The complete control of murine pregnancy from embryo implantation to parturition

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Abstract

The ovary is the main secretory source of progestin and estrogen and is indispensable to the maintenance of all events of pregnancy in mice. The purpose of this study was to control all processes of pregnancy in mice, from embryo implantation to parturition, without ovaries. The ovaries were removed before embryo implantation, and a single injection of medroxyprogesterone acetate (MPA) was given. Embryo implantation was induced by leukemia inhibitory factor, which can substitute 17 β -estradiol (E₂). Continuous exposure to E₂ was necessary at mid-pregnancy, when placentation was completed. All mice sustained pregnancy without ovaries before parturition, which was initiated by the removal of E₂ and MPA. Murine pregnancy is a complicated process involving embryo implantation, placentation, and parturition. Complete control of pregnancy was achieved with the simple treatment of MPA and E₂ after induction of embryo implantation. Here, time-dependent events in the uterus during pregnancy could be realized without the ovaries, because the initiation of each event could be stringently controlled by hormonal treatments.

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Introduction

The ovary is the main secretory source of progestin and estrogen and is indispensable to maintenance of pregnancy throughout gestation in many mammals, including cows (Estergreen *et al.* 1967), goats (Meites *et al.* 1951), dogs (Verstegen-Onclin & Verstegen 2008), and/or rabbits (Pickworth & Lamming 1967). On the other hand, an ovariectomy at mid-pregnancy or the later trimester of pregnancy does not disrupt the process of pregnancy in several mammals, including humans (Csapo *et al.* 1972), monkeys (Albrecht *et al.* 2000), guinea pigs (Bland & Donovan 1969), and/or mares (Hinrichs *et al.* 1987), whose progestin and estrogen are supplied by other tissues such as the placenta. In ovariectomized mares, a daily single injection of progesterone is sufficient to establish and maintain pregnancy after embryo transfer (Hinrichs *et al.* 1987). In addition, ovariectomized ferrets cannot sustain pregnancy with any steroid treatment (Wu & Chang 1972). The steroid requirements for pregnancy are dependent on the animal species.

The ovary is absolutely necessary for murine pregnancy (Rubinstein & Forbes 1963). In mice, serum progesterone levels increase with the progress of

pregnancy, reach a peak at gestation day 16 (D16; the first day of pregnancy (D1) is determined as the morning a vaginal plug is observed in a female that had been housed with a male the previous evening), and rapidly decrease before parturition (Murr *et al.* 1974). A transient increase in 17 β -estradiol (E₂) at D4 is essential for embryo implantation (McCormack & Greenwald 1974). Serum E₂ levels increase from mid-pregnancy to term and decline rapidly before delivery (Barkley *et al.* 1977). Ovariectomy at any time during pregnancy results in abortion in mice, even if progesterone is administered.

Here, we attempted to control murine pregnancy completely, from embryo implantation to parturition, using mice that were ovariectomized before the implantation period, with hormonal treatments.

Results

The number of implantation sites at the ninth day of experimental pregnancy day (ED9) was 10.2 ± 4.2 ($n=11$, average $\pm$ s.d.) after injection with leukemia inhibitory factor (LIF). The number of placenta or living fetuses at ED22 was verified in mice, inside which the E₂ implant and medroxyprogesterone acetate (MPA) were

Table 1 Maintenance of pregnancy in ovariectomized mice with hormonal treatments.

Removal of E ₂ (ED17) and MPA (ED20)	No. of pregnant mice	Pregnancy rates (%)	Total no. of placentas (live; ED22)	Birth rates (%)	Total no. of birth (live)	Total no. of MGs (ED24)
–	7	100	60 (53)	0	0 (0)	N/A
+	10	100	N/A	90	43 (36)	110
Normal pregnancy	4	100	N/A	100	58 (58)	69

E₂, the silicone tube containing with E₂; MPA, medroxyprogesterone acetate; MGs, the metrial glands; and N/A, not applicable.

left. All ovariectomized mice ($n=7$) had formed the placenta (8.6 ± 7.2 /head, average $\pm$ s.d.), and 53 of 60 pups (88.3%) were alive (Table 1). Nine of ten mice underwent successful parturition by removal of the silicone tube containing E₂ and MPA (Table 1). One mouse died at ED19, although the pregnancy was sustained. Forty-three pups (4.8 ± 4.0 /head, average $\pm$ s.d.) were delivered, of which 36 (83.7%) were alive (Table 1). The number of the metrial glands (11.0 ± 3.7 /head, average $\pm$ s.d.), which were counted after parturition, was consistent with the number of implantation sites at ED9 (Table 1).

Plasma levels of MPA were almost constant and not high through pregnancy (Fig. 1A) compared to those of progesterone during normal pregnancy (Murr *et al.* 1974). MPA was rapidly declined after ED20, at which MPA was removed for induction of the parturition, and not detectable at ED23 (Fig. 1A). The concentrations of E₂ became slightly higher during ED11 and ED17 after the silicone tube was implanted (Fig. 1B). Elevation of E₂ was observed after parturition (ED24, Fig. 1B).

The mammary glands were sparse among the fat tissue in ovariectomized mice (Fig. 2A) compared to that in the normal pregnant mice (Fig. 2B). Secreted milk was stored in the alveolus of ovariectomized mice, which is occasionally enlarged (Fig. 2A). The alveolar epithelium of these mice is thinner than the control (Fig. 2C and D).

Discussion

Successful pregnancy, from initiation of embryo implantation to parturition, was completely achieved artificially in ovariectomized mice by exogenous treatments according to the experimental procedure. LIF lies downstream of estrogen and is an essential factor of murine embryo implantation (Chen *et al.* 2000). Although female *Lif*-deficient mice are infertile, injection with LIF can recover the implantation process, and embryos are sustained to birth if the mother possesses ovaries (Chen *et al.* 2000). This effect is completely reflected by that of E₂ (Chen *et al.* 2000). The requirement for E₂ at the time of implantation is very low (<10 ng/head; Milligan & Finn 1997) and excess amounts of E₂ disrupt implantation. Therefore, we substituted LIF for E₂ to facilitate implantation control. Our finding suggests that LIF priming at ED6 of

pregnancy and simultaneous administration of progesterone (MPA) can sustain pregnancy at the earliest until ED11. Serum levels of MPA were almost constant after injection (Fig. 1A). This experimental model could be applied to precise analysis of trophoblast differentiation toward completion of placentation and/or vasculogenesis during placentation. Embryo implantation was also induced by other molecules such as cAMP except for E₂ in mice (Holmes & Bergström 1975). Because pregnancy was not sustained with progesterone alone after cAMP-induced implantation, it was not determined whether cAMP was sufficient for complete embryo implantation (Holmes & Bergström 1975). It might be due to failure of sustenance of pregnancy after mid-pregnancy.

Figure 1 Plasma concentrations of medroxyprogesterone acetate (MPA; A) and 17 β -estradiol (E₂; B). No significant difference is observed in MPA from ED5 to ED20. The levels of E₂ are statistically higher at ED11 (b) and ED17 (b) compared to that of ED5 (a). There is significant difference between a and b ($P<0.05$). Average $\pm$ s.e.m. ($n=3-5$). *Under detection. **The number of samples is two.

Figure 2 The mammary gland from ovariectomized mice at ED23 (A and C) and normal pregnant mice at D21 (B and D). The alveolus of the ovariectomized mice is sparse (A and C) compared to the normal alveolus (B and D). Scale bar = 100 μ m (A and B) and 50 μ m (C and D).

In addition to progesterone, estrogen, which lowers progesterone requirements, is necessary to sustain pregnancy in mid-pregnancy, during which placentation is completed in mice (Milligan & Finn 1997). In this study, we used a silicone tube that could gradually release E_2 ($\sim 20\%$ of the total amount of E_2 is released within 1 week; Okada *et al.* 2005), because a high dose of E_2 causes fetal death. The first single injection of MPA and transplantation of the silicone tube containing E_2 were sufficient to maintain placentation and the last trimester of pregnancy. The plasma concentration of E_2 was slightly higher when silicone tube was s.c. inserted (Fig. 1B). These E_2 levels were not inconsistent with that of normal mid-pregnancy, although E_2 was gradually increased toward term in normal pregnancy (Barkley *et al.* 1977). The results show that 84.3% of implantation sites formed the placenta (average implantation sites at ED9; 10.2 vs average placenta at ED22; 8.6). Several implantation sites would have been lost during mid-pregnancy in this study because spontaneous abortions occur during mid-pregnancy, even in a normal pregnancy (Kusakabe *et al.* 2008). This is a brand new model investigating the mechanism of spontaneous abortion during mid-pregnancy.

Parturition was not observed in ovariectomized mice if the silicone tube containing E_2 and MPA was left inside the mother. As the serum levels of both progesterone and E_2 decline before parturition in normal pregnancy (Murr *et al.* 1974, Barkley *et al.* 1977), the decrease in progesterone appears to be essential in parturition. Mice deficient in the prostaglandin receptor that binds to prostaglandin $F_{2\alpha}$ cannot deliver their fetuses because the corpora lutea does not regress, and progesterone levels remain high (Sugimoto *et al.* 1997). In these mice, ovariectomy could induce parturition because of the decline in the sex steroids (Sugimoto *et al.* 1997). Ninety percent of ovariectomized mice underwent successful

parturition by removal of the silicone tube containing E_2 and MPA (Table 1). The time-point of steroid removal was the start point of parturition in this study. This is a very useful model for analysis of the molecular mechanisms of parturition without functioning ovaries. The concentration of plasma E_2 was higher after parturition at ED24 ($n=2$). E_2 derivative, E_2 benzoate used in this study, itself does not have high estrogenic activity as E_2 (Whitman *et al.* 1937). This derivative dissociates benzoate rapidly after administration into the body (Dunn *et al.* 1977). The antibody for E_2 inside the EIA system does not show any cross-reactivity with it. Therefore, the removal of MPA at ED23 might promote dissociation of benzoate very rapidly to produce E_2 .

The live pups were born alive, however, they all died within a day after parturition. It was because the mammary glands of the mothers did not grow well (Fig. 2). The pups from the normal mother were transferred to the ovariectomized mother. However, the pups could not live over 48 h. Relaxin derived from the ovary is essential to develop the mammary gland (Zhao *et al.* 1999). The morphological structures of the mammary gland in ovariectomized mice were quite similar to those in the mice without a functional relaxin gene (Zhao *et al.* 1999). Previous study showed that the administration of relaxin extract from pregnant rabbit serum and/or pregnant sow ovaries improved the number of mice which reared their pups after ovariectomy at mid-pregnancy and subsequent maintenance by daily injection of progesterone and E_2 (Hall 1957).

Delivered pups from ovariectomized mother had been raised by the normal mother since immediately after parturition ($n=3$). These pups did not grow under the foster mother (died in a day). The possible reasons why the pups from ovariectomized mice could not live long are as follows: 1) the levels of two steroids, MPA and E_2 , are not high enough for the embryos to urge normal development (Murr *et al.* 1974, Barkley *et al.* 1977); and 2) the embryos require additional ovarian factors from the mother for normal development.

When we counted the number of pups in the morning after parturition, the number of delivered pups was 4.78 on an average, which was significantly less than the prospective number of implantation sites. It is possible that some pups were eaten by their mothers because their young died after birth accompanying maldevelopment of the mammary glands.

This is the first report on control of whole pregnancy in mice, from embryo implantation to parturition, by exogenous treatments without the ovaries. According to the protocol, almost all the mice could sustain pregnancy. Murine pregnancy is a very complicated process involving embryo implantation, placentation, and parturition and contains multiple cellular/tissue events. In addition to this, the timing of the initiation of each event depends on that of mating time (up to 12 h). This model is quite helpful in the analysis of

transcriptomes and proteomes in the uterus, because time-dependent events can be traced exactly at any time during pregnancy.

Materials and Methods

Animal

All experimental procedures were performed under avertin anesthesia (Sigma–Aldrich) according to the guidelines established by the Committee for Animal Welfare at Nagoya University (approval number: 2010042803). ICR mice (Japan SLC, Shizuoka, Japan) of 8–12 weeks of age were used in the experiments. Mice were housed at $23 \pm 3^\circ\text{C}$ in controlled light–darkness cycles (1200 h light:1200 h darkness) and were fed *ad libitum*.

Control of pregnancy

The protocol of the experiment is described in Fig. 3A. Mice were ovariectomized between 1730 and 1900 h on D3 (D1≡vaginal plug), and MPA (100 µl/head; Pfizer Inc, New York, NY, USA) was injected s.c. at the dorsal limber region (Fig. 3B). I.p. injection with LIF (25 µg/head), which can substitute estrogen (Chen *et al.* 2000), was given to induce embryo implantation at ED6. Because embryo implantation was occurred at D4 in normal pregnancy (McCormack & Greenwald 1974), this experimental procedure was 2 days late compared to the normal pregnancy after embryo implantation. Recombinant LIF protein was prepared according to a previous report (Terakawa *et al.* 2011). A silicone tube containing 50 µl of 25 µg/ml E_2 benzoate (Kyoritsu Seiyaku, Tokyo, Japan) was s.c. inserted at the dorsal region at ED11 (Fig. 3B), as a previous study reported that a silastic implant with 10–33 µg/ml E_2 was sufficient to maintain pregnancy during this period (Milligan & Finn 1997, Okada *et al.* 2005). The silicone tube consists of inner silicone tube (i.d. 2 mm, o.d. 3 mm, and 20 mm in length; Kaneka Medix Corp, Osaka, Japan) and outer

polyethylene tube (i.d. 3 mm, o.d. 4 mm, and 8 mm in length; Imamura, Tokyo, Japan), and both sides of edge were covered by the silicone (Shin-Etsu Chemical Co Ltd, Tokyo, Japan) after E_2 was infused (Fig. 3C). The silicone tube was soaked in sterile saline before the day of transplantation. To deliver the fetuses, the silicone tube with E_2 was removed at ED17, and MPA was removed at ED20. The expected date of delivery (EDD) was ED22.

Plasma levels of steroid hormones

Blood samples ($n=3-5$) from the mice of the experimental group were collected via the tail vein at ED5, 8, 11, 14, 17, 20, and 23 (or 24) respectively. Each plasma was isolated and stored at -80°C until the measurement. E_2 concentration was measured by the E_2 EIA kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the instruction manual. Detection limit of this assay system was 11.8 pg/ml.

Plasma levels of MPA were determined by LC-MS/MS. Ten microliters of centrifuged supernatant from the mixture of 20 µl of each plasma and 80 µl of methanol (Wako, Osaka, Japan) were applied to ACQUITY Ultra Performance LC (UPLC; Waters, Milford, MA, USA) with ACQUITY UPLC HSS T3 column (50×2.1 mm, i.d. 1.8 µm; Waters) by the following conditions: flow rate, 0.4 ml/min; column temperature, 40°C ; and mobile phase, 0.1% formic acid (Wako)/methanol containing 0.1% formic acid. UPLC was connected with Micromass Quattro Premier XE Tandem Quadrupole Mass Spectrometer (Waters). The measurement condition was as follows: method: electrospray ionization, positive-mode; capillary voltage: 3.5 kV; cone voltage: 25 V; collision energy: 15 eV; and precursor ion→product ion: m/z 387.4→327.1. The concentrations of MPA in samples were calculated on the calibration curve, which was determined by the normal murine plasma containing known concentrations of MPA.

Statistical analysis

Statistical analysis was performed by one-way ANOVA. Values of E_2 at ED23 and ED24 were excluded from the statistical analysis because the number of samples was insufficient for one-way ANOVA.

Evaluation of successful pregnancy

The number of the placenta or living pups at term of pregnancy (ED22) was counted using mice, inside which the E_2 implant and MPA were left ($n=7$). To confirm whether the fetuses were alive or not, their hind limbs were lightly pinched by the forceps immediately after the fetuses were picked out from the uterus. Delivered pups were counted on the peri-EDD ($n=10$). Mothers were sacrificed after parturition, and the number of the metrial glands, which is the number of predicted implantation sites, was counted. The mammary gland was collected at ED23 in ovariectomized mice and at D21 in normal pregnant mice respectively. Samples were fixed in Bouin's solution and paraffinized by the conventional procedure. Four micrometer section was prepared and stained with hematoxylin and eosin.

Figure 3 Experimental procedure to control pregnancy in mice (A). Medroxyprogesterone acetate (MPA) is injected (B, arrowhead) simultaneously with ovariectomy at D3. LIF administrated at ED6 induces embryo implantation. A silicone tube with 17β -estradiol (E_2) is inserted at ED11 (B, arrow; and C). E_2 and MPA are removed for parturition at ED17 and ED20 respectively. The EDD is ED22.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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