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REVIEW

Embryo implantation: A time for recalling and forwarding

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The success of embryo implantation is a critical step towards further embryo development and pregnancy outcome. The observations and investigations on embryo implantation have been over a century. A huge body of knowledge has been accumulated in anatomy, histology, ultrastructure and hormonal regulation; as well as recently in depth information about molecular signaling pathways got from studies of genomic wide gene screening and specific gene deletion. The knowledge from basic research has also substantially helped to initiate and improve the Artificial Reproductive Technology (ART) in clinical applications. Now we've known that the normal embryo implantation involves the embryo's development into an implantation-competent blastocyst and the synchronized transformation of uteri into a receptive stage. The interdependent relationship between the blastocyst and uterus involves complicated hormonal regulation and local paracrine, juxtacrine interactions. In this paper, we review some important historical findings regarding uterine receptivity and blastocyst activation, as well as some less discussed topics such as embryo spacing, embryo orientation. Further understandings on detailed mechanisms during the process of embryo implantation will help cure women infertility as well as develop new generation of non-steroids contraceptives.

embryo implantation, uterine receptivity, blastocyst activation, research history

PHYSIOLOGY

Pregnancy loss is a common pathological condition in human pregnancy, which is often seeded from early pregnancy around embryo implantation. The infertility issue is now drawing increasing public attention due to its worldwide social and economic impact. While the world is facing increasing population, there are still about 15% couples who could not have their children because of infertility. During the past decades, the development and clinical application of *in vitro* fertilization and embryo transfer (IVF-ET) have conquered many infertility problems and made new hopes for thousands of infertile couples. However, the implantation rate after embryo transfer is still disappointingly low (~30%), most possibly because the transferred embryo is not synchronized with the development of uterus (or the uterus is in the nonreceptive stage)[1]. Recently, evidence from both animal models and clinical survey supports a novel concept that the success of embryo implantation could no longer be simply judged by "implantation or not implantation". It is now recognized that the timing of implantation is also crucial to the ongoing embryo development and final pregnancy outcome. A short delay of implantation beyond the normal implantation window would result in subsequently increased pregnancy loss[2–5]. It is the quality of embryo implantation that determines the quality of ongoing pregnancy and fetal development. In this regard, in depth researches about detailed molecular mechanisms during embryo implantation are necessary to help us conquer two worldwide problems, infertility and development of novel contraceptives.

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The normal embryo implantation requires the embryo's development into an implantation-competent blastocyst and the synchronized transformation of uteri into a receptive stage. These two interdependent processes involve complicated hormonal regulation as well as local interactions between the embryo and endometrium. To date, we have been familiar with the morphological and cellular changes during embryo implantation. Also, based on recent technological advances in the bio-medical field, we could now get easy access to the database about genome wide screening information regarding the implantation process. Moreover, the increasing number of knockout mice models with implantation defects has provided us valuable information to understand the molecular events during this process. However, to unravel the very details of embryo implantation, it is also necessary to have a comprehensive understanding of the basic knowledge and research history of this field, as well as the many comparative aspects regarding different species. So, recalling the field regarding some important milestones seems necessary and could possibly open new research directions for this old yet still mysterious and fascinating field. This is what this short review wants to serve, to recall the past (primarily from studies of mice and rats) and mention some unsolved problems as well as some comparative issues for human pregnancy.

1 The concept of "implantation window"

The understanding and conception of "implantation window" were established by performing embryo transfer experiments. Here we would first introduce a commonly used term, "pseudopregnancy". Pseudopregnancy is induced by mating a female mouse with a sterile male (vasectomized male). During the first four days (morning finding of the vaginal plug is designated as Day 1) of pseudopregnancy, the hormonal environment of the uterus is similar with that of normal pregnancy, and the uterine sensitivity to embryo implantation is also similar. If Day 4 blastocysts were flushed out and transferred into the uteri of Day 4 pseudopregnant mice (synchronous transfer), embryo implantation would happen. By performing asynchronous transfer experiments, it was demonstrated that if older embryos were transferred into younger uteri (take mice for example, the Day 4 embryos were transferred into the Day 3 pseudopregnant uteri), the blastocysts would wait in the uteri and implantation could happen at similar time as synchronous transfer. However, if younger embryos were transferred into older uteri which had entered the receptive stage, the uteri would not wait for the embryo to develop into the blastocyst, but process into the refractory stage, and the embryo implantation could not happen[6–8]. The asynchronous embryo transfer experiments tell us: (i) The uterus must enter the receptive stage to initiate implantation; (ii) when the uterus enters the receptive stage, only blastocyst (not younger forms of embryos) could be accepted by the uterus; (iii) the blastocyst could wait for the uterus to become receptive but the uterus will not wait for the embryo. Usually, the time period that the uterus is receptive is called the "window" of implantation, after which the uterus enters the nonreceptive stage and becomes indifferent or hostile to the embryos[8,9]. In a pseudopregnant mouse, the uterus is receptive on Day 4 of pregnancy, turns nonreceptive during Day 5 and becomes completely refractory by the morning of Day 6[4,9]. If Day 4 blastocysts were transferred into Day 5 pseudopregnant uteri, embryo implantation could be initiated, but would lead to a significantly increased miscarriage rate and retarded embryo growth at mid-gestation. This has demonstrated that the correct timing of embryo implantation is crucial to subsequent embryo development and overall pregnancy[4]. This conclusion is further strengthened by knockout mice models with aberrantly delayed timing of embryo implantation and well designed clinical surveys[2,4,5]. In normal situations, the embryo and the uterus could synchronously develop into the implantation-competent stage in a relatively independent manner (interdependence is also implicated due to recent evidence, see later part). The following paragraphs will try to describe how the uterus and embryo are regulated to prepare for implantation respectively.

2 Hormonal regulation of uterine receptivity in a historical view

2.1 Hormonal patterns during periimplantation

For most mammalian species, implantation happens in a relatively fixed time frame after ovulation when the corpus luteum is functionally formed. As for humans, it is around the time of the luteal phase during the menstrual cycle, and for mice and rats, it is in the dioestrus phase of the estrus cycle[10]. Both the estrus cycle and the menstrual cycle are tightly regulated by hormone fluctuation. Indeed, any discussions related to embryo implantation must be started with hormone regulation,

because it is the master regulator of all the down-stream events[10]. By the 1970s, reproductive biologists had comprehensively studied the hormonal secretion and regulations for embryo implantation, and made the blueprint of what we learned today.

Regarding hormone secretion patterns around embryo implantation, we now know that it consists of three major periods in mice. (i) There is an estrogen surge before estrus, which is now called preoestrus estrogen (follicular stage in human menstrual cycle). (ii) After ovulation (also the time of mating), both estrogen and progesterone are very low (Day 2 of pregnancy). (iii) Progesterone is secreted with the formation of corpus luteum since Day 3 of pregnancy. On the morning of Day 4, a small surge of estrogen is secreted, which is now called luteal phase estrogen[10,11] (Figure 1). Following these hormonal patterns, embryo implantation is initiated at midnight of Day 4 (around 24:00). These hormonal patterns are crucial for the onset of embryo implantation, and we could manually control the uterine receptivity with exogenous hormones according to the patterns described[12,13].

2.2 Some milestones about hormonal regulation for uterine receptivity

The following discussions about hormonal regulation for embryo implantation are primarily based on the knowledge from mice and rats. Firstly, the progesterone's role for embryo implantation has long been well established[14,15]. For most mammalian species, the requirement of progesterone for embryo implantation is absolutely necessary. Recent knockout mouse models relating progesterone signal also strengthened the notion that embryo implantation could not happen without progesterone and its downstream signaling[16,17].

The role of estrogen secretion before embryo implantation was recognized much later and has been equivocal for quite a long time regarding its necessity for embryo implantation. First, we need to introduce the process of uterine decidualization which is tightly related to embryo implantation. Shortly after the initiation of embryo implantation, the stroma cells around the embryo attachment site begin to proliferate and differentiate into distinct cells (decidual cells), also with extensive angiogenesis within the region of decidualization[18]. By Day 6 of pregnancy, the implantation site could be visualized as a small tissue swelling due to the progress of decidualization. The decidualization process is proposed to provide nutrition to the developing embryo before placentation, and to protect the embryo from immune attack[18–20]. Indeed, before the invention of blue dye method (Figure 2) (Psychoyos A. 1960), the visible decidual swelling was taken for a long time as a sign to check the uterine receptivity for implantation. Early in 1908, Loeb found that at the predicted time of embryo implantation, a physical trauma in the guinea pig uterus could initiate the process of decidualization, which is very similar to that of normal embryo implantation[10]. Later, similar findings were repeated in other rodents such as rats and mice[10]. As in the early days, it was believed that this trauma-induced decidualization was the same to that induced by embryo regarding uterine sensitivity, so this method is widely used to study related factors for uterine receptivity. Latter experiment further found that, in ovariectomized mice and rats, progesterone alone could be enough for induction of decidualization by physical trauma. This has led to the notion that no other hormones were involved in implantation.

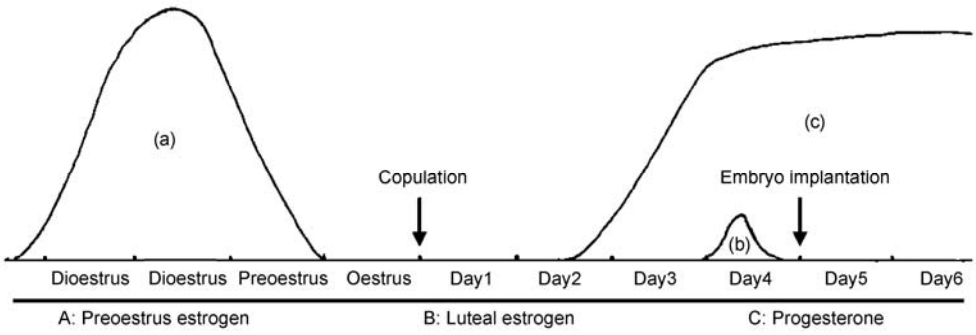

Figure 1 Hormone patterns around periimplantation in mice. Adapted from refs. [10,11].

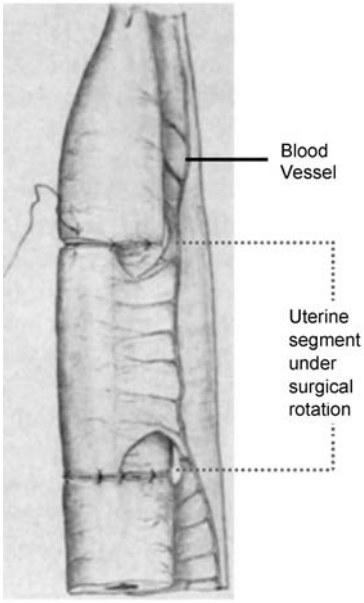

Figure 2 Illustration of surgical rotation of the uterine segment. The major mesometrial blood vessels are intact. Cited from ref. [77].

However, another phenomenon in mice and rats cast doubts on the above conclusion. It is known that if mating happens during lactation, the embryos cannot be implanted normally but stay dormant within the uterus if the newborns are allowed to suckle[10], and if more youngs are allowed to suckle, the implantation would be further postponed. Under this condition, the mother has normal corpus luteum in the ovary (secreting progesterone), and decidualization could also be induced by the physical trauma, which indicates that the uterus is indeed under progesterone priming. Why does the embryo implantation not happen? This phenomenon suggests that there might be another hormone that is necessary for the initiation of normal embryo implantation. This also indicates that the decidualization process induced by the embryo and physical trauma is not the same.

The suspicion of another implantation-related hormone was later proved, as Krehbiel (1941) showed that during delayed implantation in lactating rats, a small does of estrogen could initiate embryo implantation[10]. This finding was also proved in mice[21] and led to the hypothesis that during normal implantation, a small amount of estrogen might be secreted to sensitize the uterus. In addition, it might be the absence of estrogen that caused the delayed implantation during post-parturition lactation. Later experiments have provided more direct evidence to support this hypothesis[10]. Take mice as an example. The mice were ovariectomized at different time intervals before normal implantation, and given exogenous progesterone, then the implantation status was checked. The results clearly showed that the implantation outcome depended on the timing of ovariectomy. If the ovaries were removed after a specific time (Day 4 morning), implantation could happen. However, if the ovariectomy was processed before that time, implantation could not happen. Under this condition, the embryo will lie dormant in the uterus, and if a small amount exogenous estrogen was given, implantation could be reinitiated soon. These experiments demonstrated two important facts: (1) Estrogen is necessary for normal onset of embryo implantation; (2) before implantation, the estrogen secretion is on Day 4 morning (it was defined as luteal phase estrogen after later accurate hormone measurements developed). Moreover, it should be noticed that this manually developed model of delayed embryo implantation is similar to that observed in lactating mice. Indeed, this delayed implantation model and further derived experiments by embryo transfer into ovariectomized pseudopregnant uterus are now widely used as a powerful system to decipher the relative participation of uterus and embryo during implantation (as shown later in sections of blastocyst activation). In summary, these studies lead to the notion that normal initiation of implantation involves both the secretion of progesterone and a small surge of estrogen. These discoveries have made an overall blue print of what we have learned today regarding hormonal regulation for implantation. Recently, further discoveries were made that before implantation, the uterus is very sensitive to the dose of estrogen. While a low dose of estrogen (3 ng) prepares the uterus into the receptive stage and keeps for a longer time, a higher dose of estrogen (25 ng) quickly closes the implantation window[22]. Similar conclusion was also acquired by using oil induced decidualization[23]. Recently, it has been reported that uterine *de novo* estrogen synthesis is also necessary for the process of normal decidualization[24].

In addition to the critical role of luteal phase estrogen, the secretion of preoestrus estrogen has also been extensively studied for uterine preparation of implantation. It is now generally accepted that this phase of estrogen is to maximize the uterine sensitivity at the time of implantation[25]. This phase of estrogen is now routinely used to achieve uterine receptivity by exogenous hormone regimen[13].

2.3 Blastocyst derived estrogen initiates implantation?

It should be recognized that although estrogen secreted before implantation is critical to mice and rats, it is not a universal law for all other mammalian species. For example, in the golden hamster and the guinea-pig, the initiation of embryo implantation solely requires progesterone but not ovarian estrogen. Because of this, delayed implantation could not be induced in these species by ovaricotomy before implantation. For these species, an important hypothesis is that the blastocyst could compose estrogen to initiate the implantation[26]. There has been evidence that the hamster blastocyst shows the presence of aromatase which is responsible for the estrogen conversion[27] (It has been demonstrated that the mouse blastocyst does not express the aromatase gene Cyp19[28]). As for humans, there has not been enough evidence to support the requirement of estrogen for embryo implantation, whether human blastocyst is a source of estrogen for implantation is also not identified.

2.4 About the artificially induced decidualization

Since it has been recognized that the physical trauma induced decidualization is different from that of normal implantation regarding hormonal dependence, later a better method of intrauterine oil infusion was found to induce artificial decidualization due to its similar hormone requirement[10]. Indeed, other stimuli such as air bubbles could also induce decidualization[29,30]. Because of the relative convenience of oil infusion, it now becomes the most widely used method to induce artificial decidualization[12]. However, several recent investigations have indicated that although the oil induced decidualization was similar to that of the embryo, the underlying mechanisms were quite different in downstream gene regulation. Also, there have been reports suggesting that stimuli induced decidualization was quite different regarding decidual zone permeability[31–34].

2.5 Implantation related molecules and signaling pathway-clues from knockout mice models

In the past two decades, more and more knockout mice were generated and have provided new insights into the process of embryo implantation and decidualization. For example, the cytokine family member Lif, IL-11[35–37], prostaglandin signal related members cPLA2α, COX-2, PPARδ[4,38–40], lysophospholipid signal member LPA3 receptor[5], the progesterone receptor (PR) and its co-chaperone FKBP52, growth factor related genes HB-EGF, Areg[41], angiogenesis related genes[42,43] and some developmental genes such as Ihh, BMP2, Wnt4[44–46] are all actively involved in the process of embryo implantation and decidualization. Here we will not introduce these pathways in details. Readers who interested in these contents could refer to other related reviews in this field[11,18,47–49]. Notably, here we should mention that previously genome wide deletion of many developmental genes resulted in embryo lethality, thus precluded the study of these genes in implantation. Recently developed PR-Cre-transgenic mice bypassed such disadvantages and have been used to conditionally delete these genes in uterine cells. By cross breeding the PR-Cre and the gene-loxp mouse, it is now possible to explore the roles of many developmental genes such as Ihh, BMP2 during the process of embryo implantation[44,45]. The wide use of Cre-Loxp transgenic mouse models in the future will undoubtedly provide a good strategy to further explore the roles of many previously inaccessible genes during embryo implantation.

3 Blastocyst activation for embryo implantation and its molecular mechanisms

3.1 Blastocyst activation and its hormonal regulation

After the establishment of hormonal regulation of uterine receptivity, it was thought that the uterine receptivity was the major determinant of "implantation window", while the blastocysts' state has not drawn much attention. Until 1993, by manipulating a delayed implantation model, the Dey, SK lab found that under delayed implantation, the pseudopregnant recipient mice showed expanded implantation window if they were transferred with activated blastocysts compared with dormant blastocysts. (Dormant blastocyst was obtained from the uterus of delayed implantation. And after injection of estrogen, the blastocyst was quickly activated)[9]. This experiment has for the first time showed that the state of blastocyst is also crucial during normal implantation, creating the concept that "blastocyst activation" is a necessary step towards successful implantation. Since the blastocyst activation happened soon after the injection of estrogen, it was first assumed that the process of embryo activation was also induced by estrogen. later is was found that the estrogen receptor was indeed located

in the blastocyst[50]. However, further experiments demonstrated that the primary estrogen (E_2) is not a source for blastocyst activation, it is the catechol metabolite of E_2(4-OH-E_2) (which is converted by CYP1B1 expressed in the uterus) that activates dormant blastocysts[51]. To demonstrate the differential effects of primary estrogen and catecholestrogen on uterus and blastocysts, Dey. SK and colleagues designed a classic experiment by using an estrogen derivant two-fluoroestradiol-17b (2-Fl-E_2). 2-Fl-E_2 is a potent estrogen but can't be converted to catecholestrogen due to structure specificity. 2-Fl-E_2 could induce uterine changes similar to that of E_2, but not initiate embryo implantation in the delayed implantation model although it indeed prepared the uterus into the receptive stage. Further experiment using embryo transfer into delayed pseudo-pregnant uterus showed that implantation could happen only after the dormant embryo cultured with 4-OH-E_2 but not E_2[51]. These experiments clearly demonstrate that during mouse embryo implantation, it is the catechol form of estrogen (4-OH-E_2) that prepares the blastocyst into the implantation competent stage, while the primary estrogen E_2 prepares the uterus into receptive state. The successful implantation only happens when both the embryo and uterus are well prepared. However, it should also be mentioned that although it is now believed that 4-OH-E_2 is the primary source for blastocyst activation, we still do not know its specific receptor, and the direct underlying signaling pathway is also unclear.

3.2 Currently known signal pathways for "blastocyst activation"

Recent study using microarray analysis has identified that blastocysts at dormant or activated states showed different gene expression profiles. The altered genes include cell cycle, cell signaling, and energy metabolic pathways, suggesting that the blastocyst activation involves dynamic transcriptional events[52]. Also, some genes are confirmed to show changed expressions at protein level. For example, HB-EGF is up-regulated[52], and the endocanabinoid receptor CB1 is downregulated after blastocyst activation[53]. Here it should be mentioned that HB-EGF is actually the earliest specific biomarker detected in the blastocyst before embryo implantation[54]. The HB-EGF expression in the blastocyst soon induces epithelial HB-EGF expression at the site of blastocyst apposition, forming a "self-induction loop" as a molecular bridge linking the blastocyst and the uterus so as to initiate the onset of implantation[52]. This notion has been further strengthened by the uterine transfer of blastocyst-sized beads soaked with HB-EGF. The results showed that HB-EGF soaked beads could induce similar implantation events such as blue dye reaction, early COX-2 induction at the implantation sites[48,55]. Moreover, it has been indicated that the HB-EGF receptor family ErbB1-4 are all expressed in the blastocyst. Among these receptors, ErbB1 and ErbB4 have been showed to participate in embryo activation[56,57]. Especially, the shift expression of ErbB4 from cytoplasm to membrane location was thought to indicate the activation of blastocysts. Further functional study also showed that ErbB4 is the most important receptor in mediating HB-EGF signaling in the blastocyst[57,58]. Recent genome wide deletion and uterine specific deletion of HB-EGF both showed defects of embryo implantation, and it was demonstrated that the partially preserved embryo implantation is compensated by another member of EGF family Areg[41,59]. In summary, it is now widely accepted that the EGF signaling is important for blastocyst activation and early embryo-uterine crosstalk.

Endocanabinoid system was recently discovered as an important signal regulating various aspects of early embryo-maternal interactions. The endocanabinoid anandamide and its receptor CB1 were demonstrated as an important system synchronizing the development of uterine receptivity and blastocyst competency for implantation. The level of anandamide and its receptor CB1 were simultaneously down regulated with the acquirement of uterine receptivity and blastocyst activation, and conversely up regulated in the nonreceptive uterus and dormant blastocysts[53,60]. Further experiments found that anandamide, at lower concentration, induced blastocyst activation by mediating MAPK signaling, while at higher concentration, it inhibited blastocyst competency for implantation by inhibiting Ca^{2+} channel activity[53,61].

Most recently, it was found that Wnt signaling is another important pathway for blastocyst activation[62]. By using selective inhibitor of β-catenin-TCF/LEF pathway and adenovirus mediated overexpression of DKK1 (Wnt-β-catenin pathway inhibitor), it was showed that the inhibition of canonical Wnt signaling didn't adversely affect early embryo development, but significantly inhibited the blastocyst's ability to acquire implantation competency. Using delayed implantation model and embryo transfer, it has been further demonstrated that the blocking of wnt signaling interrupted the process of normal blastocyst activation[62].

Anyway, there is still very limited information about the detailed mechanisms of blastocyst activation. This is partly due to the difficulty to get enough samples for comprehensive analysis. With the development of micro-proteomics, it is now possible to get more valuable information from large scale gene/protein screening. Equipped with these advanced technology, coupled with classic methods such as embryo transfer and delayed implantation model, it is hopeful to get more detailed mechanisms about blastocyst activation in the near future.

4 Other aspects during embryo implantation

4.1 About embryo induced uterine receptivity

Current belief about uterine sensitivity for implantation is that the uterine receptivity automatically occurs and recedes independent of the embryo, which is mainly regulated by timely release of ovarian progesterone and estrogen. There have been very few discussions about the embryo's influence on endometrium as to change the course of uterine receptivity. Indeed, some asynchronous embryo transfer experiments have suggested that the normal timing of implantation could be changed (come in advance) by the advancely developed embryos, suggesting a role of embryonic regulation on uterine receptivity[63]. Evidence from other *in vitro* and *in vivo* models also suggested that the embryos could to some extent extend the receptivity of uterus[64,65]. More importantly, recent evidence from clinical IVF-ET practices showed interesting results that it seems the implantation of each embryo facilitates the chance of remaining embryo to implant[66,67], pointing to a new concept as "embryo induced uterine receptivity". However, the notion of embryonic regulation of uterine receptivity still lacks direct evidence, and the underlying mechanisms are almost totally unknown. Given the fact that the blastocyst (embryo) is a bio-active unit with the ability to secret various paracrine/autocrine factors, we could imagine that the embryonic regulation of uterine receptivity is at all possible, and this might become a new direction for future studies in embryo implantation.

4.2 Embryo location within the uterine lumen: embryo spacing and embryo orientation

(i) Embryo spacing. In the polyovular species such as rabbits, rats and mice, before the start of implantation, the embryos within the uterus tend to be equally spaced with each other along the uterine horn (Figure 3). This seems to help prevent the overcrowding and preclude

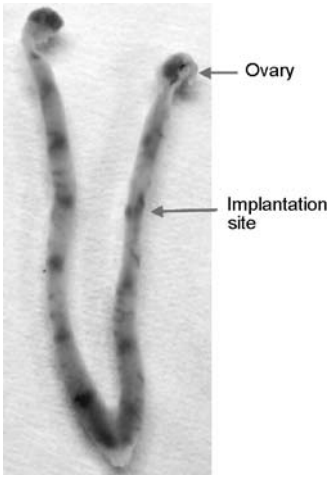

Figure 3 Illustration of embryo spacing in mice. 0.1 mL blue dye was injected intravenously 10 min before sacrifice of mice. Each blue band showed an implantation site due to increased vascular permeability. Note that each implantation site tends to space evenly along the uterine horns.

the possibility of consequent embryo loss. The phenomenon of embryo spacing has been found for more than a century, however, there is still very limited information about the underling mechanisms. In the early days, Mossman (1937) speculated that after entering the uterus, every implanted embryo formed a refractory zone around its position and made it impossible for other embryos getting close[68]. However, latter experiments by McLaren et al. suggested that this was not the truth; because it seemed that every uterine segment could serve as a potential implantation site. And then it was postulated that the observed embryo spacing was simply caused by some kind of uterine movement[69]. Later on, by series longitudinal sections at different time points before implantation, it was described that after the embryos entered the uterine horn on Day 4 morning, they tended to firstly gather together in the middle of the horn, then gradually get separated bidirectionally[70]. As for the molecular mechanisms of embryo spacing, it has long been found that the disturbance of prostaglandin signaling could cause aberrant embryo spacing, leading to embryo crowding[71,72]. Recently, it was found that cPLA2α, an enzyme providing upper sources for prostaglandin composition, once deleted, would cause aberrant timing of embryo implantation as well as disrupted

embryo spacing[4]. Moreover, the deletion of lysophospholipid receptor LPA3 caused similar results observed in cPLA2α knockout mice, LPA3 was demonstrated to intercross with prostaglandin signal and its deletion resulted in decreased COX-2 level and local prostaglandin production[5]. Further investigation demonstrated that LPA3 knockout mice showed decreased uterine contractility, which might be responsible for aberrant embryo spacing[73]. However, it should be mentioned that administration of exogenous prostaglandins to either cPLA2α or LPA3 knockout mice could not rescue the aberrant embryo spacing[4,5], suggesting that there is still a wide knowledge gap in the detailed process of how PG signaling regulating embryo spacing. Besides PG signaling, there is also sparse literature reported that some muscle relaxant drugs or disturbed hormone environment would cause aberrant embryo spacing[74–76]. Furthermore, it is also possible that the embryos could release certain kinds of factors "dictating" the on-time and on-site movements of uterus so as to get appropriate intrauterine location. These situations still need further careful examinations in well designed physiological conditions.

(ii) Embryo orientation. Comparing with the embryo spacing, the phenomenon of embryo orientation is a more difficult problem. Mossman (1937) described that for rodents like mice and rats, besides the phenomenon of embryo spacing, for each implanted embryo, the position of implantation site constantly showed an antimesometrial orientation in the lumen, with the ICM (inner cell mass) towards the mesometrial pole[68] (Figure 4). So, here we can see that embryo orientation actually contains two events: (1) About the location of embryo as a whole; (2) about the ICM's specific orientation. For the first event, it seems that uterus is the major control, because even if the embryo was replaced by other objects such as glass beads and air, they all ended up with a location in the antimesometrial pole of the lumen[52]. In the early days, scholars postulated that the antimesometrial location of the embryo was caused by earth gravity[77]. However, a later delicate experiment proved this wrong. In that experiment, a part of the rat uterine segment was surgically inverted regarding the mesometrial polarities (Figure 2), then the rats were allowed to get pregnancy and the implantation sites were examined. It was surprisingly found that all implantation sites, whether at the intact or the inverted segments, were constantly located at the antimesometrial pole re-

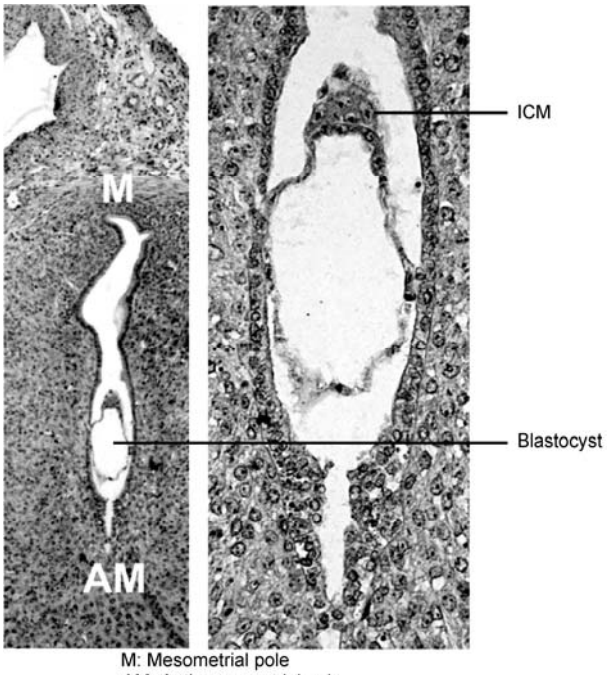

Figure 4 Illustration of embryo orientation. Left, The blastocyst is implanted in the anti-mesometrial pole of uterine lumen. Right, The orientation of ICM is towards the uterine mesometrial pole. ICM, Inner Cell Mass.

ferred to the uterus[77]. This experiment ruled out the role of earth gravity in embryo orientation, and suggested that this process was programmed by intrinsic factors of the uterus. Possibly, this process is related to the closure of the uterine lumen before embryo implantation. However, the underlying mechanisms are largely unexplored. PG signal mediated stroma edema might take part in this process[78]. As for the second phenomenon, the ICM's orientation is much more complicated. Currently, there has been even no well established hypothesis about its underlying mechanisms. Some scholars suggested that this might involve ICM migration after initial attachment and some polarity specific gene expressions[79]. It should be mentioned that in human beings, the orientation of ICM at implantation is towards the uterine wall, which is different from that of mice and rats. The physiological significance of embryo orientation might be maximally adapt the embryo to the environment it is posed. Thus in different species, different strategies are utilized. Just as Richard Assheton (1895) said when describing the phenomenon in rabbits: It is probably necessary for the development of the rabbit that it should be thus situated. It would be very awkward if the embryo became fixed in any other position. The shape of the uterus at this stage, and the shape of the blastodermic

vesicle at this stage, are so beautifully adapted one to the others to render any other position almost impossible (p.173)[77]. Further unraveling the detailed process and mechanisms of embryo orientation would require developments of more advanced techniques for *in vivo* observation.

It should be emphasized that although previous discussions of embryo spacing and embryo orientation are confined to rodents with multiple implantation sites, we should keep in mind that the very essence of embryo spacing and embryo orientation is actually a process of selective embryo location within the uterus. In fact, in single birth species such as the rehsus monkey, the location and orientation of blastocysts at implantation are also definitely regulated[77]. For human beings, the normal embryo implantation site within the uterus is indeed quite restricted to certain areas of the uterus (fundus of the uterus). Once the implantation happens at aberrant sites, it might cause miscarriage or other pathological conditions such as placenta praevia, cornual pregnancy which would cause severe situations if not properly handled. Moreover, with the increasing application of IVF-ET cycles, it is now recognized that after multiple embryo transfer, more than one implantation sites could be detected within the first trimester. However, during late pregnancy there is only one surviving embryo. This situation is now called the "vanishing twin" syndrome and is drawing increasing worldwide attention, because the survived twin has showed increased postnatal abnormities[80]. It is conceivable that the case of vanishing twin is possibly a result of embryo crowding at implantation, and the later resorption of one twin is due to the failure of competitions in space and nutrient supply, a process similar to that observed in mice with crowded implantation sites[4,5]. In this regard, deciphering the underlying mechanisms of embryo spacing and embryo orientation will help understand normal processes of embryo locating during human implantation, as well as related pathological conditions in spontaneous and IVF-ET pregnancy.

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