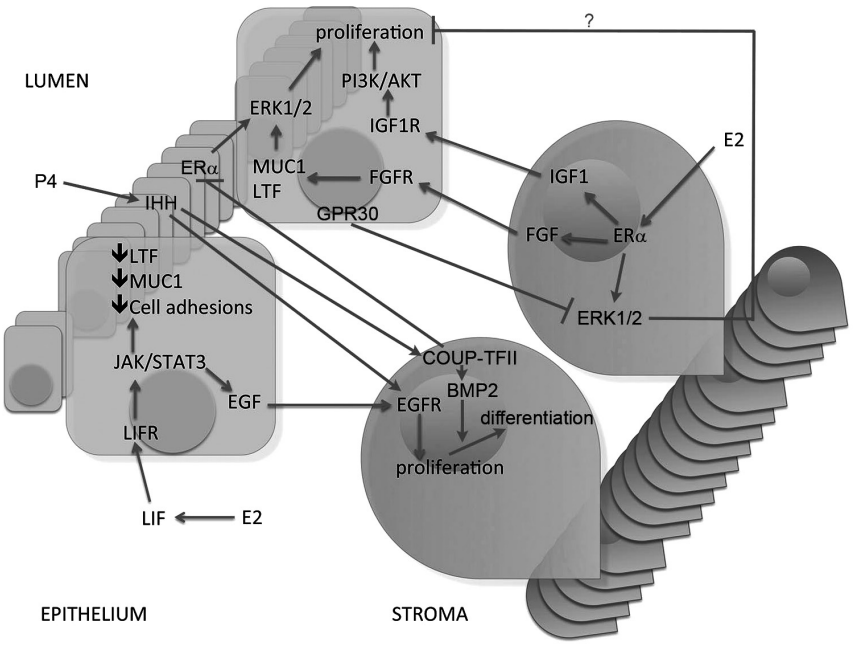

Figure 1
A schematic model for a molecular cross talk between the endometrial epithelium and stroma proposes a traditional ligand–receptor pathway during the regulation of cellular proliferation and differentiation under the direction of ovarian steroid hormones.

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during pregnancy, suggesting a role as paracrine mediators driving stromal proliferation (Fig. 1) (Song *et al*. 2000, Xie *et al*. 2007). Furthermore, *Stat3*-null mice demonstrate increased epithelial expression of estrogen-regulated genes *Ltf* and *Muc1*, which heighten estrogen signaling allowing for persistent proliferation in the luminal epithelium and a lack of proliferation in the stromal layer (Sun *et al*. 2013), indicating an a nonreceptive uterine state. Collectively, these findings demonstrate that the loss of the LIF-STAT3 signaling pathway culminates in undifferentiated uterine epithelium and is therefore nonreceptive to the embryo implantation.

Indian hedgehog signaling

Indian hedgehog (IHH), a member of the hedgehog gene family, is a progesterone-regulated factor produced in the epithelium and controls stromal function via paracrine mechanisms (Fig. 1) (Matsumoto *et al*. 2002*b*, Takamoto *et al*. 2002). Using a conditional *Ihh*d/d-KO mouse model, studies have demonstrated that in the absence of *Ihh*, a uterine nonreceptive state is achieved secondary to failure of stromal cell proliferation and vascularization along with increased estrogen signaling during the peri-implantation phase (Lee *et al*. 2006, Franco *et al*. 2010). The lack of stromal cell proliferation is in part due to *Ihh*s regulation of the EGFR in the stromal compartment, which allows the stroma to be activated by the EGFs produced by the epithelium secondary to estrogen stimulation (Franco *et al*. 2010). These observations suggest that the hedgehog signaling cascade plays a crucial role in the events occurring just before decidualization.

Chicken ovalbumin upstream promoter-transcription factor 2 signaling

Previous studies have shown that epithelial IHH stimulates chicken ovalbumin upstream promoter-transcription factor 2 (COUP-TF2) (also known as nuclear receptor subfamily 2, group F, member 2 (*Nr2f2*)), a stromal factor that mediates decidualization (Takamoto *et al*. 2002, Lee *et al*. 2006, Lee *et al*. 2010). Using *PR*-Cre to cause conditional ablation of endometrial *COUP-TF2* in mice demonstrates a defect linked to decreased expression of bone morphogenic protein 2 (BMP2), a factor produced by the stroma in response to progesterone stimulation (Kurihara *et al*. 2007). This aberration results in failure to undergo structural changes involved in decidualization. Additionally, *Coup-Tf2*-deficient mice show an increase in epithelial ERα expression and increased estrogen activity, resulting in *Ltf* and *Muc1* expression (Kurihara *et al*. 2007). Furthermore, studies have shown that the loss of epithelial ERα activity by COUP-TF2 is critical for successful progression of embryo implantation and decidualization (Lee *et al*. 2010). Overall, these studies conclude that COUP-TF2 plays a major role in epithelial remodeling and differentiation through controlling ERα activity to support the initiation of embryo implantation.

Fibroblastic growth factor/insulin-like growth factor signaling

Stromal factors that regulate epithelial function have also been identified in the intercellular communication pathways, which play a critical role in the implantation window. Specifically, fibroblast growth factors (FGFs) and insulin-like growth factor 1 (IGF1) have been proposed for stromal epithelial communication in a variety of tissues. The FGF family is a group of stromal ERα-induced paracrine factors that act on the epithelium to activate ERK1/2 signaling cascades that stimulate epithelial proliferation (Fig. 1) (Li *et al*. 2011). In this regard, based on uterine coculture experiments, evidence suggests that estrogen-mediated epithelial proliferation may involve stroma-derived factors FGF10 and BMP8a (Chung *et al*. 2015). With the FGF10 receptor, FGFR2, primarily detected in the epithelial cells in both the coculture system and the adult ovariectomized uteri, collectively these results suggest that FGF10/FGFR2 signaling may be specifically involved in the stroma–epithelial cross talk during early pregnancy. However, Filant and coworkers (2014) demonstrated that conditional ablation of *FGFR2* after birth results in abnormal basal cell appearance and stratification in the luminal epithelium, as well as subfertility that progressed to infertility. These results show the critical importance of FGFR2 in postnatal uterine development of LE and female fertility; however, further studies are needed to delineate the molecular mechanism resulting in the observed phenomenon in *Fgfr2*-null mice, which leads to complete infertility in multiparous *Fgfr2*-mutant mice. Similarly, IGF1, following estrogen stimulation, is abundantly detected in the uterus with IGF1R being identified in the epithelium (Murphy and Ghahary 1990, Kapur *et al*. 1992). A lack of IGF1 expression is observed in *Er*-KO mice stimulated with estrogen, validating these previous findings (Hewitt *et al*. 2010). The fact that IGF1R and IGF1 are abundantly expressed in the uterine epithelium suggests that IGF1 may be a paracrine mediator involved

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in the epithelial proliferation during early pregnancy. It is hypothesized that IGF1 stimulates activation of PI3/AKT pathway in the epithelium, which phosphorylates and inactivates glycogen synthase kinase 3 beta (GSK3β), allowing for epithelial proliferation (Zhu and Pollard 2007). When analyzing the role of IGF1 in *Igf1*-KO mice, Sato and coworkers (2002) demonstrated that uterine growth is supported by systemic IGF1 in the absence of local IGF1 production. This suggests that local IGF1 is not a direct mediator to estrogen effects in the uterus, but rather systemic IGF1 may be the key factor for growth.

Wnt signaling

The biological effect of estrogen can also be associated with Wnt signaling pathways. Wnt is a family of genes that encode a large group of glycoproteins that have a critical role in embryonic development and are also involved in tumorigenesis (Smalley & Dale 1999). The canonical Wnt signaling pathway, which involves regulation of β-catenin, has been the most widely studied. The activation of Wnt signaling stabilizes intracellular β-catenin by antagonizing the kinase activity of GSK3β. In the absence of Wnt signaling, GSK3β forms a multimolecular complex with axin (a bridging molecule), adenomatous polyposis coli, and β-catenin, leading to phosphorylation and then subsequent degradation via ubiquitination pathway of β-catenin. When activated, β-catenin translocates to the nucleus and forms a complex with downstream effectors such as lymphoid enhancer factor (*Lef*)/T-cell factor (*Tcf*) family that stimulates the transcription of Wnt target genes. These target genes are involved in cellular organization during embryonic development, proliferation, and differentiation as well as cell-to-cell communication and cell fate specification (Smalley & Dale 1999).

Previous studies have shown that *Wnt4* expression is upregulated at the site of embryo implantation during decidualization (Daikoku *et al*. 2004). Further studies revealed that *Wnt4* plays a key role in implantation and decidualization (Franco *et al*. 2011), and this action is mediated downstream of progesterone via β-catenin signaling pathway in uterine stromal activity with proliferation and differentiation (Rider *et al*. 2006, Li *et al*. 2013).

We previously demonstrated the presence of an ER-independent pathway of estrogen stimulation via Wnt pathway (Hou *et al*. 2004). After exposing *Erα*-KO (ERKO) mice with estrogen, prompt stabilization and localization of β-catenin in the nucleus of uterine epithelial cells were observed. This finding confirmed that injection of adenovirus-driven expression of SFRP2, a Wnt antagonist, was suppressed rapidly by estrogen during the early phase in the uterus in an ER-independent manner, since as reported by (Das *et al*. 2000), demonstrating the downregulation of β-catenin and halting of epithelial cell growth without affecting early estrogen effects (Hou *et al*. 2004). Similarly, studies have also shown that Wnt/β-catenin downstream effectors *Lef1* and *Tcf3* are upregulated in an estrogen-independent manner (Ray *et al*. 2008). Through immunofluorescence studies, *Lef1*/*Tcf3* localization was confirmed in the epithelial cells after estrogen exposure and was interestingly found to be interacting with ERα in a time-dependent manner (Ray *et al*. 2008). Furthermore, evidence was provided for an ERα and *Tcf3*/*Lef1* complex occupying a certain DNA region of estrogen-responsive gene promoters, suggesting a nonclassical induction mechanism of the Wnt/β-catenin pathway that is necessary in the estrogen-dependent gene regulation.

GPR30 signaling

GPR30 (also known as GPER1), a G-protein-coupled receptor, has been implicated in early nongenomic signaling mediated by E2. In mouse uterus, GPR30 localizes primarily in the uterine epithelial cells (Gao *et al*. 2011). Studies from *Gpr30*-KO mice appear to imply that GPR30's role in uterine biology is minimal for estrogenic growth regulation (Wang *et al*. 2008, Martensson *et al*. 2009, Otto *et al*. 2009). In contrast, using selective activation of GPR30 by G1, studies have shown that GPR30 is involved in regulating early signaling events, including the inhibition of ERK1/2 and ERα (Ser118) phosphorylation signals in the uterine stromal compartment, suggesting that a paracrine signaling is involved (Fig. 1) (Gao *et al*. 2011). However, it should be noted that this study was unable to exclude the possibility through the off-target effects of G1. Moreover, further studies should be considered to show that *Gper1*-null mice are insensitive to G1 in the above uterine effects. Overall, studies show that GPR30 can act as a negative regulator of ERα-dependent uterine growth in response to E2.

Molecular links between the phase I and phase II estrogenic responses in the uterus

Early (phase I) and late (phase II) estrogenic responses in the uterus have been recognized for more than 70 years, yet mechanisms involved in their regulation remain controversial. One concept is that an early events(s), occurring within the first 6 h, prepares the uterus for later (18–30 h) increase in DNA synthesis, cell proliferation, and protein synthesis. An alternate view is that the late growth phase is a result of the continuous presence of

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a stimulus. Discussion of either concept usually makes the assumption that all of the responses are dependent upon ligand interaction with one of the two estrogen receptor isoforms (ERα and ERβ). However, we and others have shown that *Erα*-null mice (ERKO) or wild-type mice in which ER functions are silenced by ER antagonist ICI-182,780 manifest the expression of several early genes in response to 4-hydroxyextradiol-17β or a xenoestrogen (kepone), as well as induction of early responses such as water imbibition and macromolecular uptake by 4-hydroxyextradiol-17β (Das *et al*. 1997, 1998, 2000, Hewitt *et al*. 2003, Watanabe *et al*. 2003, Hou *et al*. 2004, Ray *et al*. 2006). Furthermore, studies have also shown that ICI was able to suppress the expression of *Ltf*, a well-characterized estrogen-responsive uterine gene, in the wild-type mice after E2, indicating the effectiveness of ICI in this study (Das *et al*. 1997, 1998). Using the same effective dose of ICI (Das *et al*. 1997, 1998), we have identified two such ER-independent uterine genes *Bip* (*Hspa5*) and *Sik-SP* (*Nop58*) that are regulated by E2 in ERKO mice (Das *et al*. 2000). The bimodal nature of estrogen effects coupled with phase I ER-independent estrogenic responses and phase II mostly ER-dependent responses has ignited interest in understanding the pathways linking these two phases.

Role of Bip

Bip, also known as *Grp78* encoded by *Hspa5*, is a member of the heat-shock protein (HSP70) chaperone family, and it is induced by estrogen in an ER-independent manner as a phase I response (Das *et al*. 2000, Ray *et al*. 2006). It is a protein that resides in the endoplasmic reticulum (Fig. 2), where assembly of newly synthesized peptides occurs, and is abundantly present during cell proliferation and differentiation, particularly at the site of embryo implantation during decidualization (Simmons and Kennedy 2000). As a chaperone molecule, the role of BIP is for functional maturation of steroid hormone receptors. In the mouse uterus, it mediates estrogen-dependent responses through molecular association with ERα (Ray *et al*. 2006). Studies have demonstrated through *in vivo* and *in vitro* mouse models that suppression of *Bip* antagonizes *Erα*-mediated gene transcription and compromises estrogen-dependent phase II growth response (uterine epithelial cell proliferation) with sustained phase I responses (water accumulation and macromolecular uptake). Most interesting is the lack of growth response in the presence of ERKO state even if *Bip* is upregulated (Ray *et al*. 2007). Although this study analyzed xenoestrogen and *Bip*, it demonstrates the close relationship between

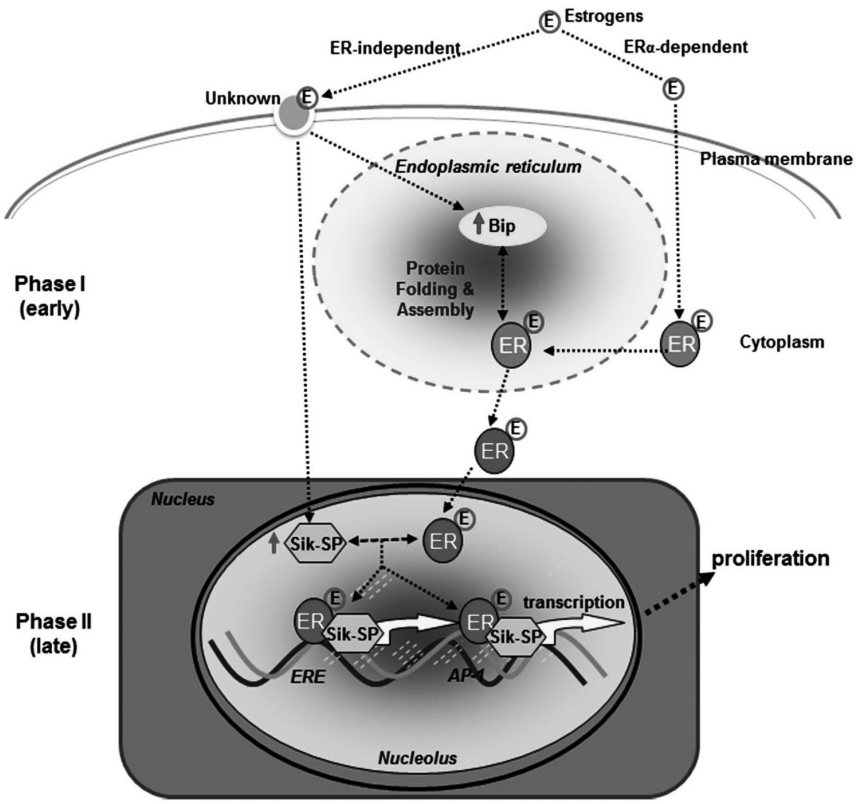

Figure 2
A schematic model for *Sik-SP*- and *Bip*-mediated estrogen signaling in uterine cell proliferation. The molecular cross talk mediated by *Bip*, in the translocation of ERα from the endoplasmic reticulum to the nucleus under the direction of estrogen. In the nucleus, distinct accumulation of *Sik-SP* and its association with ERα in the nucleolar region is necessary for the SIK-SP/ERα complex-mediated regulation of gene transcription and cellular proliferation.

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Bip and ERα in regulation of uterine growth. Together, studies suggest that the functional activation of ERα via *Bip* plays a role in coordinating phase I responses with those of phase II for regulated growth and differentiation via estrogen signaling in the mouse uterus.

Some organochlorine compounds, such as polychlorinated biphenyls, are highly persistent organic pollutants in many industrial nations. These compounds have gained attention recently secondary to their potential for adverse effects on health and reproduction. The reproductive toxicity is thought to be due to their estrogen-like properties; hence, they are categorized as xenoestrogens. The ability to bind to ERα allows for mimicking effect on target organ function, yet the mechanisms are not well defined (Das *et al*. 1997). There are, however, significant differences in coactivator recruitment and transcriptional activation in tissues exposed to xenoestrogens corresponding to distinct biological effects causing endocrine disruption. Furthermore, these compounds are effective at very low doses comparable to their level of exposure, making them very potent estrogens (Ray *et al*. 2007).

Knowing the critical role that *Bip* plays in regulation of estrogen-dependent ERα-mediated gene transcription and growth, the xenoestrogen-mediated effects with regard to the upregulation of *Bip* under certain conditions could be potentially harmful with respect to enhanced uterine estrogenicity. Specifically, the xenoestrogen kepone can induce sustainable levels of uterine *Bip* without involving ER, which in turn regulates the kepone-dependent ERα-mediated gene expression (Ray *et al*. 2007). Furthermore, with the notion that stress can regulate *Bip* expression and the ability of uterine growth via stress-induced estrogen response in mice, studies have demonstrated that endogenous *Bip* via stress-related signals contributes to uterine estrogenicity for kepone (Ray *et al*. 2007). Thus, the combination of a variety of signals in the body, such as stress, and xenoestrogens can act as a plausible risk factor enhancing estrogenicity and therefore major health concerns.

Role of Sik-SP

The nucleolus is the nuclear subdomain that primarily carries out the assembly of ribosomal subunits in eukaryotic cells. A recent study has uncovered an unexpected role of uterine estrogen signaling which involves a nucleolar protein SIK-similar protein (SIK-SP, also known as NOP58/NOP5/NOI5) (Chung *et al*. 2012). Studies have shown that the expression of uterine *Sik-SP* is tightly regulated by E2 in an ER-independent manner but is still required for the control of ERα-mediated late uterine functions (Fig. 2) (Das *et al*. 2000, Chung *et al*. 2012). Specifically, using both the *in vivo* and *in vitro* coculture approaches, studies have shown that E2-induced *Sik-SP* directly interacts with ERα to mediate ERα-dependent gene regulation and is necessary to coordinate the biphasic responses in the uterus for its appropriate growth under the direction of E2. Overall, this finding of ERα-independent early *Sik-SP* contributing to ERα-regulated events adds new insights to our understanding of nucleolar involvement in uterine estrogen signaling.

Taken together, these studies provide evidence of nonclassical pathways that mediate estrogen actions in a time-dependent fashion, possibly shedding a light on how the biphasic, phase I and phase II, estrogenic responses are molecularly linked to mediate uterine cell proliferation.

ER-independent genes associated with embryo implantation

To understand the functional significance of estrogen-induced ER-independent early uterine genes, studies were undertaken to determine whether E2 administration in the delayed implantation model in mice enhances the expression of *Bip* and *Sik-SP* at the site of implantation. Indeed, results demonstrated that these genes are specifically upregulated in the subluminal stromal cells at the site of the implanting embryo following activation with E2; however, the delayed stage of the uterus does not show any expression at the site of embryo

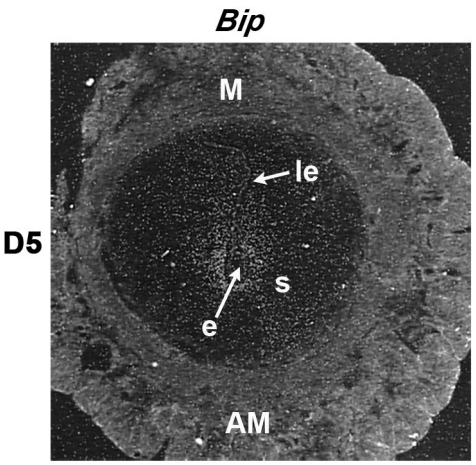

Figure 3
Analysis of expression for *Bip* mRNAs. *In situ* hybridization detects the expression of *Bip* at the site of implantation on D5 of pregnancy in mice. le, luminal epithelium; s, stroma; e, implanting embryo; M, mesometrial pole; AM, antimesometrial pole.

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(Reese *et al*. 2001, Chung *et al*. 2012). Furthermore, this induced expression is consistent with the status of expression in normal implantation sites on D5 for *Bip* (Fig. 3) and *Sik-SP* (Chung *et al*. 2012). Taken together, studies have shown that these ER-independent genes are physiologically important during the onset of embryo implantation under the direction of E2.

Conclusions

This article has served as an update of the literature describing the molecules involved in estrogen signaling in the mouse uterus during early pregnancy. We have discussed the signaling pathways that are ER dependent and ER independent as well as the molecular links that shed light into the complexity of the bimodal estrogen actions occurring in early pregnancy. Dysregulation of the cross talk between these pathways can lead to implantation failure through the inability to obtain a receptive uterine epithelium. Environmental toxins can mimic estrogen pathways; however, the mediated effects differ from normal through the enhanced estrogenicity of the uterus creating a nonreceptive uterine epithelium. Continued research into the mechanisms involved in estrogen signaling will expand our understanding of this delicate and time-sensitive event. Understanding the molecular interactions will provide the knowledge needed to improve current treatments of infertility through the exploration of new ideas, techniques, and technology.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this article.

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