—Mini Review—

Molecular mechanisms regulating embryo implantation in mammals

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Abstract: In mammals, pregnancy is an irreversible and complicated event. The mammalian uterus requires many physiological and morphological changes for pregnancy-associated events including implantation, decidualization, placentation and parturition. The failure to complete any events results in implantation failure, spontaneous miscarriage or abnormal parturition, including preterm birth. These events are primarily regulated by ovarian estrogen and progesterone (P4). P4 and estrogen are produced in the ovary throughout pregnancy in mice, but in humans, hormonal support switches from the ovary to the placenta. The first direct interaction between embryo and uterus is implantation. In humans, about 75% of unsuccessful pregnancies are believed to result from defective implantation. Therefore, a better understanding of the molecular mechanisms associated with implantation would be helpful for the further improvement of clinical treatments. Recent studies using genetically modified mice have given us considerable insight into the molecular mechanisms underlying embryo implantation. In this review, we discuss in the understanding recent advances of the molecular events during implantation, especially focusing on the roles of estrogen and P4 signaling. We also offer our thoughts on the as yet unelucidated processes in implantation to guide and stimulate further research in this area.

Key words: Implantation, Pregnancy, Embryo, Uterus

Introduction

Human infertility has developed into serious physiological and social problems all over the world, especially in developed countries. Numerous assisted reproductive technologies (ARTs), for example artificial insemination [1], *in vitro* fertilization (IVF) [2] and intracytoplasmic sperm injection (ICSI) [3], have been developed and are widely used for the rescue of human infertility. Also cryopreservation of sperm [4], oocytes [5], or embryos [6, 7] is an important technology which is routinely applied used by human infertility clinics. Despite the establishment of these technologies and great efforts by medical doctors, nurses and embryologists, current treatment methods cannot rescue the fertility of about half of couples who desire a baby. Therefore, additional research and improved knowledge of embryo implantation is required to develop new technologies to address these shortcomings.

In most mammalian species, oocytes are arrested at the metaphase-II stage before ovulation [7, 8]. Once oocytes have ovulated, they migrate to the oviductal amplulla, where fertilization occurs. Penetration of a sperm triggers the release of the metaphase-II arrest in oocytes via fertilization-associated calcium signaling [9–11]. Thereafter, the embryos transit to the uterus through the oviduct and then develop to the blastocyst stage. In the uterus, activation of extracellular regulated protein kinase occurs in the embryo giving it acquires the competence to be implanted in the uterine endometrium [12, 13]. The uterus undergoes considerable physiological and morphological changes during pregnancy. Successful pregnancy includes implantation, decidualization, placentation and parturition [12, 13]. The success of these events is indispensable for the birth of offspring. In humans, it is thought that 75% of unsuccessful pregnancies are associated with implantation failure [14], because implantation is the event of the first contact between the embryo and maternal tissue, and when implantation failure occurs, the subsequent pregnancy-associated events, such as

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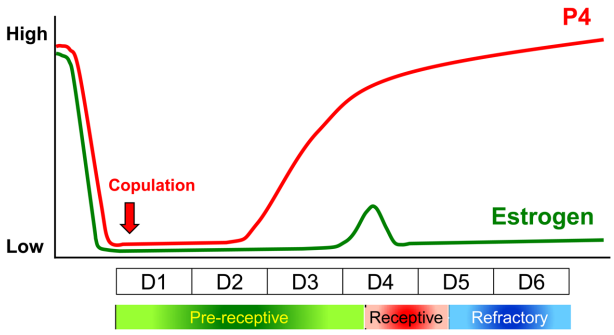

Fig. 1. Estrogen and progesterone (P4) orchestrate implantation window in mice. In mice, uterine sensitivity for accepting the embryo is composed from perceptive (Day 1–3; with the day of vaginal plug observed being defined as Day 1), receptive (Day 4) and refractory (Day 5 afternoon). On Day 4, an increase of estrogen level is observed prior to the receptive stage.

decidualization or placentation cannot take place [12, 13]. These pregnancy-associated events are primarily orchestrated by two steroid hormones called estrogen and progesterone (P4) [15]. Estrogen plays roles in the proliferation of epithelial cells, suppression of apoptosis, and regulation of the expression of lactoferrin and mucin 1 which are critical for normal uterine function [16–19]. On the other hand, P4 has roles in the suppression of epithelial cell proliferation and stromal cell proliferation via the expression of Indian hedgehog homolog (Ihh) and heart- and neural crest derivatives-expressed protein 2 (Hand2) [20–23]. Therefore, a better understanding of the molecular mechanisms associated with implantation would be helpful for the further improvement of clinical treatments. Recent studies using genetically modified mice have provided us with information for clarifying these molecular mechanisms. In this review, we provide an overview of the recent advances in our understanding about implantation, especially focusing on the roles of P4 and estrogen signaling.

A Theory of Implantation Window

In mice, uterine sensitivity for accepting the embryo is classified as perceptive (Day 1–3; with the day of vaginal plug observed being defined as Day 1), receptive (Day 4) and refractory (Day 5 afternoon) stages [12, 15] (See also in Fig. 1). Only during the receptive stage can embryos implant into the uterine epithelium. This duration of the receptive stage is also called the 'implantation window' [24]. Some research groups have demonstrated the start and end points of the implantation window using embryo transfer in mice. Earlier studies showed that when embryos were transferred to the uterus at 9:00, 14:00 or 18:00 on Day 4, successful implantation was confirmed on Day 5 [25]. Another study showed that embryos transferred at 9:00 on Day 5 also implanted but not those transferred at 21:00 on the same day [26]. These results suggest the window of implantation opens at Day 4 and is maintained until the morning of Day 5. The transition from the pre-receptive to the receptive stage is primarily regulated by estrogen and progesterone (P4). Estrogen and P4 bind to their nuclear receptors at different times, and different cell-types in the uterus regulate the uterine receptivity of mammals [27, 28]. It is well known that two types of estrogen receptors (ERα and ER*β*) and two types of P4 receptors (PR-A and PR-B (*Pgr*)) are expressed in the mouse uterus. *ERα*-knockout mice show defective phenotypes during reproductive events, including implantation but not *ERβ*-knockout mice [29]. *PR-A* and *PR-B* double knockout mice are also infertile [30], but not single *PR-B* knockout mice [31]. Based on these results, both ERα and PR-A are essential for embryo implantation in mice. During ovulation, estrogen secreted from the ovary induces proliferation of uterine epithelial cells in the uterus [17]. In the epithelial-specific deletion of *ERα* in the mouse uterus, proliferation of epithelial cells and PR distribution was not affected [17], which suggests stromal ERα has a major role in these events. At the transition from the pre-receptive (Day 3) to receptive (Day 4) stage, a rise in P4 secretion occurs in the newly formed corpus lutea. Epithelial-specific deletion of PR cannot suppress the proliferation of epithelial cells by estrogen, suggesting that the role of PR in epithelial cells is to inhibit epithelial estrogen action for successful implantation [23].

On Day 4, a rise in the estrogen level derived from the ovary is observed prior to the receptive stage, but the detailed mechanism of this still remains unclear [32]. In several species other than mice, ovarian estrogen is dispensable for embryo implantation; however, ovarian P4 is indispensable for the process in all the species studied to date [12]. Ovariectomized mice on the morning of Day 4 (just prior to the rise of estrogen for implantation) can be used as a model of delayed implantation and embryonic dormancy [25]. Continuous P4 injection can maintain this condition for several days. Once estrogen is administrated after P4 injection, implantation can be induced. These results suggest a rise in the level of estrogen is a key condition for the induction of embryo implantation. Using this model of delayed implantation, different concentrations of estrogen were examined. A high level of estrogen rapidly induced the transition to the refractory stage,

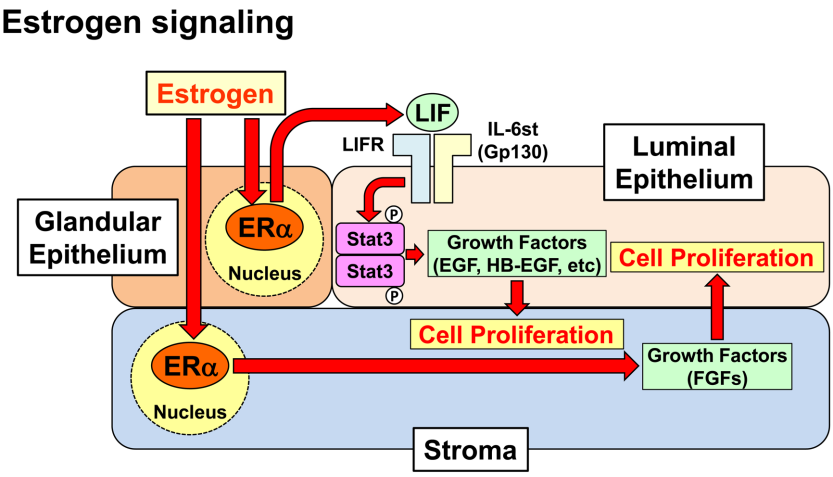

Fig. 2. Estrogen-dependent signaling in epithelial and stromal cells during implantation. Estrogen binds to its nuclear receptor, ERα, in both epithelial and stromal cells. In the glandular epithelium, LIF expression occurs and binds to the receptor (LIFR and IL-6st) in the luminal epithelium, induces activation of Stat3. Activation of Stat3 in involved in stromal cell proliferation via growth factors expression. Stromal ERα is also important for cell proliferation of the luminal epithelium via activation of Fibroblast growth factors.

bypassing the receptive stage [25]. On the other hand, injection of low concentrations of estrogen did can eventually induce the transition to the receptive stage. These results strongly suggest that an optimal concentration of estrogen is required for on-time implantation.

The transition of these stages is one-way, and recovery from the refractory stage requires the withdrawal of P4 [12, 13]. Although a similar sequence of these events occurs in humans, the menstrual cycle is longer (around 28–30 days) than the estrus cycle in the mouse (around 4 days). Also in humans, it is known that the pre-receptive stage spans the first 7 days after ovulation (early luteal stage), and transition to the receptive stage occurs in the mid-luteal stage (around 7–10 days after ovulation). After that, the uterus proceeds to the refractory stage for the remainder of the cycle (late luteal stage), until menstruation ensues [13].

Role of Estrogen Signaling in Implantation

Although estrogen and P4 signaling are both essential for embryo implantation and their signaling are complicated, a major mediator of estrogen action is leukemia inhibitory factor (Lif) [33, 34] (See Fig. 2). Lif is a member of the interleukin-6 (IL-6) family of cytokines [35]. Deletion of the *Lif* gene causes complete implantation failure in mice, suggesting LIF is indispensable for embryo implantation [34]. Lif binds to its receptor (Lifr) and IL-6 signal transducer (IL-6st), gp130 [35] (Fig. 2). At the time of implantation, both Lifr and IL-6st are expressed in the uterine epithelium [36], and mice with deletion of Lifr or IL-6st-knockout mice show embryonic lethality [37, 38]. Uterine deletion of IL-6st or its downstream target, signal transducer and activator of transcription 3 (Stat3), also causes implantation failure [39]. Recently, epithelium-specific deletion of Stat3 was shown to result in complete implantation failure followed by downregulation of epidermal growth factors (EGFs) which are essential for stromal cell proliferation [40]. In that study, it was also demonstrated that Stat3 has crucial roles for epithelial junctional reorganization via suppression of Claudin1, an integral membrane protein and a component of tight junction strands. On the other hand, stromal-specific deletion of Stat3 just decreased the number of pups due to defects in placentation followed by down-regulation of EGFs [41], suggesting the epithelial Lif-signaling pathway is important for implantation via activation of EGF signaling. In humans, it has been reported that a rise in Lif expression is seen before implantation [42] and some studies have demonstrated Lif expression is higher in fertile women than infertile women around the time of implantation [43, 44]. However, in other species except for mice, it remains inconclusive whether Lif is an indispensable factor for implantation.

A comparison of wild-type and *Lif*-null mice showed the homeobox transcription factor, *Msx1*, has an essen-

tial role during implantation [45–47]. *Msx1* is transiently expressed in the epithelium around the time of receptivity and its expression reached a maximal level on the morning of Day 4 [46], but it was not detected in the uteri of pregnant mice. Uterine specific deletion of *Msx1* resulted in partial implantation failure but double knockout of *Msx1* and *Msx2*(*Msx1/Msx2*), another member in the family, resulted in infertility due to complete implantation failure via suppression of bone morphogenetic protein 2 (Bmp2) and cyclooxygenase-2 (Cox-2) [46]. Since *Msx2* expression is upregulated in *Msx1* null, but not wild-type mice, *Msx2* has a compensatory role for *Msx1*. *Msx1*/*Msx2* are involved in the polarity of the luminal epithelium at the time of the attachment of embryos [46]. Wnt5a, a traditionally non-canonical Wnt and mediator of cell polarity, is upregulated in the epithelium and stroma of *Msx1/Msx2*-knockout mice [48]. A recent study revealed that downstream factors of Wnt5a, receptor tyrosine kinase-like orphan receptor 1 (Ror1) and Ror2, are essential for implantation and the disruption of Wnt5a-Ror signaling results in disorderly epithelial projections, crypt formation, embryo spacing and impaired implantation [49]. Another recent study showed that recombination signal binding protein for immunoglobulin kappa J region (Rbbj), the nuclear transducer of Notch signaling, confers on-time uterine lumen shape transformation by physically interacting with uterine ERα in a Notch pathway-independent manner [50]. These estrogen-dependent signaling pathways are required for normal mammalian embryo-uterus interaction.

Role of P4 in Implantation

The importance of P4 in implantation has been confirmed in all the mammalian species studied to date. Since a high P4 level is also required for later reproductive events, for example decidualization [51] and maintenance of pregnancy [52], P4 is called 'the pregnancy hormone'. *PR* null mice show some defective phenotypes including disrupted ovulation, luteinization, and decidualization [30]. Epithelial-specific deletion of *PR* does not inhibit epithelial proliferation induced by estrogen, suggesting epithelial PR is essential for the suppression of estrogen action [23]. These *PR*-null mice also showed infertility in females which was attributed to incomplete uterine receptivity due to reduced expression of Ihh. It has been shown that PR can directly bind to *Ihh* promoter, resulting in the induction of the proliferation of stromal cells [23]. Another study showed stromal PR mediates induction of Ihh in the uterine epithelium and its downstream targets in the uterine stroma [53]. Chicken ovalbumin upstream promoter-transcription factor 2 (COUP-TFII) is a downstream target of Ihh signaling and it is expressed in the sub-epithelial stroma [54]. Uterine deletion of *COUP-TFII* causes implantation failure with excessive estrogenic action in the epithelium. A P4-induced transcription factor, Hand2, is expressed in the stroma and has been reported as a regulatory factor of uterine receptivity and implantation [22]. Uterine deletion of *Hand2* resulted in excessive estrogen activity and proliferation of epithelial cells via high expression of fibroblast growth factors (FGFs) [22]. These results suggest that a major role of Hand2 in stromal cells is the suppression of epithelial proliferation. Another P4-inducible factor, FKBP52, is required for modulating PR activity [55–57]. *FKBP52* null mice show implantation failure due to impaired uterine P4 responsiveness and enhanced estrogen-like signaling. Deletion of *FKBP52* increases the sensitivity to oxidative stress followed by reduced expression of a unique antioxidant enzyme, peroxiredoxin 6 (PRDX6) [58]. However, since this type of infertility is rescued by the injection of antioxidants, it suggests that FKBP52 has a partial role in implantation.

Conclusion

Studies of genetically modified mice have identified estrogen- or P4-dependent factors have been identified as critical factors involved in implantation in mammals. However, it is necessary to consider that most of the data previously reported was from knockout mice and the gene deletion was not specific to the uterus. For example, in most of these studies, Pgr^{Cre} transgenic mice (Cre recombinase is expressed under *Pgr* promoter) are used to generate uterine gene knockout mice [59]. PR is expressed not only in uterine cells but also in ovarian cells including the corpus luteum which is a source of P4 production. It has been shown that conditional deletion of the gene causes infertility due to its deletion not in the uterine tissues, but in other tissues [52]. In addition, $Wnt7a^{Cre}$ and $Amhr2^{Cre}$ transgenic mice are used for epithelial and stromal cell-specific deletion, respectively [17, 41]. These genes are potentially important for the development of the reproductive organs and Cre is expressed in developing female reproductive tracts, suggesting that the phenotype of infertility may be a secondary effect. Recently, *Lactoferrin*-iCre (Ltf^{Cre}) transgenic mice have been developed [60]. In this line, Cre recombinase is first expressed in the uterine epithelium beyond day 30 after the birth. By using this new transgenic line, it may be able to more precisely clarify the molecular mechanisms underlying implantation.

Recently, genome editing technologies such as zinc-finger nuclease (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR/Cas9 became available for the production of knockout animals [61–63]. Very recently, it has been shown that, at least in the mouse, these technologies are available for generating conditional knockout animals using genome editing technologies [64]. If these technologies were applied to the clarification of molecular mechanism of implantation in other mammalian species, the results from various species would help to explain species-dependent differences pregnancy-associated events.

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