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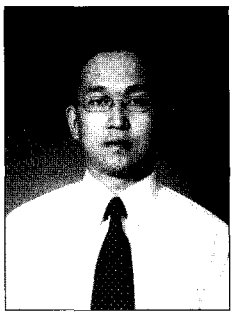

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Molecular Signaling in Uterine Receptivity for Blastocyst Implantation

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Many underlying causes of human infertility have been overcome by using *in vitro* fertilization (IVF) and embryo transfer (ET) techniques. Nevertheless, implantation rates in IVF programs remain disappointingly low despite the transfer of apparently healthy embryos. This suggests that there are problems with the differentiation of the uterus to the receptive state or with asynchronous interactions of blastocysts with nonreceptive uterus leading to implantation failures. Uterine receptivity is defined as the window of limited time when the uterine environment is conducive to support blastocyst growth, attachment reaction, and subsequent events of implantation. Uterine milieu for blastocyst implantation is classified into prereceptive, receptive, and nonreceptive phases. This process is primarily coordinated by ovarian progesterone and estrogen in a spatiotemporal manner. In mice, it has long been believed that implantation occurs only for a limited period (~24 hours) defined as the 'window' of receptivity for implantation. The uterus becomes receptive on day 4 of pregnancy or pseudopregnancy (PSP) with the initiation of the attachment reaction around midnight. These previous works, which examined implantation sites (IS) by the blue dye injection 24 h after ET, suggested that the uterus is receptive for implantation on day 4 and becomes nonreceptive by the next day.

Blastocysts Can Initiate the Attachment Reaction in the Non-receptive Uterus beyond Normal "Window" of Uterine Receptivity: While this belief has been strongly held for a long time, recently we developed an novel concept on uterine receptivity from experiments to characterize implantation phenotypes of mice deficient of enzymes involved in prostaglandin (PG) synthesis, such as cyclooxygenase-2 and cytosolic phospholipase A2 (cPLA2). Characterizing reproductive phenotypes of cPLA2(-/-) mice, we observed an interesting event that the average number of IS on day 5 of pregnancy (0900 h) is smaller than that of litter size at term. Not only was the number of IS remarkably low, but also a large number of unimplanted blastocysts was recovered from all mice examined after flushing their uteri on day 5 of pregnancy. These data led us to hypothesize that blastocysts which failed to implant at the time of implantation window (day 4 midnight) could initiate implantation later. To test our hypothesis, we performed the blue dye injection in the morning of day 6 of pregnancy rather than day 5. Surprisingly, we obtained significantly increased number of IS on day 6 of pregnancy while the size of IS was obviously smaller than that of

wild-type female mice. This demonstrates that blastocysts can implant beyond normal "window" of uterine receptivity.

To rule out the possibility that the deferred implantation in cPLA2(-/-) mice could be primarily caused by sub-optimal uterine environments due to impaired PG signaling, we performed ET with wild-type recipients on day 5 of PSP and examined IS 48 h after ET rather than 24 h. Whereas no IS were visualized by the blue dye method 24 h after ET, distinct IS were obtained from day 5 pseudopregnant recipients 48 h after ET as in day 4 pseudopregnant recipients. This result reinforced our new view on uterine receptivity that the attachment reaction may be delayed when the uterine environment is sub-optimal as in the uteri of cPLA2(-/-) female mice or in the uteri on day 5 pseudopregnant wild-type mice. With this finding, we revisited the definition of "window" of implantation with extensive ET experiments in which ET was performed on later days of PSP. While transferred blastocysts could implant on day 5 of PSP (48%) similar to day 4 (52%), they completely failed to initiate implantation 48 h after ET (0%).

When we investigated possible mechanisms by which uterus become entirely nonreceptive on day 6 of PSP, it was recognized that progesterone (P4) level sharply decreases on day 6 of PSP. Thus, to examine if exogenous P4 supplementation can experimentally sustain uterine receptivity on day 6 of PSP, we injected P4 (2 mg/mouse) daily in the morning (0900 h) from day 5 to day 7 of PSP and ET was performed on day 6. 60% (9/15) of day 6 pseudopregnant recipients showed IS as opposed to 100% (5/5) and 86% (6/7) of days 4 and 5 of pseudopregnant recipients, respectively. However, the number of IS (50/221; 23%) in day 6 pseudopregnant recipients with P4 were much lower than that of day 4 (31/60; 52%) and day 5 (44/92; 48%) pseudopregnant recipients. These results suggest that critical changes in uterine physiology occur in the uterus between days 5 and 6 of PSP and that P4 supplementation alone is insufficient to improve implantation rate.

To characterize alteration of uterine receptivity both at the physiological and molecular levels, we examined decidualization response, cell proliferation, and expression patterns of uterine receptivity markers on days 4-6 of PSP with or without P4 supplementation. As observed in ET, responses to artificial decidualization stimuli were similar in days 4 and 5 of PSP, but we were not able to induce it on day 6. [3H]thymidine incorporation experiments clearly demonstrated that stromal cells undergo proliferation as long as uterus is receptive on days 4 and 5, but not in nonreceptive uterus on day 6. In terms of gene expression, leukaemia inhibitory factor, which is induced in uterine glands prior to implantation on day 4 of pregnancy, was expressed similarly in uterine glands on days 4 and 5 of PSP. However, its expression rapidly decreased and became undetectable on day 6. Consistent with ET results, P4 supplementation partially sustained, at certain extent, characteristics of uterine receptivity in all three experiments performed. Collectively, these results demonstrate that blastocysts can initiate implantation beyond normal "window" of uterine receptivity on day 5 and even on day 6 of PSP in mice.

The Risk of Early Pregnancy Loss in Embryo Implantation with Sub-optimal Uterine Receptivity Is Increased: During the characterization of reproductive phenotypes of cPLA2(-/-) mice, we recognized that the average number of IS examined on day 6 of pregnancy is much higher than that of pups at term. Simply it suggested that some implanted blastocysts in sub-optimal uterus of cPLA2(-/-) mice may not develop normally to term. Thus, we promptly focused on this question that asynchronous interaction of blastocysts with sub-optimal uterine environments leading to deferred implantation could affect postimplantation embryo development. To elucidate the effect of deferred implantation on subsequent developmental processes, we examined the growth and development of the IS on day 12 of pregnancy (midgestational stage). While most of the IS in wild-type or cPLA2 (+/-) mice developed normally, many IS in cPLA2(-/-) mice were smaller and showed signs of resorption. The median weight of day 12 IS or isolated embryos was reduced significantly in cPLA2(-/-) mice when compared with wild-type or cPLA2(+/-) mice. Although there was only a short delay in the timing of implantation, many of the isolated embryos from cPLA2(-/-) mice exhibited retarded growth at varying degrees. Furthermore, defective development of feto-placental unit with hemorrhagic placentas and preponderance of trophoblast giant cells was frequently noted, although decidual defect was not apparent. Our results showed that a transient delay in the attachment reaction produced heterogeneous fetoplacental developmental phenotypes ranging from less severe to markedly retarded growth. Our observation of retarded postimplantation development and demise of embryos was reflected in high embryonic mortality (38% versus 2%) and the reduced number (4.8 ± 0.8 vs 12.2 ± 0.8) of pups delivered at birth by cPLA2(-/-) mice as compared to wild-type mice.

To reinforce that implantation timing affects postimplantation development, we employed ET in wild-type mice once more. Wild-type day 4 blastocysts were transferred into day 4 or day 5 wild-type pseudopregnant mice and IS were examined 8 days later after ET equivalent to day 12 of pregnancy. While resorption and retarded feto-placental growth were frequent in recipients that received ET on day 5, normal development was noted in those on day 4. The number of normal IS was significantly higher in day 4 recipients than in day 5 recipients (18/23 versus 10/42). Next, we compared the length of gestation and the number of pups delivered after ET between day 4 and 5 pseudopregnant mice. It was interesting to note that the length of gestation was similar when counted from the day of ET; 16.4 days and 16.5 days in day 4 and day 5 pseudopregnant recipients, respectively. Although there was one-day delay in initiating implantation in day 5 pseudopregnant recipients, the length of gestation did not reflect this deferral, suggesting that embryonic age after implantation is critical for determining gestation length. However, pregnancy outcome at term was quite different between these two recipient groups. The number of pups born at term was much lower for recipients receiving ET on day 5 (16/130, 12.5%) than those on day 4 (44/168, 26%). It should be recalled that IS examined by the blue dye method 48 h after ET on days 4 and 5 of PSP

was similar (52% vs 48%). This suggests that physiological discrepancy between the uterine receptivity and the embryonic stage causes embryonic death after implantation.

Clinical Implication of Blastocyst Implantation in Sub-optimal Uterine Environments in Mice: Collectively, these results from wild-type mice and cPLA2(-/-) mice clearly demonstrate that timing of implantation is a crucial determinant for normal feto-placental development and pregnancy outcome. It has a major clinical significance, as embryo implantation in sub-optimal uterus in humans beyond the normal 'window' of uterine receptivity (8-10 days postovulation) is associated with higher risk of early pregnancy losses. In other words, synchronized molecular interactions between healthy blastocyst and optimized receptive uterus are essential for successful embryo implantation followed by normal embryogenesis to term both in mice and humans. Our studies suggest that uterine physiology with respect to uterine receptivity for implantation between humans and mice may be more similar than previously understood. With respect to the similarity of uterine receptivity for blastocyst implantation between humans and mice, in-depth research for implantation with mouse models will reveal puzzles of embryo implantation in humans with ethical issues. Recently, we also have shown that estrogen is a critical determinant that specifies the duration of the window of uterine receptivity for implantation in mice. Our findings underscored a possible adverse effect of high level of estrogen induced in controlled ovarian hyperstimulation for human IVF-ET on implantation of transferred human embryos. Currently, we are trying to examine the adverse effect of hyperstimulation of ovarian follicles in superovulation on uterine receptivity for blastocyst in mice.