ORIGINAL

Activin effects on follicular growth in *in vitro* preantral follicle culture

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Abstract : As the follicular environment transits from being activin dominant to inhibin dominant during folliculogenesis, it is assumed that activin plays an important role in the early stage of follicular growth. We examined the effects of activin on morphological, biochemical and molecular changes in isolated preantral follicles. Preantral follicles were mechanically isolated from 14-day old female C57BL/6 mice. Each follicle was cultured and observed for 14 days using an *in vitro* follicle culture system containing FSH, FSH + activin A and FSH + inhibin in the culture medium. We subsequently examined FSH receptor (FSH-R) mRNA expression in isolated follicle cultures with or without activin on days 0 and 2. Activin was observed to significantly stimulate follicle enlargement on days 2, 4, 6 and 8, accelerate morphological changes and increase estradiol levels in culture medium on days 4, 12 and 14. In contrast, inhibin did not alter follicular growth. Additionally, activin stimulated the expression of FSH-R mRNA in isolated granulosa cells. It was demonstrated that activin stimulated the growth of preantral follicles, mainly during the early stage of folliculogenesis, by inducing FSH-R expression, in an isolated follicle culture system. J. Med. Invest. 66 : 165-171, February, 2019

Keywords **:** *follicle, activin, in vitro follicle culture, preantral follicle, FSH receptor*

INTRODUCTION

Gonadotropins are important regulators of follicular development. Follicle stimulating hormone (FSH) receptor is expressed on granulosa cells in primordial follicles in some instances (1). However, early follicular development is not gonadtropin-dependent and certain local factors stimulate the growth of preantral follicles, resulting in the induction of sufficient levels of FSH receptor expression in granulosa cells. However, little is known about how follicles grow from the primordial stage to the antral stage, and preantral follicle development is an important issue for reproductive science with clinical implications.

Female FSH receptor (FSH-R) knockout mice showed an arrest of follicular development at the preantral stage (2, 3), suggesting that FSH is essential for preantral follicles to proceed to antral follicles. Various local factors in the TGF-β family, such as activin and its intracellular signaling molecules (Smads), regulate the development of preantral follicles in an FSH independent manner. Activins are heterodimers (activin AB ; βAβB) or two homodimers of the β-subunits (activin A ; βAβA, activin B ; βBβB) of inhibin subunits, whereas, inhibins are heterodimers of the α-subunit and one of the two β-subunits. Activins bind two types of receptor, i.e. activin receptors type I and type II (4, 5). Follicles at the early follicular stage synthesize more activin more than inhibin, according to analyses of inhibin/activin subunit mRNA and protein expression (4, 5). Furthermore, follicular fluid transits from an activin rich to an inhibin rich environment during follicular development, suggesting that activin plays an important role in early stage follicular growth (6-8). Activin induces expression of FSH-R in the absence of FSH (9, 10), although FSH-R is also induced by FSH itself (11). These findings suggest that activin could play a key role in gonadotropin-independent follicular development during early follicular development, especially in the preantral stage.

Activin stimulates granulosa cell proliferation in ovarian tissues (12) and accelerates follicular enlargement in *in vitro* follicle culture with FSH in rats, goats and cats (13-18). It was also reported that the inhibin/activin βB subunit, which forms a homodimer in activin B, is essential for early folliculogenesis (19). However, there are no detailed reports of morphological, biochemical and/or molecular transitions, which could be studied in an *in vitro* follicle culture, in the same experiment. Additionally, inhibin is released by granulosa cells into the general circulation and suppresses FSH secretion in the pituitary, thereby counteracting the effects of activin. Inhibin may also affect the ovary itself because several studies have reported its local enhancement of androgen production in theca cells (20) and suppression of FSH-induced FSH-R promoter activity (21). However, it is unclear whether inhibin itself has any effect on follicular development as a local paracrine/autocrine factor. Thus, we examined the effects of activin and inhibin on morphological, biochemical and/or molecular alterations during follicular development in isolated preantral follicles using an *in vitro* preantral follicle culture system.

MATERIAL AND METHODS

Animals

Female C57BL/6 mice, 14 days old, were obtained from Charles River Japan, Co. (Yokohama, Japan). Experiments were conducted in accordance with the ethical standards established by the Committee on Animal Care and Use of Tokushima University.

Chemical substances

Recombinant human follicle stimulating hormone (rh-FSH) was obtained from Ares-Serono (Geneva, Switzerland). Recombinant human activin A (rh-ActA) was obtained from Genzyme/Techne

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(Cambridge, MA, USA). Bovine inhibin (b-inhibin) was kindly provided by Professor Y. Hasegawa (Kitasato University, Kanagawa, Japan) (22, 23).

Experiment 1 : Morphological and biochemical examinations in in vitro preantral follicle culture

In vitro follicle culture was performed according to the method of Cortvrindt *et al.* (13, 24). Twenty-five mice were used for experiment 1. Mouse ovaries were removed after cervical dislocation under ether anesthesia and were collected in L15 medium (Gibco BRL, Tokyo, Japan) with 100 IU/mL penicillin, 100 mg/mL streptomycin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 10% fetal bovine serum (FBS) (Moregate Bio Tech, Brisbane, Australia) on ice. Under a stereoscopic microscope, adipose tissue around the ovaries was cleanly removed and follicles were dispersed using 27 gauge needles. Preantral follicles of diameter between 100-130 μm were selected and pooled. The follicles had intact basal membranes and one or two layers of granulosa cells with a few adhering theca cells. Culture dishes (60 mm petri dishes : Falcon®, Becton Dickinson, Franklin Lakes, NJ, USA) contained 20 culture medium droplets (volume of each droplet was 10 μL) and were covered with 5 mL of mineral oil (Sigma, Bornem, Belgium). Each follicle was placed individually in a droplet. Follicles were cultured for 14 days under the following conditions : culture medium containing 100 mIU/mL rh-FSH (GF, n=153), culture medium containing 100 mIU/mL rh-FSH and 100 ng/mL rh-ActA (GFA, n=150), culture medium containing 100 mIU/mL rh-FSH and 1,000 ng/mL b-inhibin (GFI, n=97). Because ActA had been usually used in past studies, it was also used in this study. The culture medium consisted of alpha-minimal essential medium (alpha-MEM) (Gibco BRL, Tokyo, Japan), 5% FBS, 5 μg/mL insulin and 10 μg/mL transferrin (Sigma-Aldrich, Tokyo, Japan). Follicles were cultured in an incubator with 5% CO_2 in air and 100% humidity at 37°C. Day 0 represents the day on which the culture was started. On day 1 of culture, 10 μL of culture medium was added to each droplet. Half of the medium was refreshed and the medium was collected on every other day after day 2 of culture, and pooled at −20°C until the concentration of estradiol was measured by enzyme immunoassay (EIA ; Tosoh AIA, Toyama, Japan). Diameters and morphological structures of the cultured follicles were observed using an inverted microscope every other day beginning on day 2 of culture. The morphological structures of the cultured follicles were classified according to the classification of Cortvrindt *et al.* (24) (Figure 1). N (No change) ; an oocyte with a zona pellucida, surrounded by one or two layers of granulosa cells, a basal membrane and a single layer of theca cells. L (Low growth) ; partial outgrowth of granulosa cells through the basal membrane. H (High growth) ; outgrowth of granulosa cells through the basal membrane and in all laps around the basal membrane. A (Antrum formation) ; formation of an antral-like cavity. D (Dispersed) ; granulosa cells are dispersed and the follicular structure is disrupted. E ; (Extruded) ; ovum is extruded from follicles and follicular structure is disrupted. D and E are not represented in Figure 1 and are combined in the analysis ; both morphologies represent the end of life stage for a follicle.

Experiment 2 : Effect of activin on the expression of FSH receptor mRNA in granulosa cells of cultured preantral follicles

Five mice were used for experiment 2. Experiment 2 compared three treatment groups : the D0 group was isolated from ovaries and not cultured (n=30), the FD2 group was cultured for 2 days with 100 mIU/mL rh-FSH (n=38), and the FAD2 group was cultured for 2 days with 100 mIU/mL rh-FSH and 100 ng/mL rh-ActA (n=36). Granulosa cells from each follicle were mechanically isolated using 27 G needles under an inverted microscope. Expression of FSH receptor (FSH-R) mRNA in the granulosa cells of individual follicles was estimated by reverse transcriptase polymerase chain reaction (RT-PCR).

RT-PCR analysis

Total RNA was extracted using an RNeasy Micro Kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer's protocol. The RNA was stored at −80°C until use. Complimentary DNAs were synthesized using a Sensiscript™ Reverse Transcriptase kit (Qiagen K.K.). PCR primers used in this study are listed in Table 1. The PCR program for FSH receptor consisted of 15 min at 95°C, 50 cycles of 10 seconds at 95°C, 30 seconds at 64°C and 30 seconds at 72°C, concluding with 15 seconds at 95°C, 15 seconds at 60°C and 15 seconds at 95°C. The PCR program used for β-actin consisted of 15 min at 95°C, 50 cycles of 10 seconds at 95°C, 30 seconds at 58°C and 30 seconds at 72°C, concluding with 15 seconds at 95°C, 15 seconds at 60°C and 15 seconds at 95°C. PCR products were separated by electrophoresis in a 1% agarose gel and viewed under ultraviolet light.

Statistical analysis

Differences in follicle diameter and estradiol concentration among the groups were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer post-hoc test. Difference in follicular morphology and FSH-R mRNA expression were analyzed using a chi-square test. Values are expressed as mean ± standard deviation (SD), and statistical significance was defined as $p < 0.05$ or $p < 0.01$ in all analyses.

RESULTS

Experiment 1

The mean diameter of follicles in the GFA group were significantly larger than that of GF and GFI groups on days 2, 4, 6 and 8 of culture ($p < 0.01$), and the GF and GFI groups progressed to the same diameter as the GFA group on day 10 (Figure 2). The rate of

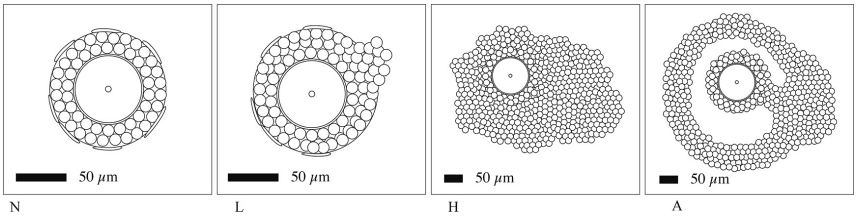

Figure 1. Schematic diagram of the classification of cultured follicle morphological structures, in accordance with report of Cortvrindt *et al.* (Cortvrindt *et al.* 1996). A : N (No change) ; An oocyte with a zona pellucida surrounded by one or two layers of granulosa cells, a basal membrane and a single layer of theca cells. B : L (Low growth) ; Partial outgrowth of granulosa cells through the basal membrane. C : H (High growth) ; Outgrowth of granulosa cells through the basal membrane and those surround the basal membrane. D : A (Antrum formation) ; Formation of an antral-like cavity.

Table 1. PCR primers and amplicon sizes for mouse FSH receptor and beta-actin

Gene	Primer		Product (bp)
Beta-actin	5' primer	5'-OH TCATGAAGTGTGACGTTGACATCCGT-3'	285
	3' primer	5'-OH CTTAGAAGCATTTGCGGTGCACGATG-3'	
FSH receptor	5' primer	5'-OH ATCACACATGCCATGCAACT-3'	199
	3' primer	5'-OH GTACGAGGAGGGCCATAACA-3'	

follicle diameter increase (gain in 2 days) was significantly different between the GFA and GF or GFI groups only on days 2 and 4 (data not shown). The concentrations of estradiol in the culture medium of the GFA group were significantly higher than in the GF and GFI groups on days 4, 12 and 14 of culture ($p < 0.01$ on days 4 and 14, $p < 0.05$ on day 12) (Figure 3). The proportion of follicles where granulosa cells grew through the basal membrane (Low growth ; L) on day 2 of culture was significantly higher in the GFA group (78.1%) than in the GF group (1.3%) (Figure 4). Thereafter, development of High growth (H) and Antrum (A) morphologies in the GFA group occurred earlier than in the GF group by almost 2 days. However, the development of H and A morphologies in the GFA group were not as dramatic as the development of the L morphology in the GFA group. There were no significant differences in diameter, morphological structures of the cultured follicles and concentration of estradiol in the culture

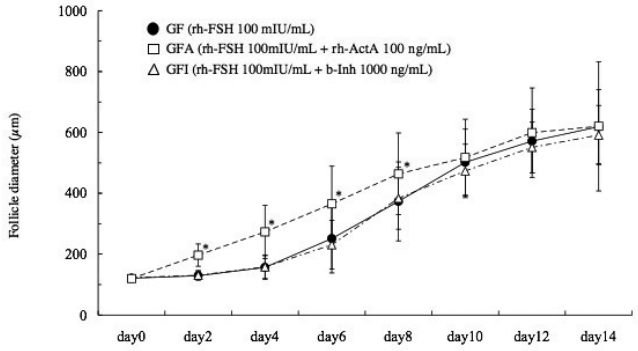

Figure 2. Alterations in the diameter of preantral follicles derived from immature mice (14-day-old) cultured in the presence of rh-FSH (100 mIU/mL) (GF), rh-FSH (100 mIU/mL) + rh-ActivinA (100 ng/mL) (GFA), and rh-FSH (100 mIU/mL) + b-Inhibin (1,000 ng/mL) (GFI). Diameters of the follicles were observed using an inverted microscope every other day beginning on day 2 of culture. The mean diameter of the follicles was significantly larger in the GFA group than in the GF group on days 2, 4, 6 and 8 of culture. However, the difference in follicle diameters between the GF and GFA groups did not increase after day 4. *$p < 0.01$ vs. GF (one-way ANOVA followed by the Tukey-Kramer post-hoc test).

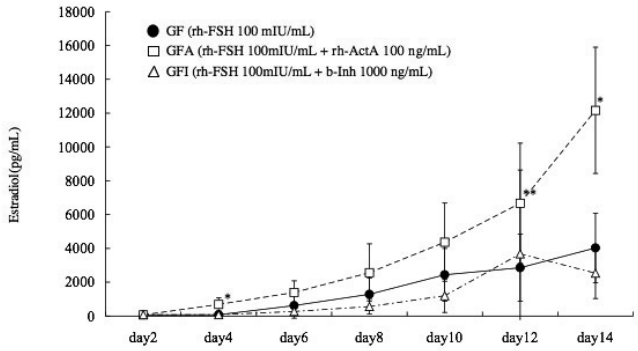

Figure 3. Changes in the concentrations of estradiol in medium cultured in the presence of rh-FSH (100 mIU/mL) (GF), rh-FSH (100 mIU/mL) + rh-ActivinA (100 ng/mL) (GFA), and rh-FSH (100 mIU/mL) + b-Inhibin (1,000 ng/mL) (GFI). Half of the culture medium was replaced with fresh medium and collected for measurement of estradiol every other day from day 2 of culture, and pooled at –20°C until examination. The concentration of estradiol in the medium was measured using an enzyme immunoassay (TOSOH AIA, Toyama, Japan). The concentration of estradiol in culture medium was significantly higher in the GFA group than in the GF group on days 4, 12 and 14. *$p < 0.01$ and **$p < 0.05$ vs. GF (one-way ANOVA followed by the Tukey-Kramer post-hoc test).

medium between the GFI and GF groups (Figure 2, Figure 3, Figure 4).

Experiment 2

FSH receptor mRNA expression was detected by RT-PCR analysis in granulosa cells of *in vitro* follicle culture. The agarose gel showing FSH-R mRNA amplicons from the D0, FD2 and FAD2 groups is provided in Figure 5. The proportion of follicles expressing FSH-R mRNA was significantly higher in the FAD2 group (94.4%, 34/36) than in the FD2 (63.2%, 24/38) and D0 (56.7%, 17/30) groups ($p < 0.01$) (Figure 6).

DISCUSSION

In this study, we showed that activin altered preantral follicle behavior by increasing their diameter, accelerating morphological changes up to the antral stage, increasing estradiol secretion, and enhancing FSH-RmRNA expression in granulosa cells, which was actually derived from the wall of isolated preantral follicles. As all of the data were shown using the same *in vitro* follicle culture system during the follicular growth of individual preantral follicles, these findings successfully integrate the current knowledge of activin effects as a local factor on early follicular development.

Primary follicles do not respond to gonadotropin, and follicular growth before the preantral or early antral stages is thought to be regulated by local factors independently of gonadotropins (25, 26). Various local factors are expressed in ovarian somatic cells (granulosa and theca cells) and oocytes, which regulate folliculogenesis in a stage-specific manner (27). Several factors from the transforming growth factor β (TGF-β) superfamily appear to play important roles in folliculogenesis (28). One such member, activin is produced in granulosa cells from the early follicular stage, while

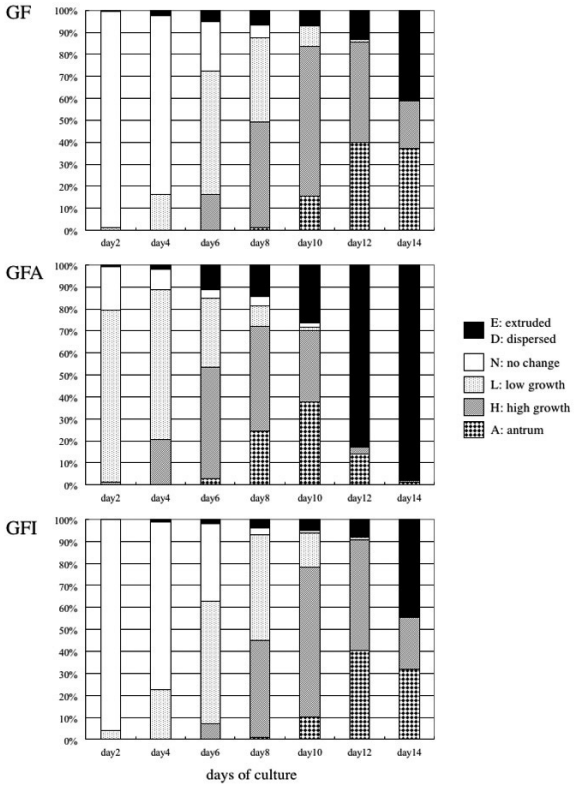

Figure 4. Alterations in the morphological structures of preantral follicles cultured for 14 days in the presence of rh-FSH (100 mIU/mL) (GF), rh-FSH (100 mIU/mL) + rh-ActivinA (100 ng/mL) (GFA), and rh-FSH (100 mIU/mL) + b-Inhibin (1000 ng/mL) (GFI). Morphological changes of the cultured follicles were observed every other day. The proportion of follicles with granulosa cell outgrowth through the basal membrane (Low growth ; L) on day 2 was significantly higher in the GFA group (63.2%) than that in the GF group (1.8%) ($p < 0.01$). Thereafter, the appearance of High growth (H) and Antrum (A) in the GFA group preceded that of the GF group by almost 2 days. *$p < 0.01$ vs. GF (one-way ANOVA followed by the Tukey-Kramer post-hoc test).

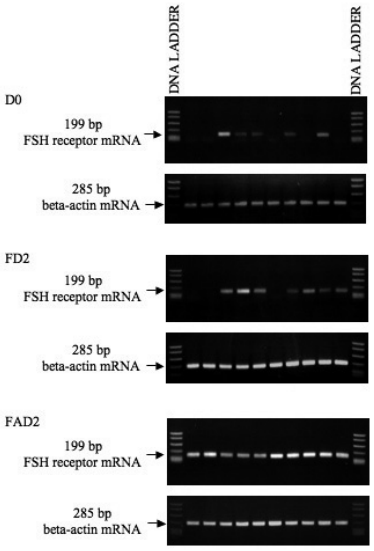

Figure 5. RT-PCR analysis of FSH receptor mRNA expression in granulosa cells of follicles in D0 (n=10), FD2 (n=10), and FAD2 (n=10). An agarose gel showing representative samples from each group (n=10 each) is presented in the figure.

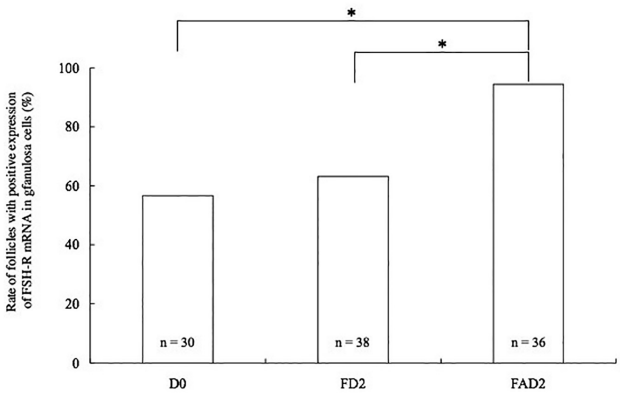

Figure 6. RT-PCR analysis of follicles expressing FSH receptor mRNA in granulosa cells. D0 ; follicles in D0 (n=30). FD2 ; follicles in FD2 (n=38). FAD2 ; follicles in FAD2 (n=36). The proportion of follicles expressing FSH receptor mRNA was significantly higher in FAD2 (94.4%) than in FD2 (63.2%) and in D0 (56.7%). *$p < 0.01$ (chi-square test).

inhibin/follistatin, which counteract the effects of activin, become dominant in granulosa cells according to follicular growth (6-8). From the primary follicle stage, inhibin/activin βA and βB subunit mRNAs (29) and activin receptor type IA, IB, IIA, IIB mRNAs and type IB, IIA, IIB proteins are already expressed in rat ovary (30). Therefore, activin could regulate follicular growth from the primary follicle stage. The autocrine/paracrine actions of activin on granulosa cells were reported as follows ; induction of FSH-R (9, 10) and LH receptor (31), enhancement of FSH induced aromatase activity (32), suppression of follicle atresia and premature luteinization (32). Our analysis of follicular diameter in *in vitro* preantral follicle cultures has clearly demonstrated that activin stimulates follicular growth almost 4 days earlier than the other groups, which is fundamentally in accordance with previous reports (13, 16, 18). Four days later, follicles in the non-activin groups started growing at the same rate as the activin group, and progressed to plateau at the same diameter by day 10 in culture. In the morphology transition, the activin group showed a dramatic change into low or high growth on days 2 and 4, indicating proliferation of the granulosa cell layer ; whereas the non-activin groups exhibited a similar change on days 6 and 8, which is a four day delay that is similar to the delay observed in diameter changes. Furthermore, an approximately four day difference was observed for changes in estradiol concentrations between the activin group and the other groups. This was observed in the first half of the culture period, which is equivalent to the preantral follicle stage. Our data indicated that activin initiated the growth of early preantral follicles. It has been reported that activin receptor type II-deficient female mice have small ovaries and their follicular development is arrested at the early antral stage (33). Furthermore, follistatin transgenic female mice show an arrest of folliculogenesis at various stages of primary or secondary development and/or follicular atresia without corpora lutea (34). Combining these reports with our findings, it appears that activin has an indispensable role in folliculogenesis in the early antral follicle.

FSH-R knockout mice showed follicular arrest at the preantral stage (2, 3), although FSH-R is expressed in granulosa cells at the primary follicle stage (34). Therefore, enhanced expression of FSH-R would be essential for preantral follicles to transit to antral follicle. Activin, which is present in preantral follicles, induces FSH-R in granulose cells (9, 10). In the present study, activin induced the expression of FSH-R mRNA on day 2 of the culture of granulosa cells derived from preantral follicles, whereas FSH-R mRNA was scarce in preantral follicles immediately after collection from 14 day-old mice. The expression of FSH-R is thought to initiate the growth of early preantral follicles in the activin group four days earlier than the non-ancivin groups in our experiment. The non-actvin groups would express endogenous activin under natural control between day 4 and day 6, thereby initiating follicular growth four days later than the activin group. These results indicate that activin induces sensitivity to FSH in preantral follicles by inducing FSH-R expression in granulosa cells of the preantral follicle. Once the follicle acquires sensitivity to FSH, FSH accelerates follicular growth to the antral stage and induces granulosa cells to express aromatase, resulting in greater estrogen production.

Estradiol levels in the activin group showed a marked elevation in the latter half of the culture period, especially in days 12 and 14, although morphological finding, such as dispersion of granulosa cells and ovum extrusion, observed in this period were inconsistent with normal follicular development. This phenomenon might be a result of exposing follicles that have already transited into antral follicles to supraphysiological levels of activin. The activin group was subjected to high levels of activin, which might induce excessive FSH-R, and is expected to exhibit higher expression of aromatase and greater production of estrogen. In comparison, the non-activin groups would undergo a physiological transition from the preantral to antral stage in which endogenous activin expression would be decreased in the latter half of the culture period. Activin is needed for the growth of preantral follicles ; therefore, it might be beneficial to induce such phenomenon *in vitro*. However, excess exposure to activin during the antral follicle stage might induce non-physiological changes or potentially disrupt the follicle structure. This information is important for future clinical applications of follicle culture.

Inhibin is a feedback signal from the ovary to pituitary. Inhibin is produced in granulosa and luteal cells, and suppresses FSH secretion in pituitary gonadotrophs in opposition to the effects of activin (35). Although ovaries are the source of serum inhibin, the effect of inhibin on follicular growth is obscure. Two contradictory reports investigated the intraovarian roles of inhibin *in vivo* (36, 37), with inhibin reported to be a follicle maturation factor (36) or an inducer of follicular apoptosis (37). There are only a limited number of studies describing a possible local action for inhibin *in vitro*. For example, inhibin was reported to enhance androgen production in theca cells (20) and suppress FSH-induced FSH-R promoter activity (21). In the present study, exogenous inhibin did not affect follicle growth in the preantral and antral stages in *in vitro* follicle culture. The concentration of b-inhibin used in the present study was 10 times higher than the dose reported to inhibit FSH-induced FSH-R mRNA expression in rat granulosa cells (21) and 300 times higher than the dose reported to inhibit activin A-induced glycogenolytic glucose output in rat hepatocytes (22). Although it is possible that FSH added in medium might be so strong and that it overwhelms the suppressive effects of inhibin on preantral follicle. Therefore, the effect of inhibin on follicular growth might not be significant, if it exists at all.

In conclusion, activin accelerated the growth of preantral follicles, increasing their size, morphological stage and estradiol synthesis, as well as the expression of FSH-R mRNA in isolated granulosa cells. Furthermore, all of the data in this study was obtained using the same *in vitro* of preantral follicle culture system. These results integrate the fragmented information regarding the effects of activin on follicular development and confirm the physiological role of activin in the ovary being the trigger for initiation of follicular growth, especially during the early stage of preantral follicle development.

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DISCLOSURES

Conflict of interest : The authors have no conflicts of interest to declare.

Human and Animal Rights : This article does not contain any experiments involving human subjects. All of the institutional and national guidelines for the care and use of laboratory animals were followed. The protocol for the research project was approved by a suitably constituted ethics committee.

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