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ORIGINAL PAPER

Placental activin A is required for follicular development during the second half of pregnancy in the golden hamster (*Mesocricetus auratus*)

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Abstract Numerous antral follicles develop during the second half of pregnancy in the golden hamster even though LH and FSH are maintained at basal levels. To investigate the possible hormone actions of activin A associated with follicular development, pregnant golden hamsters were placentectomized on day 6 of pregnancy and animals were sacrificed at day 8, 10, 12, or 14 of pregnancy. There was a drastic decrease in the plasma concentrations of activin A from day 10 of pregnancy in the operated group compared to the controls. Positive immunohistochemical staining of inhibin/activin subunits βA and βB in the syncytiotrophoblast of the placenta revealed the source of activin A, AB, or B. The number of healthy follicles did not change until day 12 between the operated and the control groups, but decreased in numbers in the operated groups thereafter. The decreased concentrations of inhibin A, B, and estradiol-17β in the operated groups at day 10 and 12 correlated well with the number of mature follicles in response to hCG treatment. In conclusion, we revealed that activin A secreted from the placenta induces folliculogenesis to maintain the high levels of estradiol-17β needed to induce uterine dilatation for fetus growth and impending parturition.

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Introduction

The golden hamster (*Mesocricetus auratus*) has several unique characteristics during pregnancy that are quite different compared with other rodent species. During the second half of gestation in the golden hamster, numerous antral follicles develop [1, 2], but all large antral follicles degenerate at the end of pregnancy. Post-partum ovulation, as seen in rats [3], does not occur in the golden hamster [4], and large antral follicles in the golden hamster do not reappear until the end of lactation. The proliferation of numerous antral follicles and the circulating pattern of gonadotropins and ovarian hormones during the second half of pregnancy in the golden hamster are also markedly different compared with the pattern in the pregnant rat [5–8]. At the time of follicular emergence, circulating luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are maintained at low basal levels. Therefore, the mechanisms behind hormones regulating follicular dynamics during the second half of pregnancy in the golden hamsters are still not clear.

It is well known that progesterone is the hormone essential to maintain pregnancy. But at later stages in pregnancy, estrogen is also a key hormone which maintains pregnancy and expels the fetuses. It is known that the placenta of the golden hamster does not possess aromatase activity [9], so the main sources of estrogens during pregnancy are the antral follicles. It is believed that in the golden hamster, the antral follicles that develop in the second half of pregnancy supply enough estrogens to maintain pregnancy, leading to parturition. We previously

reported that during this period, large amounts of activin A are secreted from the placenta [10]. Our previous studies also suggest that this activin A is the factor that is involved in developing the antral follicles that emerge at the same time during the second half of pregnancy.

Inhibins and activins are structurally related dimeric proteins first identified in ovarian follicular fluid [11, 12]. Inhibins are heterodimers consisting of a common subunit and either a βA or βB subunit, resulting in inhibin A or inhibin B, respectively [13]. Activins are dimers of the β-subunits, with three forms currently identified: activin A (A-A), activin B (B-B), and activin AB (A-B) [14]. Activins are important proteins that have several physiological functions, and that may act in the early development in the formation of the mesoderm [15, 16]. But activin is also well known as an important factor in reproduction, such as the promotion [17, 18] and inhibition [19] of preantral follicle growth, stimulation of FSH secretion [12, 20], and augmentation of the number of FSH receptors [21, 22].

In the second half of the pregnancy in golden hamsters, even though LH and FSH are maintained at low basal levels, the numbers of follicles observed are more than twice the number during the estrous cycle. In the present study using placentectomized animal models, we investigated activin A, which may be responsible for follicular development in the second half of pregnancy in golden hamsters.

Results

Plasma concentrations of LH, FSH, inhibin A, inhibin B, activin A, progesterone, and estradiol-17β after placentectomy

Plasma concentrations of LH, FSH, inhibin A, inhibin B, activin A, progesterone, and estradiol-17β are shown in Fig. 1.

The plasma concentrations of LH were maintained at basal levels, with no significant changes observed between the control and experimental groups (Fig. 1d). The levels of FSH showed a similar tendency to remain at the basal level, with the exception of a significantly low FSH concentration in the experimental group compared with the control group on day 10 of pregnancy ($P < 0.05$) (Fig. 1e).

The plasma concentrations of activin A significantly decreased in the experimental groups compared to the control groups at day 10, 12, and 14 of pregnancy. On day 12 and 14 of pregnancy, the levels of activin A in the experimental groups decreased 27.9% and 15.6% compared with the control group, respectively ($P < 0.01$) (Fig. 1c).

The plasma concentrations of inhibin A and inhibin B in the control group abruptly increased from day 10 and reached maximal levels on day 10 and 12 of pregnancy, respectively. Thereafter, plasma concentrations of both inhibin A and B decreased on day 14 of pregnancy. In the experimental groups, the changes in both hormone concentrations correlated closely, on days 10 and 12 of pregnancy, the concentrations of inhibin A and B significantly decreased compared to the control group (Fig. 1a, b).

The plasma concentration of estradiol-17β showed significantly higher levels in the control compared to the experimental groups after day 10 (Fig. 1f). Circulating concentrations in progesterone levels, however, did not change except on day 12, when the control was significantly higher than the experimental group ($P < 0.01$) (Fig. 1g).

Immuno-histochemical studies of inhibin subunits in the placenta

The results of the immuno-hisotchemistry shows definitively the presence of inhibin/activin subunits α, βA and βB in the placenta are shown in Fig. 2, respectively. Positive staining of inhibin (a) α, (b) βA, and (c) βB subunits were all found in the cytoplasm of syncytiotrophoblast in the placenta.

The follicular number, size, and morphological changes after placentectomy

Histologic examination of ovarian serial sections was performed to determine the follicular number, size, and morphologic changes (Fig. 3). The follicular numbers of both control and experimental groups rapidly increased in the second half of pregnancy. The number of healthy vesicular follicles doubled in both groups on day 8 and 10 of pregnancy. Although there were no significant differences between the control and experimental groups in the number of healthy follicles on day 12, the number of atretic follicles in the experimental group showed a significant decrease compared with the control group ($P < 0.05$). At day 14 of pregnancy, numbers of atretic follicles rapidly increased in both groups, and in over 50% of the follicles, we observed suspensions of granulosa cells in the antrum, and deformation of the granulosa layer or oocytes.

Follicles were classified according to their sizes, as shown in Fig. 4. There were no significant differences on either day 8, 12, or 14 of pregnancy for all of the follicular sizes. But on day 10 of pregnancy, S-sized healthy vesicular follicles showed a significant decrease in the

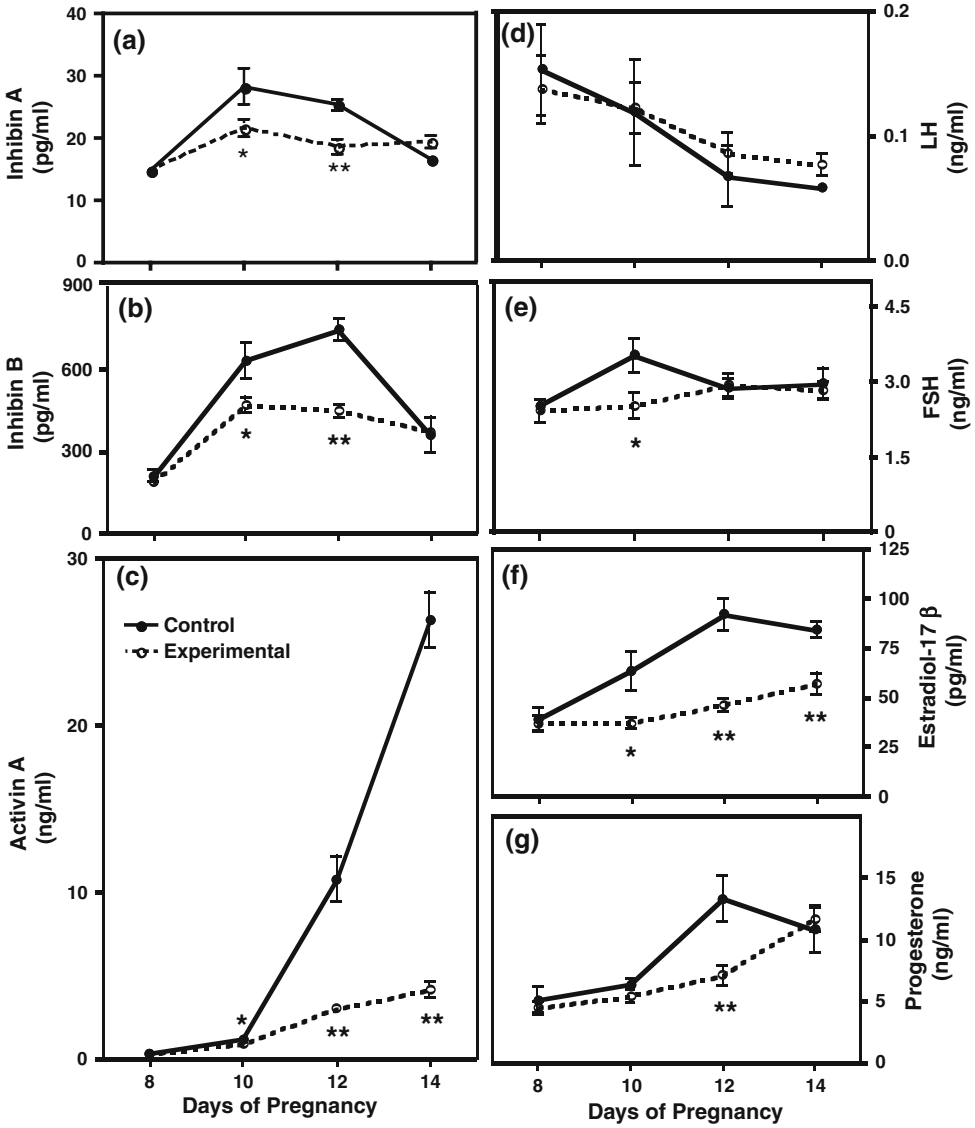

Fig. 1 Changes in plasma concentrations of (**a**) inhibin A, (**b**) inhibin B, (**c**) activin A, (**d**) LH, (**e**) FSH, (**f**) estradiol-17β, and (**g**) progesterone of the control groups (●) or the experimental groups (○) during the second half of pregnancy in the golden hamster. On day 6 of pregnancy, the number of embryos was reduced to four in each of the experimental groups. Each value represents the mean ± S.E.M. of five to seven observations. *$P < 0.05$, ** $P < 0.01$, significantly different from the control groups (Student's *t*-test)

experimental groups ($P < 0.05$). Moreover, there were no differences in the L-sized vesicular follicles, but the numbers decreased in the experimental groups.

Induction of ovulation after placentectomy

To evaluate the number of healthy mature follicles that have developed during pregnancy, we chose an ovulation induction model in response to hCG. Table 1 describes the number of oocytes ovulated using i.v. treatment of 10 IU of hCG. All of the hCG-treated animals ovulated. On day 12 of pregnancy, both of the experimental and the control group showed the highest numbers of ovulated oocytes. On days 10 and 12 of pregnancy, the number of oocytes decreased significantly, especially on day 10 ($P < 0.01$). There was only about one-half the number of oocytes in the experimental groups compared with the control groups.

Discussion

To reveal the mechanisms underlying the rapid development of antral follicles in the second half of pregnancy in the golden hamster, we artificially decreased the number of placentas to four in pregnant golden hamsters as a model animal, and investigated follicullogenesis and endocrine functions.

In the second half of pregnancy, there was a significant reduction in the plasma concentrations of activin A in the experimental group compared with the control group. Our immunohisochemical approach suggests that the inhibin/activin subunits βA and βB localize in the syncytiotrophoblast of the placenta, indicating secretion of activin A, AB, or B from these cells. From these results, in the second half of pregnancy in the golden hamster, we postulate that placenta is a potent source of activin A, consistent with our previous report [10].

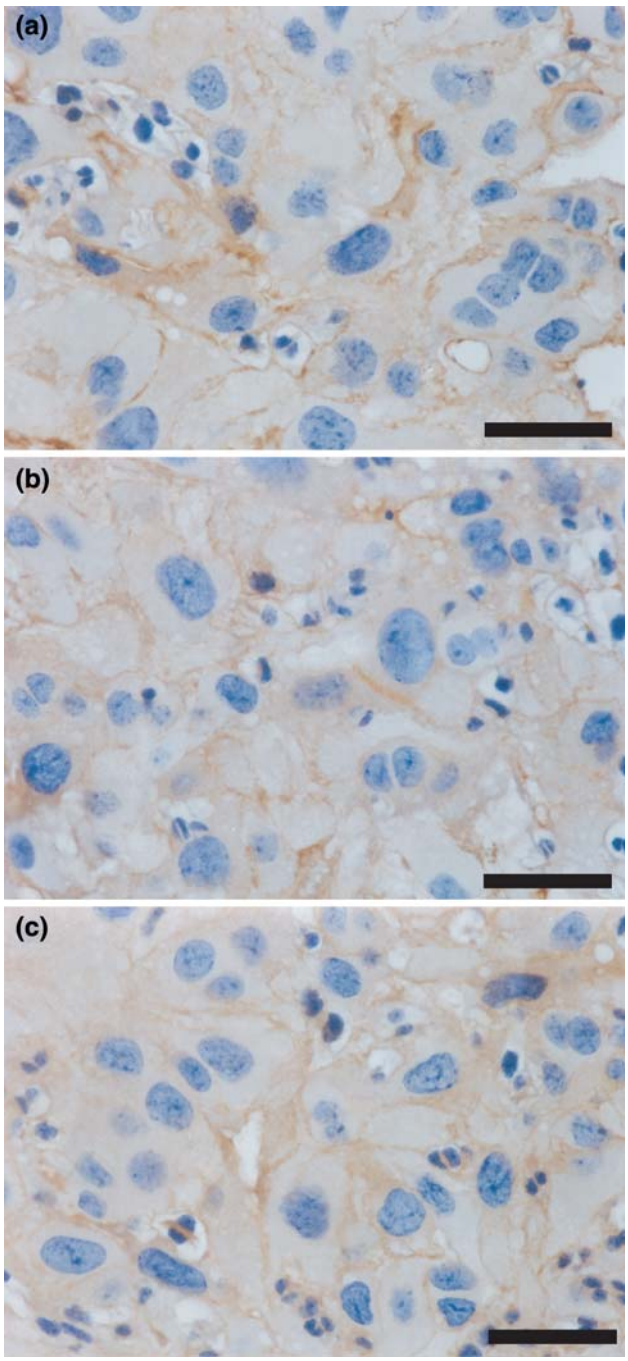

Fig. 2 Immunostaining of inhibin/activin subunits α, βA, and βB in the placenta of a pregnant golden hamster on day 12. Panels (**a**), (**b**), and (**c**) show staining of inhibin/activin α, βA, and βB subunits, respectively. Bar = 50 μm

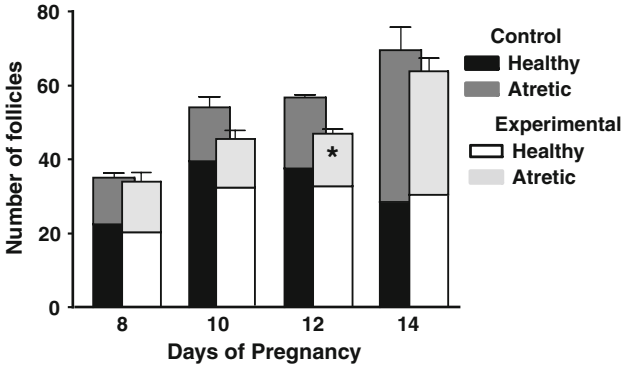

Fig. 3 Changes in total number of healthy and atretic follicles, and control groups or the experimental groups during the second half of pregnancy in the golden hamster. Each value represents the mean ± S.E.M. of five to seven observations. $*P < 0.05$, significantly different from the control groups (Student's *t*-test)

Activin was discovered to be a secretagogue factor of FSH [12, 23, 24]. Previous reports show that administration of activin A induces FSH secretion [20, 25] in vivo, so we thought that placental reduction leading diminished circulating levels of activin A, would suppress the secretion of FSH. But the plasma concentrations of FSH were maintained at a basal level except on day 10 of pregnancy, when FSH levels decreased significantly. In the experimental groups on day 14 of pregnancy, although the levels of activin A decreased by 15.6% compared with the control group, there were no significant differences in the plasma FSH levels. It is reported that the major target organs of activin A are the uterus and the ovary, as well as the pituitary [26, 27], and activin activates receptor type 1(ActR-I) and type 2 (ActR-II/ActR-IIB) [28]. Activin exerts a vast array of actions, and is reported to be regulated in paracrine/autocrine mechanisms [29]. In addition, circulating activin appears to be bound to follistatin, an activin binding protein [30, 31]. Although we did not measure follistatin, we postulate that in the second half of pregnancy in the golden hamster, high levels of circulating activin A are bound to follistatin. Most of the activin A that is secreted from the placenta then may affect neighboring organs such as the uterus or the ovary, but not the pituitary and FSH secretion. Since ovariectomy of rats in early infantile life does not consistently result in changes in levels of circulating FSH or FSH-β mRNA [32, 33], this suggests that ovarian-derived activin may not play a role in regulating the pituitary secretion of FSH.

The concentrations of estradiol-17β in the experimental animals significantly decreased on day 10 of pregnancy compared to the controls. In the golden hamster, there is no aromatase activity in the placenta [9], and androgen and estrogen were not detected in the extracts of the placental homogenates [34]. These reports reveal that in the gestational periods of the golden hamster, the major sources of estradiol-17β are the follicles of the ovary, and not the placenta. The decrease in estradiol-17β reported in the present study is thought to be from the decrease in the number of follicles or from the atretic degradation of the follicles, resulting in the hypo-secretion of estradiol-17β.

Even though the plasma concentrations of FSH and LH are maintained at basal levels [10], the numbers of the

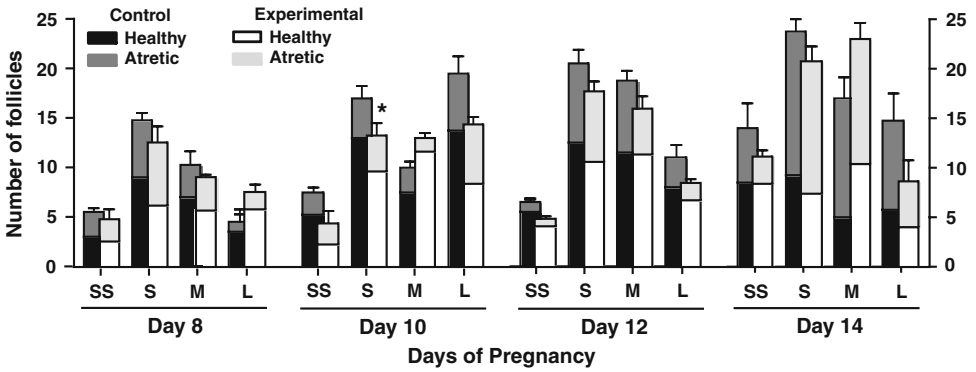

Fig. 4 Histologic classifications of follicular size and conditions of the control groups or the experimental groups during the second half of pregnancy in the golden hamster. The average diameter of follicles that were under 300 μm was categorized as SS, S for 300 μm to 400 μm, M for 400 μm to 500 μm, and L for follicles over 500 μm, respectively. On day 6 of pregnancy, the number of embryos was reduced to four in each of the experimental groups. Each value represents the mean ± S.E.M. of five to seven observations. $^{*}P < 0.05$, significantly different from the control groups (Student's *t*-test)

Table 1 Induction of ovulation by 10 IU human chorionic gonadotrophin (hCG) administered on various days during pregnancy in the golden hamster

Day of Pregnancy	Number of oocytes	
	Control	Experimental
Day 8	16.0 ± 1.6	12.8 ± 0.4
Day 10	33.8 ± 2.4	18.4 ± 1.3**
Day 12	38.8 ± 1.2	24.8 ± 1.9**
Day 14	25.2 ± 2.7	19.8 ± 0.4

Each value represents the mean ± S.E.M. of five observations. ** $P < 0.01$, significantly different from the control groups (Student's *t*-test). The hamsters were placentectomized on day 6 of pregnancy to reduce the embryos into four in each of the experimental groups

follicles in this study increased from day 8 to 10 of pregnancy. There are reports that activin A induces FSH receptors in granulosa cells of follicles in vitro and in vivo [21, 22, 25, 35]. In the experimental groups, there was a significant decrease in activin A, and the number of healthy follicles decreased commensurately, implying an inhibition of FSH receptor induction. But the numbers of follicles did not show any significant differences between control and experimental groups in contrast to changes in the serum concentrations of estradiol-17β. So we believe that activin A takes part not only in follicular maturation, but also in the secretion of estradiol-17β. In the operated groups there was an overall decrease in follicle size on day 10 of pregnancy. On day 10 of pregnancy, the FSH concentrations also decreased significantly in the experimental group. It is suggested that this decrease in FSH affected the follicle size.

Additionally, the induction of ovulation by hCG treatment led to significant decrease in follicles ovulating in the experimental groups on days 10 and 12 of pregnancy. Greenwald [36] has reported that the number of follicles larger than 415 μm in diameter was correlated with the number of ovulations induced by hCG during gestation in the golden hamster. In humans [27], and monkeys [37, 38], inhibins are secreted from the placenta, as well as from the follicle as reported by Ohshima et al. [10]. Plasma inhibin concentrations did not change after placentectomy in the second half of pregnancy in the golden hamster, which follows as inhibins are thought to be secreted from the follicle but not in the placenta in this species. Previous studies also support the changing pattern of plasma concentrations of inhibin, which corresponded well with the number of large preovulatory antral follicles for inhibin A, and small and large antral follicle for inhibin B [10, 39–41]. In the present study, plasma concentrations of inhibin A, inhibin B, and activin A showed significant decreases on days 10 and 12 of pregnancy in the experimental animals. The hormonal pattern correlated well with follicle number after hCG induction (Table 1), especially for the L-sized follicles (Fig. 4). This suggests that reduction in activin A results in altered folliculogenesis, especially in the large mature antral follicles.

When compared with the number of hCG-ovulated follicles and healthy follicles of 400 μm in diameter or more (M and L sizes) in the histologic sections on days 10 and 12 of pregnancy in Fig. 4, the numbers of healthy follicles were not different whereas, the treatment caused significant differences in the number of the ovulating oocytes. This may be due to a low degree of maturation of the large antral follicles when in a low plasma activin A environment. The differences in the maturation of the follicles may be due to the number and the affinity of LH receptors in the follicle. In early folliculogenesis, LH receptors exist only in theca interna cells but as the follicle matures, FSH induces the expression of LH receptor in the granulosa cells [42]. Activin is reported to induce LH receptors as well as FSH receptors [43, 44], and furthermore up-regulates aromatase activity [45, 46]. From these reports, activin A induced not

only FSH receptors but also LH receptors in the follicle and up-regulates aromatase activity which resulted in the follicle maturation and enhanced the sensitivity to the hCG ovulation treatment in the control animals.

In conclusion, the present study clearly demonstrates that activin A during the second half of pregnancy in the golden hamster, is secreted from the syncytiotrophoblast in the placenta and possibly up-regulates LH and FSH receptors of the follicles in the ovary (Fig. 5). Basal levels of LH and FSH can then induce folliculogenesis up to doubling the number of follicles compared with the number seen during the estrous cycles as to maintain high levels of estradiol-17β. The secreted estradiol-17β is important to induce the enlargement of the uteri, fetal growth, and uterine contractions or dilatation for impending parturition. Thus, these results strongly suggest that activin A is a key hormone that can induce folliculogenesis during the second half of pregnancy in the golden hamster.

Materials and methods

Animals

Adult female golden hamsters (*Mesocricetus auratus*), maintained on a 14 h light: 10 h dark schedule (lights on

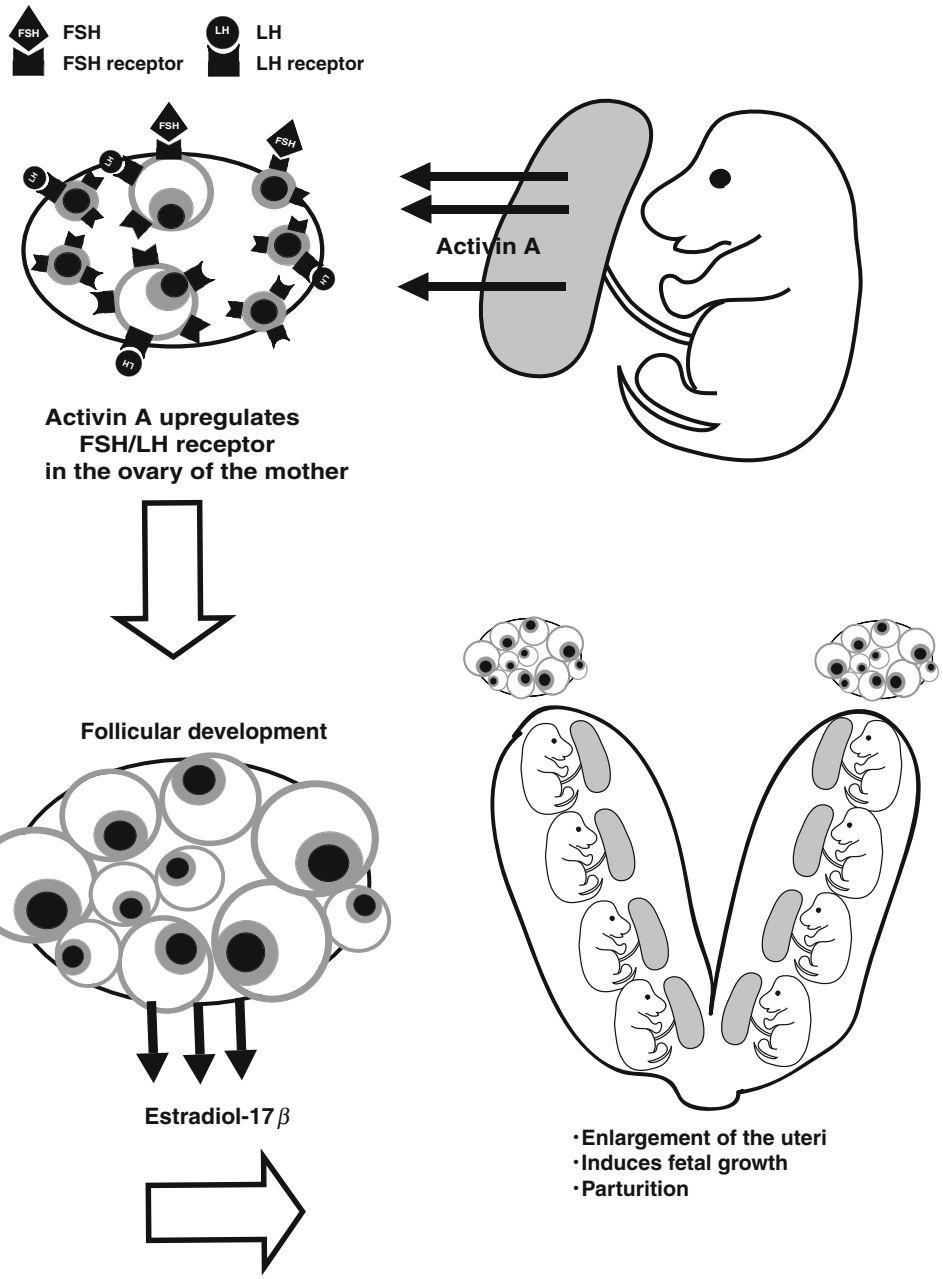

Fig. 5 Schematic figure of the hypotheoried mechanism of follicular development during the second half of pregnancy in the golden hamster. Activin A secreted from the syncytiotrophoblast in the placenta during the second half of pregnancy in the golden hamster, up-regulates LH and FSH receptors, assisted by basal gonadotropins and LH and FSH secreted from the pituitary, this induces folliculogenesis and doubles the number of follicles compared with the number seen during the first half of pregnancy. In the granulosa cells of the developing follicles, aromatase expression is up-regulated by FSH and LH, and high levels of estradiol-17β are secreted, which stimulate enlargement of the uteri, fetal growth, and uterine contractions or dilatation for the impending parturition

from 0500 h to 1900 h) were mated after two consecutive 4-day estrous cycles. Female hamsters were placed with males on the evening of day 4 (day of proestrus), and the presence of sperm in the vagina was designated as day 0 of pregnancy. In our colony, most hamsters delivered on day 15 of pregnancy. Food and water were available ad libitum. All experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals prepared by Tokyo University of Agriculture and Technology.

Experimental design

Animal models

At day 6 of pregnancy, golden hamsters were placed under ether anesthesia and number of placentas were counted, and animals underwent placentectomy ($n = 7$ at each pregnancy), by making crush wounds using forceps, reducing the placentas to 4 (2 in each uterine side) as experimental group, or were sham-operated as control group ($n = 5$ at each pregnancy). At day 8,10,12, or 14 of pregnancy, groups of animals were decapitated and trunk blood was collected. Blood samples were centrifuged immediately at 1,700 g for 15 min at 4°C, and plasma was separated and stored at −20°C until assayed. The ovaries and placenta (at day12 of pregnancy only) were also collected and fixed in 4% paraformaldehyde (Sigma Chemical Co, St Louis, MO, USA) in 0.05M PBS, and embedded in paraffin for the observation of histology and immunohistochemistry.

Radioimmunoassay of LH, FSH, progesterone, and estradiol-17β

Concentrations of LH and FSH in plasma were measured using NIDDK RIA kits for rat LH and FSH. Iodinated preparations were rat LH-I-5 and FSH-I-5. The antisera used were anti-rat LH-S-9 and anti-rat FSH-S-11. The intra- and interassay coefficients of variation were 2.1% and 6.7% for LH and 3.7% and 14.6% for FSH, respectively.

Concentrations of progesterone and etradiol-17β in plasma were measured by double-antibody RIA systems using ^{125}I-labeled radioligands as described previously [7]. Antisera against progesterone (GDN; [47] and estradiol-17β (GDN 244; [48] were kindly provided by Dr. G. D. Niswender (Colorado State University, Fort Collins, CO, USA). The intra- and inter-assay coefficients of variation were 3.5% and 15.4% for progesterone, and 2.7% and 6.2% for estradiol-17β, respectively.

Enzyme-linked immunoabsorbent assay (ELISA) of inhibin A, inhibin B, and activin A

Concentrations of inhibin A, inhibin B, and activin A were measured by ELISA (Serotec, Oxford, Oxon, UK) as described previously [10, 40, 49]. Amounts of inhibin A and inhibin B were expressed in terms of recombinant human inhibin A and inhibin B, respectively. Amounts of activin A were expressed in terms of recombinant activin A.

Immunohistochemical detection of inhibin α, inhibin/activin βA, and βB subunits in the placenta of pregnant golden hamsters

The paraffin-embedded placental tissues were serially sectioned at a thickness of 6 μm and placed on poly-L-lysine-coated slide glasses. After tissue sections were deparaffinized with xylene, they were subjected to antigen retrieval by autoclaving in 0.01M sodium citrate buffer (pH6.0) at 121°C for 15 min. The sections were then incubated in 0.3% H_2O_2 in methanol at room temperature for 1h , followed by incubation with block solution of 0.5% casein-Tris saline (CTS) (0.05M Tris–HCl with 0.15M NaCl, pH 7.6; CTS) at 37°C for 1h to quench nonspecific staining. Then the sections were incubated overnight at 4°C with rabbit polyclonal first antibodies in CTS. The antibody against each inhibin subunit was anti-[Tyr^{30}]inhibin α-chain (1–30)-NH_2 conjugated to rabbit serum albumin, anticyclic inhibin βA (81–113)-NH_2, and anticyclic inhibin βB (80–112)-NH_2. The inhibin α subunit peptide was kindly provided by Dr. N. Ling (Neuroendocrine Inc., San Diego, CA, USA) and the anticyclic inhibin βA (no. 305–24-D) and anticyclic inhibin βB (no. 30-25-D) were kindly provided by Dr. W. Vale (Salk Institute for Biological Studies, La Jolla, CA, USA). The use of these antibodies against inhibin subunits in the golden hamster has been described previously [50, 51]. The sections were then treated with 0.25% (v/v) biotinylated secondary antibodies (Elite ABC kit; Vector Lab. Burlingame, CA, USA) in CTS for 1h at room temperature, and were subsequently incubated with 2% (v/v) avidin-biotin complex (Elite ABC kit) in CTS for 30 min at room temperature. The reactions were visualized by treating with 0.025% 3,3′-diaminobenzidine tetrachloride DAB, (Sigma Chemical Co, St Louis, MO, USA) in 10m M Tris-buffered saline containing 0.01% H_2O_2 for 1–30 min.

Histological study for ovaries

The paraffin-embedded ovarian tissues were serially sectioned at a thickness of 10 μm and placed on poly-L-lysine-coated (Sigma Chemical Co, St Louis, MO, USA) slide glasses (Dako Japan Co, Kyoto, Japan). They were

stained with haematoxylin and eosin for observation of follicular stages. The follicles were classified by size and condition (healthy or atretic). The diameter of the follicles that were under 300 μm was categorized as SS, S for 300 μm to 400 μm, M for 400 μm to 500 μm, and L for follicles over 500 μm, respectively. Any observation of granulosa cells suspended in the antrum, or deformation of the granulosa layer or of the oocytes classified the follicles as atretic; whereas, morphologically normal follicles were classified as healthy.

Ovarian follicular profiles during the second half of pregnancy

To determine the number of large healthy antral follicles in the ovary, groups of animals were given 10I U human chorionic gonadotropin (hCG) (2200I U/mg; Sankyo Zoki Co., Tokyo, Japan) dissolved in 0.2m l saline, injected into the jugular vein with the animal under ether anesthesia at 17:00 of day 8, 10, 12, or 14 of pregnancy ($n = 5$ each group). Animals were killed by decapitation at 18 h after hCG injection, and then the oviducts were examined for oocytes. Numbers of oocytes were counted under a light microscope (Leica, Wetzlar, Germany).

Statistics

All data were expressed as mean ± S.E.M. One way ANOVA was performed, and the significance between two means was determined by Student's t-test. A value of $P < 0.05$ was considered to be statistically significant.

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