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Cadence of procreation: orchestrating embryo-uterine interactions

Jeeyeon Cha and Sudhansu K. Dey*

Division of Reproductive Sciences, Cincinnati Children's Research Foundation, Cincinnati, OH 45229

Abstract

Embryo implantation in eutherian mammals is a highly complex process and requires reciprocal communication between different cell types of the embryo at the blastocyst stage and receptive uterus. The events of implantation are dynamic and highly orchestrated over a species-specific period of time with distinctive and overlapping expression of many genes. Delayed implantation in different species has helped elucidate some of the intricacies of implantation timing and different modes of the implantation process. How these events are coordinated in time and space are not clearly understood. We discuss potential regulators of the precise timing of these events with respect to central and local clock mechanisms. This review focuses on the timing and synchronization of early pregnancy events in mouse and consequences of their aberrations at later stages of pregnancy.

Keywords

Embryo implantation; embryonic diapause; uterus; MSX; temporal

Introduction

In mammals, the beginning of a life commences with the union of a sperm and an egg through the process of fertilization. The fertilized egg then undergoes several rounds of mitoses to form a blastocyst. These developmental events in the embryo are synchronized with proliferation and differentiation of specific uterine cell types guided by ovarian estrogen and progesterone (P₄) in a spatiotemporal fashion to render the uterus receptive for blastocyst implantation. These events are sequential and dynamic, conferring activation of both embryonic and maternal genes in a timely and coordinated fashion that set up reciprocal interactions between these two entities requisite for successful implantation. Failure to orchestrate these coordinated interactions at scheduled times lead to defective or

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*Corresponding author at: Division of Reproductive Sciences, Cincinnati Children's Research Foundation, Cincinnati, OH 45229. Tel: +1 513 803-1158; Fax: +1 513 803-1160, sk.dey@cchmc.org (S. K. Dey).

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unsuccessful implantation. Implantation across eutherian species occurs within a precise and transient time frame known as the window of uterine receptivity to implantation (window of implantation). The onset and duration of this window varies across species. Defects around the time of implantation may compromise pregnancy outcome by steering adverse ripple effects through the remaining course of development [1].

The window for implantation is transient

Rodent models have helped us to better understand the mechanisms that direct uterine receptivity and nonreceptivity. In mice, the active states of the uterus with respect to implantation are classified as prereceptive, receptive, and refractory (nonreceptive); these states are defined by observations of uterine responses to transferred blastocyst in pseudopregnant mice and are generally directed by ovarian P₄ and estrogen secretion [2, 3]. While the uterus is prereceptive on days 1–3 (day 1 defined as finding the vaginal plug), it becomes receptive to implantation on day 4 of pregnancy or pseudopregnancy and lasts for only ~24 hours. By late day 5, the uterus becomes refractory to implantation with gradual degeneration of blastocysts until the estrous cycle is reset after hormonal withdrawal. Similarly, the uterus is receptive for a short period spanning 7–9 days after ovulation (cycle days 21–23) during the mid-luteal phase in women. After this time, the uterus becomes and remains refractory (nonreceptive) for the remainder of the luteal phase.

Stages of implantation progress in a discrete sequence

When the mouse embryo reaches the blastocyst stage, it gains the ability to attach to the receptive luminal epithelium once the uterus has been primed with P₄ and superimposed by a small amount of estrogen. If this condition of embryo-uterine synchrony is met, engagement of cell adhesion molecules at the uterine luminal and blastocyst trophoctoderm epithelial surfaces initiates the implantation process. These adhesion molecules then transduce signals necessary to sustain embryonic and maternal contributions to support fetal development [4].

For implantation to ensue, the uterine luminal epithelial closure is essential on day 4 of pregnancy (day of uterine receptivity) in mice. This luminal epithelial closure is P₄ dependent, but independent of embryonic participation, since this occurs in pseudopregnant mice with uterine steroid hormonal milieu similar to that of pregnant females during the periimplantation period [5]. In rodents, the process of implantation is classified into three stages: apposition, adhesion/attachment, and penetration [6, 7]. Close apposition of the blastocyst trophoctoderm with the luminal epithelium within a specified implantation chamber (crypt or nidation) is followed by the adhesion stage. This latter stage initiates further intimate and molecular exchanges between the two epithelial cell types, leading to the attachment reaction. The attachment reaction is coincident with localized increased endometrial vascular permeability at the site of the blastocyst, as determined by the visualization of blue bands along the uterine horn after an intravenous injection of a macromolecular blue dye (uterine blue reaction) [3]. In mice, successive events spanning the luminal closure to the attachment reaction occur starting from day 3 afternoon and are complete by day 4 of pregnancy [8, 9].

The attachment reaction in mice and rats occurs on the evenings of day 4 and 5, respectively, and day 6½ in rabbits [10–12]. This reaction is assumed to occur approximately on day 8 in humans and baboons, day 9 in macaques, and day 11 in marmoset monkeys [13, 14]. In large animals, the attachment reaction occurs on day 13 in pigs, day 16 in sheep, day 19 in goats, and day 20 in cows [2]. Finally, penetration involves the invasion by the trophoctoderm through the epithelium into the stromal bed. Stromal cell differentiation to specialized decidual cells (decidualization) becomes robust with the demise of the luminal epithelium at the attachment site.

Spatial orientation for implantation: crypt formation

Blood vessels enter the uterus from the mesometrium, assigning a mesometrial-antimesometrial (**M-AM**) axis to the uterus. In mice, implantation occurs within a crypt (nidus) towards the antimesometrial pole of the uterus, and discrete implantation sites are spaced evenly with respect to adjacent sites along the uterine horn. How the uterus and embryo communicate to spatially coordinate implantation is not fully understood. Mouse blastocysts are oriented with their inner cell mass (ICM) directed towards the mesometrial pole, whereas the ICM in humans is directed towards the antimesometrial pole. Initially, mouse blastocysts are situated into crypts with random orientation of their ICMs. The underlying mechanism by which the orientation of a blastocyst is directed at the time of implantation remains elusive.

Implantation strategies are diverse across eutherians

In 1884, Bonnet classified implantation based on histological analyses of cell-cell interactions between the blastocyst and uterus, grouping strategies into three categories: central (rabbits, ferrets, and marsupials), eccentric (mice, rats, and hamsters) and interstitial (guinea pigs, chimpanzees, and humans) [15]. Nearly one century later, Schlafke and Enders classified implantation in certain species into intrusive, displacement, and fusion types from their ultrastructural studies [16]. Humans and guinea pigs show an intrusive type of implantation in which trophoblast cells penetrate through the luminal epithelium, reaching and extending through the basal lamina. In contrast, rodents exhibit a displacement type of implantation: the luminal epithelium is freed of the underlying basal lamina, facilitating the passage of trophoblasts through the epithelium. In rabbits, the fusion type of implantation allows trophoblast cells to unite with the luminal epithelium to form symplasma.

Interestingly, implantation is shallow in large animals, such as pig, sheep, cow and horse in which the entire trophoblastic surface of elongated blastocysts makes the attachment contacts along the luminal epithelial surface [17]. In these animals, less-invasive blastocysts with remarkable elongation occurring on day 12 of pregnancy exhibit longer free-floating status within the uterus than in species with more invasive conceptii. The growth of extra-embryonic tissue contributes to this elongation, enabling the embryo access to an efficient supply of nutrition from uterine secretions until the attachment reaction occurs. Trophoctoderm invasion through the luminal epithelium and basal lamina into the stroma is required to provide nutrition to the developing embryo by establishing a vascular connection with the mother. This process varies considerably from species to species with respect to

timing (heterochrony) and cytological features [16]. The significance of diverse implantation strategies and timing displayed by different species suggest diversification of species-specific adaptation. However, one common feature is the enhanced endometrial vascular permeability at the site of blastocyst attachment in many animals examined. It is believed that this increased permeability also occurs in the endometrial bed in human implantation, but has not been experimentally documented due to ethical restrictions.

Implantation requires coordinated action of ovarian hormones

The master regulators that specify the transient window of uterine receptivity are primarily the ovarian hormones P_4 and estrogens. While both hormones are crucial for implantation in mice and rats, ovarian estrogen is not essential for implantation in many species such as pigs, guinea pigs, rabbits, and hamsters. Ovarian P_4 alone can support implantation in these species; it is thought that embryo-derived estrogen contributes to implantation [3, 18–22]. It would be interesting to determine whether preimplantation estrogen secretion by the ovary or embryo plays a crucial role in human implantation [1].

Heterogeneous uterine cell types uniquely respond to fluctuating ovarian P_4 and estrogen levels. In mice, preovulatory estrogen secretion promotes epithelial cell proliferation on day 1 of pregnancy. In contrast, rising P_4 levels from newly formed corpora lutea provoke extensive stromal cell proliferation when superimposed with preimplantation ovarian estrogen secretion on the morning of day 4. This stromal cell proliferation is associated with the cessation of epithelial cell proliferation, leading to epithelial cell differentiation in preparation for implantation [23]. In pseudopregnant mice, the steroid hormonal milieu in the uterus is similar due to the presence of the newly formed corpora lutea following ovulation. Thus, the hormonal milieu and associated changes in pseudopregnant uteri on days 1–4 are quite similar to normal pregnancy, and the transfer of blastocysts into a pseudopregnant uterine lumen during the receptive phase elicits normal implantation and decidualization.

Time-sensitive critical genes for implantation

Several genes have been identified as critical for uterine receptivity, implantation, or decidualization. However, some genes show overlapping expression patterns spanning more than one event during early pregnancy, making it difficult to delineate their roles during particular events. The expression of muscle segment homeobox gene *Msx1* is unique, since it has a transient peak expression in the luminal epithelium at the receptive phase on day 4, but is not expressed in the uterus thereafter for the remainder of pregnancy. Mice with uterine deletion of *Msx1* show deferred implantation outside the normal window with compromised pregnancy outcome, while mice with uterine deletion of both *Msx1* and *Msx2* exhibit implantation failure since *Msx2* compensates for the loss of *Msx1* in *Msx1*-deleted uteri [24]. Normally, the uterine luminal epithelium transits from its higher polar to a less polar state approaching implantation; epithelial cells become more cuboidal with corrugated apical surface with loss of apicobasal polarity, conducive to blastocyst attachment [24]. The presence of heightened luminal epithelial apicobasal polarity in the absence of *Msx* genes creates a barrier for trophectoderm attachment and penetration into the stroma. Although

regulation of *Msx* in the uterus is not clearly understood at this time, it appears that *Msx1* is cross-regulated with leukemia inhibitory factor (Lif), another critical factor for implantation [24]. The unique, transient expression of *Msx* suggests its distinctive role in coordinating gene expression prior to implantation.

At present, heparin binding EGF-like growth factor (HB-EGF) is considered the first molecular link between the blastocyst and receptive uterus for attachment and is an important paracrine and juxtacrine mediator of embryo-uterine interactions during implantation. It is expressed in the luminal epithelium exclusively at the site of the blastocyst several hours before the attachment reaction that occurs on the evening of day 4. Its expression on the afternoon of day 4 is coincident with the downregulation of *Msx1* and persists through the attachment phase. Other critical factors that are expressed prior to attachment to facilitate embryo-uterine interactions may also be involved but remain to be identified. A plethora of other known critical factors including transcription factors, growth factors, morphogens, cytokines, and signaling molecules are also expressed in a spatiotemporal manner in the uterus around the time of implantation (Figure 1) and play stage-specific or overlapping functions spanning more than one stage in early pregnancy events [1].

Deferred implantation compromises pregnancy outcome

The complex and tightly regulated dynamicity of pregnancy renders it vulnerable to disruption if the timing of early events veers off course. Significant or subtle aberrations at critical stages during the periimplantation period may immediately terminate pregnancy or perpetuate adverse effects throughout the remaining course [1]. Therefore, late-stage defects could be the result of disturbances incurred at an earlier stage. In fact, gene-deleted mouse models provide credence to the fact that implantation beyond the normal window (deferred implantation) or an aberration in an early event leads to compromised pregnancy outcome.

Adverse ripple effects from deferred implantation were first observed in mice missing *Pla2g4a*, which encodes enzyme cPLA2 α [25]. cPLA2 α generates arachidonic acid from membrane phospholipids for prostaglandin (PG) synthesis via cyclooxygenase-1 (Cox1) or Cox2 encoded by *Ptgs1* and *Ptgs2* respectively. *Pla2g4a* is expressed in the uterus in a similar pattern as *Ptgs2* at the time and site of blastocyst attachment. In *Pla2g4a*^{-/-} mice, implantation timing is deferred beyond the normal window, generating adverse ripple effects reflected in embryo crowding, stunted fetoplacental growth, conjoined placentae, increased resorptions, and reduced litter size [25]. Since cPLA2 α is also expressed in the human endometrium, it is possible that this enzyme plays a similar role in humans [26].

Mice deleted of *Lpar3*, a receptor for lysophosphatidic acid (LPA3), showed similar phenotypes as *Pla2g4a*^{-/-} females [27]. The remarkable similarity in reproductive deficiencies between *Pla2g4a*^{-/-} and *Lpar3*^{-/-} females is attributed to reduced levels of Cox2-derived PGs. This is consistent with the finding that *Ptgs2*^{-/-} mice, depending on the genetic background, also manifest deferred implantation and adverse ripple effects during the subsequent course of development [28]. These findings suggest that the LPA3-cPLA2 α -Cox2 signaling axis is a key determinant for on-time implantation and any aberration in this

signaling pathway would defer the initial phases of implantation, therefore perpetuating adverse effects through the remainder of pregnancy.

Recent studies also show that conditional deletion of two transcription factors, *Klf5* or *Msx1*, in the uterus gives rise to deferred implantation with poor pregnancy outcome [24, 29], although the expression pattern of *Klf5* is different from that of *Msx1*. Interestingly, *Msx1* is primarily expressed in the uterine epithelium primarily on day 4 morning with undetectable expression following blastocyst attachment. In contrast, *Klf5*, a member of the zinc-finger family of transcription factors, is first expressed in the epithelium prior to and during blastocyst attachment and then the expression switches to the stroma with the loss of epithelial expression. The spatiotemporal expression patterns of these two critical transcription factors associated with uterine receptivity and blastocyst attachment timing provide strong support that these temporal events are crucial for the establishment of normal pregnancy, its progression and success. These genetic results corroborate the findings of a physiological study that utilized blastocyst transfer experiments. If blastocysts were transferred into the pseudopregnant uteri of wild-type recipients beyond the anticipated time of implantation, adverse ripple effects were reflected along the course of pregnancy [25].

Deferred implantation as seen in gene-deleted mice is distinct from traditional delayed implantation or embryonic diapause imposed by lactational stimulus or experimentally induced hormonal conditions [25]. Pregnancy progressively deteriorates under conditions for deferred implantation. In contrast, blastocysts undergo dormancy and uteri become quiescent to implantation for extended periods of time under delayed implanting conditions, but maintain the potential to resume implantation when the external conditions are favorable (see below).

Embryonic diapause in mammals

Delayed implantation (embryonic diapause) is a process by which implantation is disengaged from parturition without the loss of blastocyst viability and retention of its ability to initiate implantation, leading to the birth of offspring. Delayed implantation occurs when the embryo attains a state of suspended animation with provisional arrest in blastocyst growth and metabolic activities within a synchronously quiescent uterus. The incidence of embryonic diapause is widespread and has been reported to occur in nearly 100 mammals in seven different taxonomic orders [30, 31]. In most species, the developmental arrest occurs at the blastocyst stage during diapause, and the maternal endocrine milieu that directs embryonic diapause is diverse across species [32].

Embryonic diapause can be classified as facultative and/or obligatory [31] (Figure 3). Facultative diapause is present mostly in rodents and marsupials (wallabies and kangaroos) under maternal stress, including lactation, insufficient nutrition or drinking water, or harsh weather conditions [31]. The length of facultative diapause is thought to synchronize parturition with favorable environmental conditions conducive to neonatal and maternal well-being and survival [32]. Seasonal cues, such as photoperiod, synchronize reproductive events in some mammals, and play an important role in terminating diapause to initiate implantation [33]. The pineal gland-derived hormone melatonin is considered to direct

photoperiodic regulation of diapause via prolactin, since implantation could be induced by treatment with prolactin or dopamine antagonists [34, 35] while dopamine agonists can further prolong diapause [34, 36]. In contrast, obligate diapause occurs during every gestation of a species and is commonly seen in mustelids, bears, seals, and some wallabies [31]. Members within the mustelid family display variable periods of embryonic diapause which can exceed even 350 days in the fisher (*Martes pennanti*), or be as brief as three weeks in the mink (*Mustela vison*) [31, 33].

Delayed implantation normally does not occur in certain species including sheep, hamsters, rabbits, guinea pigs, or pigs. Interestingly, a recent interspecies embryo transfer study found that embryos from non-diapausing sheep endured dormancy when transferred to delayed-implanting mouse uteri. When these dormant blastocysts were transferred back into the donor sheep uterus, they underwent activation and implantation with the birth of apparently normal lambs [37]. The authors argue that embryos from a species normally incapable of undergoing diapause are competent to enter diapause if the uterine environment permits this event; whether this is applicable to other non-diapausing species remains to be tested. The authors also conjectured that all mammals inherently possess the capacity for embryonic diapause if the maternal environment is conducive to such an event. Whether humans and subhuman primates are capable of undergoing delay is under debate [38].

Experimentally induced delayed implantation

In mice and rats, ovariectomy or hypophysectomy on days 4 and 5 of pregnancy, respectively, prevents preimplantation estrogen secretion and results in delayed implantation with blastocysts becoming dormant within the quiescent uterus [10, 39]. This delayed condition can be maintained for many days by continued treatment with P₄. There are reports that dormant blastocysts can survive as long as several weeks in utero, but their viability and developmental competency are inversely correlated with the length of dormancy [40, 41]. A single injection of a small dose of estrogen (as low as 3 ng) can rapidly initiate blastocyst activation and implantation in P₄-primed mice undergoing delayed implantation [10, 42, 43]. In mice, there is a threshold dose of estrogen - higher doses of estrogen close the window of receptivity more rapidly while lower doses of estrogen (<3ng) maintain receptivity for a longer period [44] (Figure 2).

In these species, lactational delay can be induced if a mother is allowed to mate with a male promptly after delivery when post-partum ovulation occurs. Fertilized embryos develop to blastocysts and remain dormant while the mother nurses the newly born pups. The length of delay is tightly adjusted by the number of suckling pups [45]; removal of suckling stimulus rapidly initiates blastocyst activation and implantation. Suckling stimulates prolactin secretion from the pituitary, which in turn suppresses ovarian estrogen secretion. Thus, an appropriate dose of exogenous estrogen can also initiate implantation during lactational delay in mice [46].

Traits of dormant embryos

During diapause, mouse blastocysts are 'metabolically dormant' and slowly expand without much mitotic proliferation [47, 48]. DNA synthesis and cell proliferation cease within 2–3

days of delayed implantation, but a low level of RNA synthesis is still maintained (reviewed [49]). Estrogen injection reactivates dormant blastocysts within 12 h, and implantation resumes by 18 h. A global gene expression analysis in mice identified molecular pathways that distinguish the dormant state of blastocysts from their activated state [50]. This analysis identified major functional categories of altered gene expression levels that included pathways for cell cycle, calcium signaling, adhesion molecules, energy metabolism, and autophagy. HB-EGF is also expressed in activated blastocysts along with their potential ErbB receptors [50], suggesting the importance of HB-EGF signaling in exiting diapause and initiating implantation.

An intriguing feature of dormant blastocysts is the activation of autophagy [41]. Autophagy is a major cellular catabolic pathway by which macromolecules and organelles are recycled [51]. Dormant mouse blastocysts are reported to utilize this pathway to prolong their survival in utero, and inhibition of the autophagy pathway is associated with reduced blastocyst survival and compromised developmental competency in the ovariectomized mouse model of delayed implantation [41]. These findings suggest that a complex molecular network regulates blastocyst dormancy and activation, and that the delayed implantation models in mice and other species could be exploited to better understand the molecular signaling that emanates from the uterus and influences blastocyst dormancy and vice-versa.

Uterine regulation of embryonic diapause

The uterus plays a significant role both in the initiation and termination of delay. In mice, estrogen is an absolute requirement for activation of dormant blastocysts in vivo in a P₄-primed delayed uterus. In contrast, catecholestrogen 4-hydroxyestradiol (4-OH-E₂), a CYP1B1-generated metabolite of estradiol-17 β (E₂), is required for the activation of dormant blastocysts in vitro [52]. It is thought that E₂ prepares the uterus for implantation after P₄-priming, whereas the uterine P₄₅₀ enzyme CYP1B1 converts E₂ into 4-OH-E₂ locally to activate blastocysts. Indeed, CYP1B1 was shown to be expressed in the uterus close to the site of implantation [53]. The synthetic estrogenic compound 2-fluoro-E₂ (2-Fl-E₂) cannot be converted to a catecholestrogens [52, 54]. Intriguingly, 2-Fl-E₂ induced estrogen-responsive gene expression in the uterus but failed to induce implantation in the delayed implanting mouse uterus primed with P₄ [52].

Since P₄ priming is a requirement for embryonic activation and implantation after E₂ exposure in mice, we asked how long the priming effect of P₄ could be sustained and whether the uterus can retain the long term effects of P₄ once primed with this hormone. This hypothesis was tested by identifying the minimum duration of P₄ priming for effective implantation in ovariectomized mice after its withdrawal for several days with subsequent E₂ administration. Indeed, the long-term “memory” effect of P₄ on implantation was sustained and potentiated as compared with those females without P₄ priming, suggesting that the uterus is programmed efficiently by the first exposure to P₄ and for several days retains “P₄ memory” effects conducive to implantation after its withdrawal [55].

While mice with systemic deletion of leukemia inhibitory factor (*Lif*) are infertile due to implantation failure, they also exhibit a few features of delayed implantation, with embryos

assuming morphological characteristics of delay after day 5 [56]. However, this “delay” is not molecularly similar to experimentally induced delayed implantation. For example, *Ptgs2* is normally expressed in the luminal epithelium and underlying stromal cells at the site of the blastocyst with initiation of the attachment reaction. In experimentally induced, estrogen-deprived delayed implantation, *Ptgs2* expression is absent in both the epithelium and stroma, whereas in *Lif*^{-/-} females, *Ptgs2* expression is present and restricted to the epithelium [56]. In this respect, the maternal environment does not truly endorse blastocyst dormancy in *Lif*^{-/-} females. Intriguingly, although estrogen cannot induce implantation in these mice the exogenous administration of LIF can do so. Therefore, it is possible that either LIF or catecholesterogen can activate the blastocyst for implantation perhaps by two different mechanisms, since efficiency of LIF-induced implantation is potentiated by supplementing a small dose of E₂. Whether LIF and catecholesterogen work via distinct pathways or converge onto a common activating pathway remains unknown. LIF is expressed in the uterine glands just prior to and after implantation in the mink, as in the mouse [57, 58]. It would be interesting to see whether LIF or catecholesterogen also plays a role in delayed implantation in other species.

Interestingly, mink embryos in diapause co-cultured with uterine cell lines displayed the capacity to reinitiate embryonic development in vitro, providing evidence that the uterus maintains diapause in this species [59]. Maternal factors responsible for pausing embryonic development across species have not been clearly defined. For example, in the spotted skunk uterus, low levels of protein synthesis were observed during delay and were followed by resumption of protein synthesis a few days prior to implantation [60]. Recent studies in the mink have also identified uterine polyamine synthesis as an initiator for embryonic activation from diapause, since polyamine deprivation in utero can induce reversible arrest of embryonic cell proliferation [61].

Studies in species with obligate delay have shown similar gene expression patterns to those seen in the traditional rodent delayed models. EGF and EGFR are present in the spotted skunk uterus both during delay and post-implantation phases of gestation, and EGF activity is significantly higher during implantation [62]. COX-2 is similarly not present in the uterus during diapause in the mink [63] or skunk [64], but is expressed in the trophoblast and endometrium of both species during implantation.

Persistent expression of *Msx1* in the mouse uterus under various inducers of delayed implantation and its downregulation upon blastocyst activation also suggests a role for *Msx* genes in embryonic diapause [24]. *Msx1* was found to be critical for embryonic diapause since mice with uterine deletion of *Msx1* and *Msx2* revealed compromised blastocyst survival with failure of the uterus to become fully quiescent or to resume activation after termination of delay. Interestingly, persistent uterine expression of *Msx* family members was correlated with diapause in the American mink and Australian tammar wallaby, while in the mouse this expression occurs with rapid downregulation in tandem with impending blastocyst activation and implantation [49]. These results identify *Msx* genes as potential molecular determinants of embryonic diapause across species (Figure 3).

Future reflections

Are periimplantation events and associated uterine gene expression coordinated by a “molecular clock”?

Pregnancy events spanning fertilization to parturition are highly regulated across time and space. Only with correct timing and coordination of embryo-uterine interactions does normal implantation occur to direct decidualization, organogenesis, and placentation as planned to produce healthy offspring. In mice, periimplantation events are well-characterized with changes in distinct and overlapping uterine gene expression patterns occurring on the scale of hours (Figure 1). The mechanism by which these events are synchronized in a timely manner during pregnancy with gene expression is not known, but the level of precision and orchestration of gene expression suggests that a molecular “clock” is involved. Do parallel systems regulate discrete events, or does a central or local clock regulate a sequence/series of events?

There is evidence that the suprachiasmatic nucleus (SCN) in the hypothalamus, which regulates clock gene expression and oscillation, can affect female fertility by altering the levels and patterns of pituitary gonadotropins and ovarian steroid hormones via the hypothalamus-pituitary-ovarian (HPO) axis; however, the manner in which this regulation is executed at the uterine level is far from clear [65, 66]. There are reports that some members of the clock gene family are expressed in the rodent uterus in a rhythmic pattern during the estrous cycle and their absence is detrimental to female fertility, but these studies were performed in nonpregnant uteri or are based on systemic deletion of clock genes, respectively [67–70]. For example, Takahashi's group has shown that mice missing the *Clock1* gene show a surge of dysregulated LH which is reversed by administration of vasopressin in the intracerebroventricular region [71–73]. This would suggest that clock1's role is critical for regulating LH levels, the aberration of which will affect downstream LH targets including ovarian function and associated steroidogenesis, and in turn negatively affect pregnancy outcome. Identification of their cell- and stage-specific expression and critical roles in uterine biology during pregnancy warrants further investigation.

Circadian gene expression was shown to be present in many tissues including the uterus in a cyclical and anti-phasic manner. Based on the analysis of clock mutant mice, orphan nuclear estrogen receptor- α (ERR α) was reported to display a circadian rhythm in the uterus, and this transcription factor was considered as an output gene of the circadian clock oscillator [70]. The expression of clock-regulated genes, such as *Bmal1* and *Rev-erba*, also show diurnal oscillations in the uterus, and there is evidence that rhythmic expression of *Bmal1*, *Cry1*, and *Per2* becomes out of phase in *Clock1* mutant mice [70, 74]. Interestingly, mice with systemic deletion of *Bmal1* showed defective pregnancy outcome due to aberrant P₄ synthesis by the ovarian corpus luteum. However, P₄ administration partially rescued the infertility phenotype [75], and a later report observed reduced embryo maturation to the blastocyst stage within the oviduct [76]. It would be interesting to further evaluate defects in embryo development in *Bmal1* mutant females. Nonetheless, it would be important to determine the stage-specific functions of clock genes, such as *Bmal1*, and their spatiotemporal expression patterns in the uterus during pregnancy.

These results suggest that some adverse effects on female fertility are the reflection of central hormonal dysregulation originating in the SCN. Since embryo implantation can occur in a uterus primed with P₄ and estrogen in the absence of pituitary, ovary, and/or adrenal glands [49, 77], it can be argued that either these steroids locally set up a “molecular clock” for uterine function or the uterus is not under the control of a circadian clock via the SCN. The temporal and cell specific expression patterns of clock genes in the uterus, and their conditional deletions in this tissue, may offer some explanation as to whether clock genes functionally operate at the local level in early pregnancy events. Using delayed implanting mouse models, it would be interesting to see whether P₄ and/or E₂ can regulate clock gene expression in the uterus in a spatiotemporal manner. Overall, a clear link between traditional clock genes and uterine physiology has yet to be demonstrated [65]. Therefore, by combining these methods and utilizing delayed implantation models, it may be possible to determine the regulation of uterine clock gene expression and whether a temporal link exists between these genes and those that control uterine receptivity.

A further question is the sequence of events that precisely mediate timely embryo-uterine interactions through the pre-receptive to receptive to attachment phases prior to the observance of localized vascular permeability (blue bands) at the sites of blastocyst implantation. Although we have identified HBEGF as a molecular link between implantation-competent blastocysts and their attachment within the uterus [11], a careful screening for the molecular players spanning these transitions has not yet been observed or tested due to the relatively difficult nature of the experimental approaches and previously limited technology. Advances in technologies, such as in situ mass spectrometry, live imaging, single cell analysis, and RNASeq may help to identify these changes at more precise times. Using finely-tuned screening processes to determine when and how clock genes are expressed will be informative and necessary in order to identify a potential regulatory circuitry for sequential gene expression.

Delayed implantation models could be powerful approaches to study and manipulate the triggers for embryo implantation, particularly in certain Mustelid species and the tammar wallaby which show prolonged embryonic diapause lasting more than 11 months with the blastocyst suspended in dormancy. How do these dormant embryos defy traditional development, and quite possibly aging? Are there differences in the lifespan of offspring that had undergone shortened versus prolonged diapause in the same species? Mammalian target of rapamycin complex 1 (mTORC1) signaling is implicated in aging and lifespan and its repression by rapamycin has shown anti-aging effects. Does the repression of mTORC1 signaling explain the low metabolic dynamic of diapausing blastocysts and thereby extend and preserve the viability of dormant blastocysts? Since autophagy is involved in the survival of dormant blastocysts under the delayed conditions in mice, and since repression of mTORC1 promotes autophagy, it is possible that these two pathways have important implications in diapausing embryos. Therefore, the study of embryonic diapause under various conditions may provide unique models for studying aging and longevity.

Comparative studies should be pursued more extensively to understand the mechanisms underlying the sequential regulation of pregnancy events since various species use distinct temporal and spatial strategies for implantation. Although diverse hormonal requirements

for diapause and reactivation are seen in different diapausing species, *Msx* transcription factors appear to be a common molecular link in various species [49]. It is a promising candidate since it is transiently expressed during the receptive phase, is repressed just prior to implantation, and does not return in the uterus for the remainder of pregnancy. Indeed, mice with uterine deletion of *Msx* genes failed to sustain experimentally induced delayed implantation and embryo survival [49]. Although a molecular interplay is evident in bringing the uterus to the receptive state, how uterine receptivity spontaneously transits to the refractory state is poorly understood; how *Msx* genes are repressed may provide clues to the initiation of the refractory state. Continued studies may help better understand the upstream and downstream targets of *Msx* genes in the regulation of diapause and implantation.

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Highlights

1. Implantation in eutherian mammals is a highly complex process and requires reciprocal communication between the embryo and uterus.
2. Implantation is dynamic and highly orchestrated with distinctive and overlapping expression of many genes.
3. Delayed implantation in different species provides a wealth of information to understand different strategies for implantation.
4. This review focuses on the timing and synchronization of early pregnancy events.

Periimplantation gene expression in the uterus

Day of pregnancy	1	2	3	4	5	6	7	8
Gene				0800-0900h 1600-1800h 2300-2400h				
HBEGF	LE			LE	S			
Msx			LE+GE					
Lif			GE		Stroma			
Gp130/Stat3			LE					
IL-1 β	LE				S			
Cox2	LE				LE+Stroma			
Cox1			LE+GE					
cPLA2 α			LE+GE		Stroma/Decidua			
LPA3			LE	Stroma?				
PPAR δ -RXR					Stroma/Decidua			
Klf5	LE				Stroma/Decidua			
Ihh			LE+GE					
Bmp2					Stroma/Decidua			
FKBP52					Epithelium and Stroma			
Wnt5a	S		LE+Stroma		Stroma/Decidua			
Wnt4					Stroma/Decidua			
SGK1			LE+GE					
Hoxa10/11					Stroma/Decidua			
AR				LE				
Coup-TFII				Stroma				
Hand2					Stroma/Decidua			

Figure 1. A schematic diagram depicting the distinctive and overlapping expression of various transcription factors, morphogens, cytokines and signaling molecules around the time of implantation in the mouse uterus

Key signaling pathways for uterine receptivity, implantation, and decidualization in the context of cell types and temporal expression. BMP2, bone morphogenetic protein 2; cPLA2 α , cytosolic phospholipase A2 α ; COUP-TFII, chicken ovalbumin upstream promoter transcription factor-2; Cox1, cyclooxygenase-1; Cox2, cyclooxygenase-2; gp130, glycoprotein 130; Hand2, heart- and neural crest derivatives-expressed protein 2; HB-EGF, heparin-binding epidermal growth factor-like growth factor; Hoxa10/11, homeobox A10/11; IHH, Indian hedgehog; KLF5, Kruppel-like factor 5; LIF, leukemia inhibitory factor; LPA3, lysophosphatidic acid receptor 3; MSX1, Muscle segment homeobox 1; PPAR- δ , peroxisome proliferators-activating receptor δ ; RXR, retinoid X receptor; SGK1, serum- and glucocorticoid-inducible kinase 1; STAT3, signal transducer and activator of transcription 3; Wnt4/5a, Wntless-Type MMTV integration site family members 4/5a. LE, luminal epithelium (blue); GE, glandular epithelium (yellow); LE + GE (green); S, stroma (pink); LE + stroma (purple).

Figure 2. A potential scheme showing threshold of estrogen levels for the window of receptivity in the P₄-primed uterus

While estrogen at low levels below a threshold extends the window of uterine receptivity for implantation, levels above the threshold rapidly close this window, leading the uterus into a refractory state (Adapted from [43]).

Figure 3. Schematic of regulation of diapause by *MSX* in mouse, mink and tammar wallaby Despite variation in the proximate stimuli and endocrine milieu to induce diapause in these species, the diverse pathways perhaps converge to work through *MSX* in the uterus, which regulates dormancy of blastocyst in diapause. Notably, blastocysts with an inner cell mass each are present in eutherian mammals but not in marsupials. Genetic and molecular studies in mice have shown inability of uteri to undergo true delay in the absence of uterine *Msx* genes and maintain blastocyst viability, potentially via upregulation of *Wnt5a*. Prolactin (PRL), progesterone (P₄) and estradiol-17 β (E₂). Larger font size indicates higher levels of hormones (Adapted from [48]).