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A sequence of events in the uterus prior to implantation in the mouse

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Abstract I reviewed a series of events in the mouse uterus before implantation on Day 4 of pregnancy (the sperm positive day is counted as Day 1). Major events are spacing of embryos along the uterine horns, shedding of the zona pellucida, and closure of the uterine lumen. How subtle they may be, there appear to exist interactions between intrauterine blastocysts and the uterus which is regulated by ovarian steroids. Spacing of embryos along the uterine horn is not random, but they are rather evenly distributed along the entire horn. The mechanism of even distribution of embryos needs clarification, although studies indicate that adrenergic nerve activity, prostaglandins, and other molecules appear to be involved. Shedding of the zona pellucida involves trypsin-like proteinase lysis of the zona. Through the opening created by zona lysis, blastocyst gets out of the zona by repeating expansion-collapse movements. Closure of rat uterine lumen is reported to be the result of absorption of uterine fluid through uterine glands. This needs to be confirmed in other species of rodents. Since these events influence blastocyst implantation, we need more detailed information on their regulatory mechanisms in order to improve the rate of healthy implantation of transferred embryo.

Keywords Embryo spacing · Zona shedding · Luminal closure

Capsule This review paper summarizes a sequence of events that occurs in the mouse uterus on Day 4 of pregnancy.

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Leonov at LHRRB

When our group with Roy O. Greep and John Biggers' group were sharing the 4th floor of the Laboratory of Human Reproduction and Reproductive Biology (LHRRB) building at Harvard Medical School, I heard a rumor that FBI agents were on the floor and they were setting telephone line taps.—It was still the time when the relationship between the U.S. and USSR was not friendly. Soon we learned that Boris Leonov, a Russian scientist, was coming to work in Biggers' laboratory. Leonov was from the All-Union Scientific Institute of Obstetrics and Gynecology, Moscow. He found prostaglandins (PGs) are involved in mammalian embryonic development and came to work with John Biggers. Leonov's work with John demonstrated that endogenous PGs are involved in blastocyst expansion and may play a role in implantation [1].

A series of heavy snow falls buried the Boston area last winter. We also had similar heavy snow falls in the winter of 1977–8. After a very heavy snow fall in that winter I walked along Brookline Avenue to the medical school area to take pictures of the "snowscapes" at Harvard Medical School. While I was taking some pictures on Shattuck Street, I saw Leonov came along and greeted each other, and it was my last chance to see Boris Leonov. Some years after Leonov left LHRRB, Racowsky and Biggers published a paper on PG using mouse and rabbit embryos [33]. They found strong evidence for PG synthesis by rabbit blastocyst, but not by the mouse.

For the celebration issue for John Biggers' 90th birthday and his accomplishment, I have chosen to write a review of the chronological events between pre-implantation embryonic development and implantation, hopefully to fill the gap between John's research area and my research interest area. Although there is a recent very good review on embryo spacing [5], it is hard to find a paper describing the sequence

of events in the uterus during this short period of early pregnancy. PGs play important roles in these events.

Sequence of events on day 4 of mouse pregnancy

Pre-implantation embryo development through the blastocyst stage is almost completed by the time embryos enter the uterus. This happens early in the morning of Day 4 (counting the sperm positive day as Day 1). Implantation of blastocysts takes place in the night of Day 4 in the mouse. Therefore, I will describe here the sequence of events in the mouse uterus on Day 4 of pregnancy, i.e., embryo spacing, shedding of the zona pellucida, and closure of the uterine lumen.

Entrance of embryos into the uterus

In mice fertilized eggs reach the uterus about 72 h after copulation (0:00–2:00 h of Day 4), in the morula or early blastocyst stage [28]. Rat fertilized eggs develop in the oviduct and mostly attain the blastocyst stage and enter the uterus by 6:00 p.m. of day 4 [44]. Although there is a clear difference between the mouse and rat in the time of entrance into the uterus and implantation, I will add some rat data to the mouse information because rather accurate physiological information is available in the rat.

In the mouse Restall and Bindon [34] studied location of embryos in the uterus at 2-h intervals and showed that as the embryos enter the uterine horn, they reach in the middle segment where they were seen to be grouped together around 10:00 h on Day 4 and, in general, spacing begins equally in both directions from the center of each horn. The embryos spread along the length of each horn between 10:00 and 16:00 h on this day. In the rat blastocyst distribution rather precisely takes place between 15:00 and 19:00 h on Day 5 [29]. What is the mechanism for the embryos to get together at the center of the horn? Does it happen in other species of rodents? Why do they gather at the center of the horn first? It is very interesting to find out what regulates the sequence of uterine horn movements for gathering and dispersing of the embryos during this period of time.

Uterine muscle movement

Pusey et al. [29] showed that myometrial activity of the uterus play important role in embryonic spacing because the spacing was disturbed when uterine muscle activity was disturbed by relaxin during early pregnancy in the rat. Since it has been reported that uterine horns elongate before and during implantation in mice [8] and pigs [27], proper timely combination of contraction of the inner circular muscle tissue and elongation of the outer longitudinal muscle tissue is important for proper distribution of embryos along the length of uterine horn.

Legrand et al. [13] showed that a noradrenergic transmission via action on myometrial post-synaptic α_1-adrenoceptors is involved as a regulatory mechanism of uterine motility for distribution and spacing of blastocysts in the rat uterus. Chen et al. [4] injected adrenergic drugs peritoneally to mated mice in the morning and afternoon of Day 4 of pregnancy, and found the treatment resulted in disturbance of normal distribution of implantation sites, and there were crowded implantation sites. As these adrenergic drugs stimulate β_2-adrenergic receptors the treatment appears to have caused transient relaxation of uterine smooth muscles, via suppression of the muscle contractility through canonical cAMP-PKA activation.

Mohamed et al. [18] found expression of Wnt signaling in the uterus at 2 stages during early pregnancy. They found at first wnt/β-catenin at around 15:00 h on Day 4 transient activity in circular smooth muscle cells at regularly-spaced intervals corresponding to blastocysts. The Wnt activity bands require the presence of blastocysts in the horn, suggesting that blastocysts emit signal to express the wnt activity in the smooth muscles. These bands later disappear. Second appearance of wnt/β-catenin is in luminal epithelial cells at antimesometrial side on Day 5. Later the activity spreads throughout the luminal epithelium at implantation sites, but disappeared on Day 6. It is very interesting to find what role Wnt plays in uterine muscle movement and luminal epithelium prior to and during implantation. Recognition of the presence of blastocysts in the lumen by the uterus needs to be clarified. McLaren and Michie [16] transferred albino mouse blastocysts into the uterine lumen of mated native black-eyed host mice before the native embryos come down to the uterus, and examined how these two groups of blastocysts implant. There was not a single one of these 40 such transferred uterine horns where the average position of the native, black-eyed, embryos lower down the horn than that of transferred albino embryos. Thirty out of 40 uterine horns (75 %) showed all native embryos implanted on the oviduct side of the horn and all of the transferred embryos implanted on the cervical side. However the remaining 10 uterine horns showed some mixture of the two groups of embryos suggesting that they gathered (probably at the center of the horn?) and then moved towards both directions as suggested by Restall and Bindon [34].

The epithelium is considered as an important regulator of smooth muscle contraction, and releasing epithelium-derived factors, particularly prostaglandins are considered to play important role in governing smooth muscle contraction [35]. Thus, the presence of a blastocyst in the luminal epithelial prior to be transported the site of implantation may involve embryonic signal transmission to the underlying luminal epithelial cells and this may be transmitted underneath to the circular smooth muscle layer.

Spacing of blastocysts along the uterine horn

As mentioned above mouse embryos enter from the oviduct into the uterus in the morning of Day 4 and spread rather evenly along the uterine horn within several hours. The regulatory mechanism of the even distribution of blastocysts along the uterine horn in polytocous mammals has been the point of interest of many investigators. One of the early investigators was Mossman [19]. He wrote "in a bicornate uterus, as in the pig, the embryos nearer the oviduct are always slightly more advanced in development during the first few weeks than those nearer the cervix", assuming that "the uterus is about equally favorable throughout for attachment", "what seems to happen is that the first embryo suddenly strikes up some sort of a physiological relation with the mucosa, which then renders the immediate neighborhood refractory to any other embryo". "This sphere of physiological influence apparently fades out at a variable distance, depending on species, and so other embryos moving by at length to reach another favorable area where the same process recurs." This Mossman concept was examined closely by McLaren and Michie [16] using the mouse model: first they examined whether implantation is serial as Mossman assumed. They weighed embryos along the uterine horns and found that the average weight of embryos was lowest at the middle and the embryos near the ovary and cervix were heavier than those at the middle, confirming the results obtained by Hashima [9]. Second, they checked overcrowding embryos in one uterine horn by removing one ovary. These embryos implanted and intervals between two implantations decreased. Based on these experiments and other evidence, McLaren and Michie rejected the Mossman theory.

Although the experiments of overcrowded implantations in a rodent uterine horn give an idea that the endometrium of any parts of the horn are equally functional, we have to recognize that there are areas more sensitive than other portions to a systemically given steroid within the same uterine horn. Psychoyos [30] treated impregnated rats with chlorpromazine (daily sc injection from Day 3 through Day 10 to induce delay in implantation). In this experiment Psychoyos demonstrated that a threshold dose of estrogen induces implantation of blastocysts in relatively high sensitive areas of the uterine horn, but not of those in the remaining less-sensitive areas. Those blastocysts that remained unattached did implant later by a second injection of estrogen. The results indicate that one uterine horn is not uniformly sensitive to circulating steroid hormones, and some segments are more sensitive than the other. Another case of un-uniform uterine sensitivity for implantation is seen in parous uterus. In many species of mammals, the utetine segments where the placentas attached in the previous pregnancy remain refractory for the presence of blastocysts in the following pregnancy. This is due to the previous decidualization [21]. Paria et al. [25] confirmed the refractoriness of the previous placental scars (nodules) in the mouse, and showed the refractory segments were covered with thick Muc1, a good indication of refractoriness in this species. We have no information as to whether the muscle movement of these placental nodule segments is same as that in receptive areas.

Since indomethacin, a prostaglandin inhibitor, blocks implantation, delay implantation or reduce the number of implantation in the rat, mouse and hamster [6, 11, 12], prostaglandin plays an important role in implantation. When Wellstead et al., [39] injected indomethacin to mated rats at 9:30 h. and 12:30 h of Day 5 spacing of embryos was disturbed, resulting in many co-joined placentas when examined on Day 16 or Day 22. Ye et al. [42] reported that deficiency of lysophophatidic acids receptor3 (LPA3) resulted in altered spacing of blastocysts and delay in implantation. A similar alteration in spacing was observed in cytosolic phospholipase A2alpha deficient mice [38], PG treatment did rescue delay in implantation, but crowding of embryos was not cured. Ye et al. [43] showed that activation of the thromboxane A2 receptor (TP) partially rescued implantation crowding in LPA3 deficient mice.

In order to obtain information regarding uterine site of prostaglandin action in embryo spacing, Yang et al. [41] studied expression of PG receptors during periimplantaion perios in the mouse, They claim that expression of two types of PG receptors (EP_3 and FP) were primarily localized in the circular muscles, and that uterine contractions that serve for embryo spacing are the actions of the inner circular myometrial layer. Detailed information is referred to the recent review by Chen et al. [5].

Paria et al. [24] impregnated blastocyst-size beads with a variety of growth factors and inserted in the mouse uterine lumen in the morning of Day 4 of pregnancy. They found the beads impregnated with bone morphogenetic protein2 (BMP2) or BMP4 behaved just like blastocyst in spacing and native blastcysts implanted in the uterine horn close to the cervix. BMPs belong to the large transforming growth factor super family and these are secreted proteins. It is awaited to examine how these proteins regulate muscle movement that adjust spacing of implantation sites.

Shedding of the zona pellucida

Loss of the zona pellucida begins late on Day 4, by 23:00 h [22] or mostly takes place between 14:00 and 16:00 h, at the latest by 2:00 h on Day 5 [34]. Thus, blastocyst spacing preceeds the zona shedding and the shedding takes place toward the end of spacing, suggesting the regulatory mechanism of both of these events may involve same factors, probably prostaglandins. Orsini and McLaren [22] stated that "the advent of Pontamine Blue reactivity (increase in capillary

permeability at implantation site) in the uterus coincides in time very closely with the loss of the zona." Although progesterone is necessary for in-time shedding of the zona, and even precocious zona shedding takes place by progesterone in the rat [40], progesterone alone does not usually induce implantation in rats and mice and pre-implantation estrogen increase [31, 45] is also essential for induction of implantation. Shedding of the zona pellucida is usually delayed by about 24 h under the hormonal conditions where implantation is delayed, and when embryos were retained in the oviduct by ligating the uterooviduct junction shedding of the zona was delayed by 12 h. Based on these results Orsini and McLaren [22] found that some uterine factors are involved in hatching of blastocyst from the zona pellucida. Bitton-Casimiri and Psychoyos [2] made a cinematographic observation of rat blastocysts in vitro and found blastocysts keep rhythmical repeating contraction and expansion movement and squeeze themselves out of the zona through a crack.

Mintz [17] considered that some active process is involved in the passage of the blastocyst out of the zona. Bowman and McLaren [3] showed in vitro that mouse zona pellucida is lysed by trypsin or pronase, but not by other enzymes such as collagenase. They thought that zona lysine is secreted by the uterus at the time of implantation. Perona and Wasserman [26] found a limited area of mural trophectoderm of early blastocyst produces strypsin, a trypsin-type proteinase, and suggested that strypsin lysis of overlying region of the zona pellucida result in opening of the zona for blastocyst hatching. Embryonic hatching enzyme strypsin is expressed in endometrial glands during implantation and its expression is under the influence of progesterone [23].

Closure of uterine lumen

McCormack and Greenwald [15] collected peripheral blood between 8:00 am and 9:00 am on each day of pregnant mice and measured progesterone and estradiol by radioimmunoassay. They found a rise in progesterone level on day 3, and continued increase towards mid-gestation. Smith et al. [37] made a very careful study on the hormonal pattern during early pseudopregnancy . They measured the serum concentration of progesterone every 2 h intervals and found progesterone level starts to rise from 11:00 h of Day 2 in the rat. Assuming the progesterone secretion starts increasing from the afternoon of Day2, Psychoyos found out that the uterine endometrium must be exposed to progesterone for at least 48 h and estrogen at the end of the progesterone to render it receptive for blastocyst implantation. The uterus becomes receptive in the evening of Day 4 in the mouse and in the evening of Day5 in the rat. A similar progesterone secretion pattern indeed supports the Psychoyos finding for uterine receptivity. Huet-Hudson and Dey [10] examined how long progesterone priming is needed for establishment of the uterine receptivity in the mouse. They ovariectomized mice and treated with steroids as the receipents of blastocysts transfer and they found just a single injection of 2 mg progesterone is sufficient for induction of implantation with a trigger dose of estrogen. Thus, in the mouse, exposure to progesterone for 24 h is sufficient for induction of the luminal closure. In the rat Shelesnyak [36] found the uterine lumen at 17:00 on Day 5 is narrow and slit-like and the epithelial cells of opposite sides of the lumen were in direct apposition to each other. This is a histological appearance of the luminal closure approximately several hours before the initial stromal cell transformation to decidua.

Quinn et al. [32] examined rat luminal epithelial endocytotic activity to determine if the luminal closure is the result of endocytotic fluid uptake. Because tracer ferritin up take was minimal at the time of uterine closure, they concluded that uterine luminal closure is not induced by luminal epithelial endocytosis. Laser scanning confocal microscopy of the distribution of an extracellular marker within rat uterine glands showed that the endometrial glands change their fluid handling characteristics under different hormonal conditions. Under progesterone dominance, the glands showed a sodium-dependent fluid absorption. This study by Naftalin et al. [20] is the first study that the fluid absorption by uterine glands may provide the mechanism for closure of the uterine lumen and immobilization of the blastcyst necessary for implantation. Filant and Spencer [7] produced mice that lack uterine glands by treating neonatal mice with progesterone from postnatal day 2 through 10. These progesterone-induced uterine gland knockout (PUGKO) mice have luminal epithelium, but no glands. They displayed normal estrous cycle and mating behavior. Free blastocyst in were found in the uterine lumen on Day 5 and 8. Thus implantation failed in uterine gland knockout mice. This result clearly demonstrates that uterine glands are indeed necessary for implantation. However, the authors did not examine if there was closure of uterine lumen in pregnant or speudpregnant PUGKO mice. I believe these PUGKO mice are an ideal model system to investigate uterine fluid absorption by endometrial glands.

Conditions that disturb normal embryo spacing

There are different types of uneven implantations: McLaren and Michie named two implantation sites very closely attached as "close pairs." In experimental conditions we can see implantation sites crowded in a portion of the uterine horn either on the oviductal or cervical end. Analysis of these uneven spacing by normalizing with a specific molecule, may lead to clarification of the mechanisms involved in evenly spaced implantations. There appears to be at least two main factors we have to consider: uterine smooth muscle movement

and closure of uterine lumen. Treatment of early pregnant uterus with muscle relaxing agents (e.g., relaxin, nicotine) tends to result in implantation near the oviduct end. Those uteri which lack proper action of PG-related molecules [e,g,. LPA3 (−/−), cPLA2 (−/−)] tend to crowd embryos toward the cervical end. In case of adrenomedullin deficient mouse [14], we need to examine whether uterine closure may be involved in the skewed implantation.

As to closure of uterine lumen, excess uterine fluid (such as saline injection) might delay the timing of uterine closure while the uterine receptivity process is progressing without delay, thus, a delay in uterine closure might create a discrepancy between availability of blastocysts for close attachment to the luminal epithelium and the receptive endometrium at its peak.

Summary

A series of events that occur in the mouse uterus on Day 4 of pregnancy was summarized in Table 1. Starting with their entrance into the uterus, pre-implantation embryos interact with the uterus each other in order to initiate proper embryo implantation in the uterus. There appear to present two sequences of events: uterine sequence of events and embryonic sequence of events. These two sequences influence each other, and synchronization of these *two sequences of events* may be important for preparation for healthy implantation. Failure of the synchronization may results in uneven spacing of embryos along the uterine horn and delay in uterine luminal closure. The events in the mouse uterus on Day 4 of pregnancy present an ideal model system for obtaining detailed information to improve the success rate of healthy implantation of transferred embryo.

Table 1 Events that occur in the mouse uterus on day 4 of pregnancy

Time on day 4	Events
0:00 h	Embryos enter the uterus
Starting at 10:00 h	Embryos gather at middle uterine segment
14:00 h through 16 h or later	β_2-adrenergic receptor mediated uterine muscle contractions as well as contractions by prostaglandin related molecules, BMP etc. participate in spacing and spreading of embryos along the uterine horn
	Uterine circular smooth muscles transiently express Wnt in response to blastocysts
	Shedding of zona pellucida at the end of spacing
	Increased progesterone induces absorption of uterine fluid through uterine glands, leading to stromal edema, closure of uterine lumen and enclosure of blastocysts in the luminal cleft or pit, resulting in apposition of blastocyst with luminal epithelium

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