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REVIEW

# Molecular Biology of the Ovarian Follicle

Toshiaki TANAKA*, Eimei SATO**, Tatsuo TAKEYA*** and Koji ITAHANA*

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## 1. Introduction

Mammalian oocytes are surrounded by several layers of cells and grow in ovarian follicles: inner layers of granulosa cells are separated from blood vessels and theca cells by a vasement membrane lining the follicle. Periodical secretion of pituitary gonadotropins such as follicle stimulating hormone (FSH) and leuteinizing hormone (LH) induces oocyte maturation and, after ovulation of matured oocytes, ovarian follicles undergo profound changes in morphology and physiologic properties. Theca interna cells and granulosa cells transform into small and large luteal cells, respectively [1] and blood vessels invade the luteinizing granulosa cell layers to nourish cells, giving rise to corpora lutea ultimately (Fig. 1). Although a large

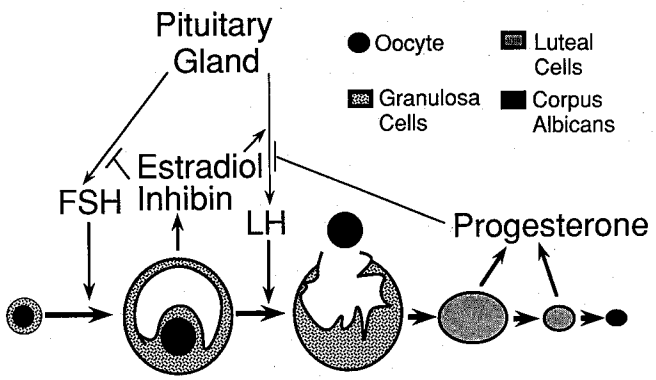



Fig. 1: An endocrine relationship between pituitary gland and ovarian follicles. FSH stimulates the maturation of ovarian follicles. Ovulation induced by LH is a signal for granulosa cells to transform to luteal cells and form the corpus luteum. Gonadotropins from pituitary gland such as FSH and LH stimulates feedback regulation by estradiol, inhibin, and progesterone.

* 田中利明、板鼻康至:Laboratry of Molecular Genetics, Institute for Chemical Research, Kyoto University, Uji, Kyoto 611, Japan
** 佐藤英明: Department of Reproductive and Developmental Biology, The Institute of Medical Science, The University of Tokyo, Minato-ku, Tokyo 108, Japan
*** 竹家達夫:Advanced Institute of Science and Technology (Nara), Takayama, Ikoma, Nara 630-01, Japan

number of follicles are present in a given ovary, only a limited number of follicles are selected and prevailed to further respond to the pituitaly gonadotropins during a given cycle. The way of this selection has been a puzzle for a long time. selected and prevailed to further respond to the pituitary gonadotropins during a given cycle.

Ovarian granulosa cells have attracted profound attentions in various points for past several decades; in particular, they show dramatic transformation to luteal cells in coupled with ovulation and they play essential roles in oocyte development and maturation [2-5]. Granulosa cells from mammalian species can be cultured in vitro in media containing serum [6] or occasionally in serum-free media [7-9] and, under appropriate conditions, granulosa cells retain the ability of progesterone synthesis and luteinization, making this system attractive for not a few cell biologists [6,9-13].

Granulosa cells can be classified into three groups based on their locations inside the follcile [14]. The mural granulosa cells are localized next to basement lamina and are presumed to be most active in various functional aspects. Closer to the antral cavity are the antral granulosa cells, and finally granulosa cells surrounding the oocyte are called cumulus cells. Although these three groups of granulosa cells are derived from common precursor cells in the process of ovary formation, they are diverged enough to respond differently to various stimuli. Besides this stratified structure of granulosa cells, each granulosa cell, cumulus cells and oocytes are connected each other through "gap junctions" [15-19]; this structure enables the transportation of metabolites such as nucleotides [19], sugars [20], amino acids, small peptides [21,22] and low molecular weight substances such as cAMP and Ins(1,4,5)P3 [23,24].

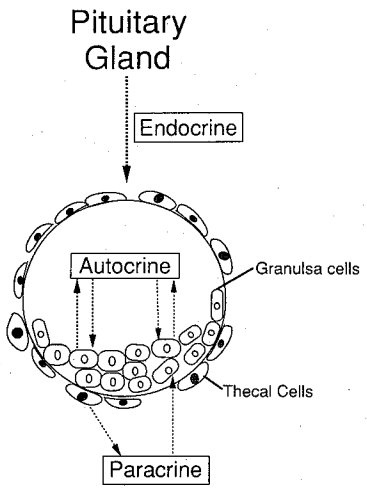



Figure 2: Granulosa cells as a model for hormonal target cell

Ovarian follicles were used to be speculated simply as being regulated by gonadotropins directly, that is "endocrine control". Recently, however, it becomes clear that the development and maturation of ovarian follicles can't be fully achieved without controls carried out in "paracrine or autocrine" fashion through granulosa cells (Fig. 2). This review will focus

on signaling pathways unique to granulosa cells with the aim of understanding the events in ovarian follicles at the molecular level.

### 2. Steriod hormones and steroidogenesis

Steroid hormones play key roles in the development and maturation of ovarian folicles. Granulosa cells and luteal cells produce and secrete two major ovarian steroid hormones:estradiol and progesterone. Estradiol, together with FSH, induces FSH receptors on granulosa cells [14] and also the secretion of gonadotropin releasing hormone (GnRH) at hypothalamus which causes the subsequent release of LH from the pituitary [25,26]. Under the continuous presence of LH, granulosa cells become luteal cells after ovulation which secrets some estradiol, but its predominant secretion is progesterone. Progesterone is known to have an activity to inhibit follicle maturation, thereby these cells prevent more follicles to mature.

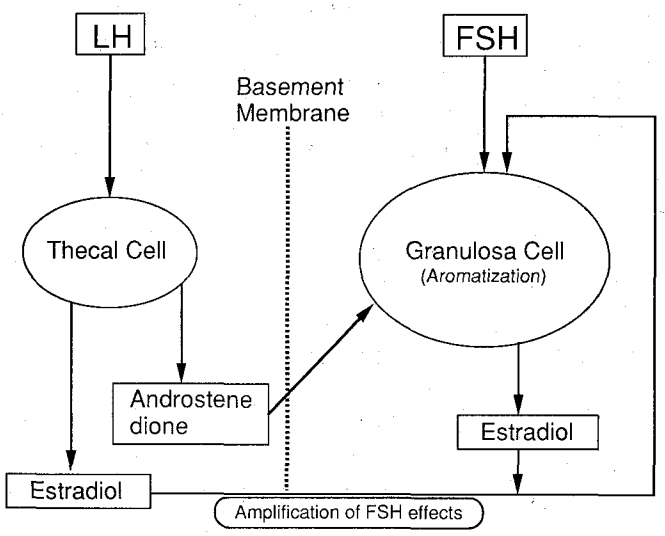



Figure 3: "Two cell, two gonadotropin theory"

In contrast to progesterone whose synthesis is completed within granulosa cells or luteal cells, estradiol requires another cells, theca cells, to go through its synthesis, which has been refered to "two cell, two gonadotropin theory" [27,28] (Fig.3); a cascade reaction (Fig.4) starts by synthesizing androstenedione in thecal cells in which process 17 $\alpha$ -hydroxysteroid dehydrogenase (generally abbreviated as HSD) plays key role and its activity is repressed by LH surge [29]. Androstenedione is then transfered into granulosa cells through basal membrane and is converted to estradiol by P450 aromatase whose activity has been shown to be regulated by FSH [9,29,30].

Progesterone is produced within granulosa cells and luteal cells (Fig.4). The side-chain

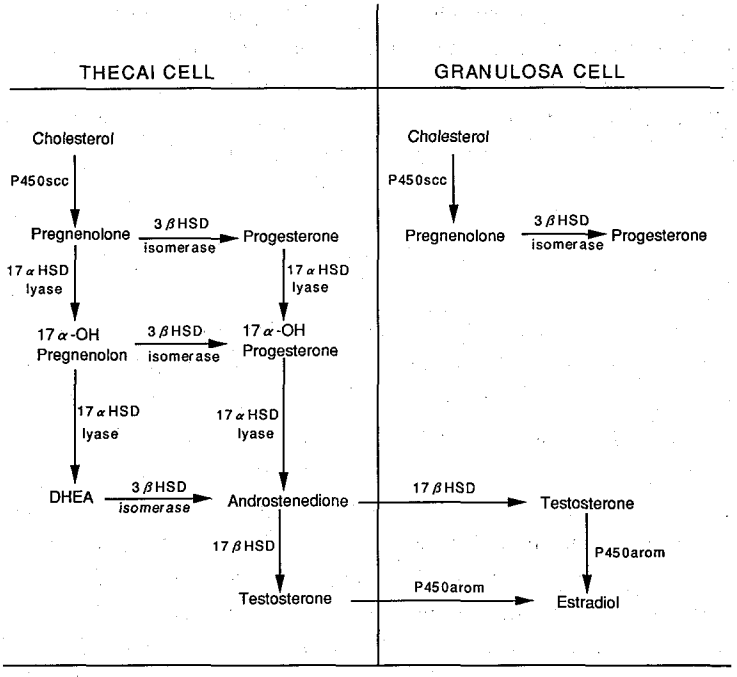



Figure 4: Production of steroid hormone on ovarian follicles. HSD: Hydroxysteroid dehydrogenase is abbreviated.

cleavage of cholesterol is the first and the rate-limiting step in progesterone biosynthesis and this conversion from cholesterol to pregnenolone is catalyzed by mitochondrial enzymes: consisting of the cytochrome P450scc and it's ancillary electron transport proteins, adrenodoxin and adrenodoxin reductase [31,32]. These enzyme activities have been found to be regulated by gonadotropins at transcription level [33-37].

Gonadotropins transduce these signals through transmembrane receptors (described below) to which G-proteins are coupled [38-40]. In fact, it is known that ganadotropin induces intracellular cAMP [41-44] and that chemicals such as forskolin, 8-boromo-cAMP, dibutyryl adenosine 3',5'-monophosphatase induce three mitochondrial genes, p450scc, adrenodoxin, and adrenodoxin reductase [33-37,45], resulting in the production of progesterone [46-48]. On the other hand, after luteinization by the administration of LH or human chorionic gonadotropin (hCG), the activity of mitochondrial scc system is maintained by cAMP-independent mechanism [29,34,49,50] to compensate the constitutive synthesis of large quantity of progesterone in corpora lutea. The regulatory mechanisms of progesterone synthesis, therefore, are defferent between granulosa cells and luteal cells.

cAMP is believed to function through cAMP dependent protein kinase (A-kinase) which is composed of two catalytic subunits (C $\alpha$ and C $\beta$) and two regulatory subunits (RI and RII). The RII subunit is induced in granulosa cells by FSH and estradiol at transcription level [51,52]. It may also be regulated by LH; the expression of RII mRNA significantly increases in granulosa cells till LH surge and decreases dramatically after LH surge. Since estradiol and forkolin [29] can induce the mRNA, cAMP is assumed to regulate the expression of the RII

gene. On the other hand, only minor changes have been observed in RI and C $\alpha$ subunit [29]. This regulatory mechanism of the RII subunit contributes to keep catalytic subunits under the tight control of the R subunit and cAMP, so that follicles can be maintained at synchronized state in cAMP-mediated events such as ovulation and luteinization which occur only in responce to the LH surge and significant increase of intracellular cAMP concentration [29].

### 3. Receptors

***FSH receptors***: The expression of the FSH receptor has been examined mainly by binding activity *in vitro* and *in vivo* [53,54]. The activity appears first in granulosa cells around the primary follicle stage [14,54,55], at which oocytes complete the growing, and the hormone activity increases during prepubertal development [56]. The expression of the FSH receptor is reported to be regulated by estradiol in association wiht FSH *in vivo* [54,57] but not by estradiol alone [54,58].

Through an estrous cycle, FSH binding capacity remains fairly constant with minor fluctuations compared to the LH binding capacity [59,60], and the numbers of FSH receptors decline as follicles mature (in porcine) and ovulate (in human; dominant folicle) [61]. Although the regulatory mechanism of this reduction of FSH receptors is unclear yet, progesterone which is secreted in the process of luteinization is presumed to play a role, at least in porcine *in vitro* system [62]. Further, down regulation of FSH receptors on granulosa cell surface has also been observed by exposing cells continuously to FSH *in vitro* [63,65]; this may have a role to progress the cycle by regulating responsiveness to FSH.

A cDNA clone of the FSH receptor has been isolated from rat sertoli cells [65] and the receptor molecule has been predicted to be highly related to the TSH receptor [66] and the LH receptor [39] which belong to a family of G protein coupled receptors. In fact, it is well known that FSH treatment of granulosa cells increases intracellular cAMP concentration. Further, by using the isolated cDNA clone as a probe, the expression of FSH receptor mRNA was examined [67]; a limited expression of the FSH receptor mRNA was detected in granulosa cells in small follicles and the copy number substantially increased after pregnant mare serum gonadotropin (PMSG) stimulation. The expression of FSH receptor was declined by further treatment of human chorionic gnadotropin (hCG), and this might correspond to a phenomenon observed *in vivo*; the FSH receptor mRNA declines after preovulatory LH surge.

***LH receptors***: The LH receptor is a G protein-coupled transmembrane receptor as described and increases intracellular cAMP concentration upon LH stimulation as expected. LH receptors are known to be induced by FSH *in vivo* [54] and *in vitro* [68,69]. These findings are consistent with the reports that LH receptors can be detected only in granulosa cells from large preovulatory follicles [54,55,70] and that, in contrast, granulosa cells isolated from immature hypophysectamized and estrogen-treated rats showed little LH/hCG binding activity [14].

Quite a few growth factors or hormones have been identified as effectors of the expression of LH receptors. Among them, platelet derived growth factor (PDGF) [71], insulin like growth factor 1 (IGF-1) [72,79], insulin [73,74], estradiol [54,69,75], progesterone [69] and androgens [69] are known to induce LH receptors in association with FSH. On the other hand, epidermal growth factor (EGF) [71,76], basic fibroblast growth factor (bFGF) [71,77] and glucocorticoids [78] antagonize FSH.

How does each effector regulate the expression is not clear yet. However, the possible involvement of cAMP has been proposed by the observation that its analogs (8-Br-cAMP, dbcAMP) [48,80-82] or inducing agents [83,84] of cAMP (cholera toxin, prostaglandin E2, forskolin) could stimulate the expression of functional LH receptors. Synthetic analogs of cAMP generally induced less LH receptors than physiological dose of FSH, suggesting that cAMP independent mechanism, in addition to cAMP dependent mechanism, might be involved in the induction of LH receptors [14]. With regard this, the commitment of C kinase in the pathway has been implicated [77,85].

In contrast to the FSH receptor mRNA which is confined to granulosa cells, LH receptor mRNA can be detected widely in theca cells, granulosa cells and corpora lutea [67]. Moreover, multiple sizes of the LH/CG receptor transcript were detected in gonadal cells, although the significance of this observation has not been elucidated yet [86-89].

***Other receptors***: In addition to gonadotropin receptors, receptors for several other effectors have been reproted to be present in granulosa cells and luteal cells. Prolactin (PRL) is known to be an important luteotropic agent and to inhibit the induction of 20 $\alpha$ -hydroxysteroid dehydrogenase (20 $\alpha$ -HSD) which catalyzes the conversion of active progesterone to less active 20 $\alpha$ -dihydroprogesterone, resulting in the acumulation of progestorone in serum which is essential for maintenance of pregnancy and fetal well being [90,91]. Although immature granulosa cells has few prolactin binding sites prior to stimulation with FSH [14,92], fully grown granulosa cells in large follicles and luteal cells in corpora lutea have a signifucant amount of specific PRL binding sites [14,93,94]. In fact, it was reported that an exposure to exogenous FSH induce PRL receptors in granulosa cells *in vivo* [13,92] and *in vitro* [92,85]. This induction of PRL receptors by FSH is also cAMP dependent, because cAMP analogs [83] and cholera toxin [95] can mimic the induction by FSH. Once induced, the continued presence of FSH, LH or hCG is required to maintain the PRL receptor level [95]. With this regard, it is proposed that Lh/hCG and PRL influence the binding capacity each other positively [14].

GnRH, whose primary target site is the pituitary glands to sitmulate hormonal secretion, has been proposed, surprisingly, to have binding site on granulosa cells and play a role for follicular maturation as well. GnRH inhibits the induction of progestrone synthesis by FSH in granulosa cells and luteal cells [96-98], and also inhibits the induction of LH and PRL receptors *in vitro* [97,98], thereby GnRH seems to have an activity as an antagonist of FSH. On the other hand, a treatment of granulosa cells with GnRH alone can stimulate steroidgenesis [99,100], follicular maturation [101] and ovulation [102,103].

### 4. Non-Steroidal regulators

***Growth Factors***: Several peptide growth factors have been identified as essential regulators for the development and maturation of ovarian follicles. Among them, EGF and bFGF are known to have negative effects for follicular maturation:e.g., they suppress the effect of the induction of LH receptors in cultured granulosa cells [71,76,77]. Generally speaking, ligands which increase intracellular cAMP concentration promote the maturation and enhance the synthesis of LH receptors, PRL receptors, progesterone and estradiol. In contrast, ligands which reduce intracellular cAMP concentration repress the maturation. However, both EGF

and bFGF may exert their inhibitory effect, not only by repressing cAMP formation or reducing cAMP concentration [76,77,104], but by activating the calcium and phospholipid dependent protein kinase C [105].

EGF causes the reduction of estradiol biosynthesis [14], while it stimulates progesterone synthesis [106], indicating that the estradiol and progesterone synthesis are regulated through distinct mechanisms [14]. bFGF, on the other hand, inhibits both progesterone synthesis [107] and estradiol synthesis in bovine granulosa cells [108,109]. Since estradiol is important for ovarian follicular maturation, EGF and bFGF may regulate follicular maturation by reducing both estradiol synthesis and the LH receptor level in granulosa cells.

The induction of receptors for EGF and bFGF are shown to be regulated by gonadotropins; FSH treatment induces the binding sites for both EGF and bFGF in cultured rat granulosa cells without changing their binding affinities [110,111], while LH/hCG reduces the binding sites for EGF. Likewise, treatment with cAMP-inducing agents which mimic FSH function such as 8-bromo-cAMP, forskolin, and choleragen stimulte EGF receptor expression, whereas GnRH or 12-O-tetradecanoyl-phorbol 13-acatate (TPA) which antagonize FSH reduce the expression of the EGF receptors [110]. The induction of EGF and bFGF receptors by FSH imply the presence of autoregulation mechanism for the follicular maturation; FSH promotes follicular maturation, while EGF and bFGF antagonize FSH functions.

PDGF, another peptide growth factor, in known to be secreted by platelet during clot formation and therefore, this growth factor has been proposed to have a role during folliculer rupture and subsequent formation of the corpus hemorrhagicum [71]. In contrast to the inhibitory activities of EGF and bFGF, PDGF has been shown to enhance the FSH stimulation of intracellular cAMP production, steroidogenesis, and LH receptors [71,76,112]. PDGF exerts its effect by maintaining FSH-sensitive adenylate cyclase and reducing phosophodiesterase activity [76].

Recent studies on insulin-like growth factors (IGFs) and its binding proteins (IGFBPs) have revealed regulatory systems in ovaries. Two kinds of IGF, IGF-1 and IGF-2, and six kinds of IGFBP have been identified in serum and other body fluid including ovarian follicular fluid [113-115]. IGF-1/IGF-1 receptor system is also shown to be produced in ovarian granulosa cells and induced by FSH [116-118]. IGF-1, like insulin, is known to promote FSH-mediated differentiation of granulosa cells and induce cAMP production, steroidogenesis, LH receptor and inhibin [72,73,119-121]. Therefore, FSH and IGF-1 synergize to further stimulate follicular maturation. IGF-1, however, at the same time, stimulates the expression of IGFBPs which antagonize FSH and IGF-1, implying the presence of a feedback mechanism among these three factors. IGF-2 mRNA, in the other hand, is detected in cultured human granulosa cells and inducible by FSH and hCG [122], the role of IGF-2 in follicles is not yet clear though.

IGFBP-2 and IGFBP-3 are reported to be repressed by FSH, a derivatives of cAMP and 8-Br cAMP [123]. IGFBP-3 is also regulated by prostaglandin $E_2$ and $F_{2\alpha}$ in luteinized granulosa cells [124]. IGFBP-4 and 5 have a role to reduce the production of estradiol and progesterone [125] and the expression of IGFBP-4 and 5 is regulated by FSH, which negatively controls at transcription level and also stimulates the production of a protease for degradation of IGFBP-4 and 5 [125].

Transforming growth factor-$\beta$ (TGF-$\beta$) has been reported to be produced in murine corpora lutea and to mediate luteotropic action of PRL [126]. TGF-$\beta$ has also been identified

in cultured rat granulosa cells and found to increase both basal and FSH-stimulated inhibin $\alpha$ and $\beta$ A-subinit mRNA level [127]. Furthermore, TGF-$\alpha$ inhibits forskolin-induced 17$\alpha$-hydroxysteorid dehydrogenase activity and androstenedione production in bovine thecal cells [28], thereby TGF-$\beta$ plays a role in ovarian follicles.

***Meiosis Arresting Factor* (*MAF*)**: Mammalian follicular fluid has been reported to contain an activity capable of preventing spontaneous dissolution of germinal vesicle (GVBD) of oocytes and similar activities have been reported to be secreted by granulosa cells [3,128]. Oocyte maturation inhibitor (OMI), one of such inhibitor of GVBD [129], has been isolated from porcine follicular fluid and shown to be a heat-stable peptide with wide species specificities [129]. Another factor, which has been speculated to be secreted by granulosa cells and localized at cell surface, has been identified by Sato *et al* [2] and called meiosis arresting factor (MAF).

Müllerian inhibitory factor (MIF) is also known as an inhibitory factor and is detected in both follicular fluid and garanulosa cells by immunohistochemical research [130-132]. Although the ways these factors affect on the resumption of meiotic arrest are still unclear, they may exert their activities by regulating intracellular cAMP concentration of oocytes, because only the declining of cAMP concentration has been consistent phenomenon associated with the resumption of meiosis [133,134].

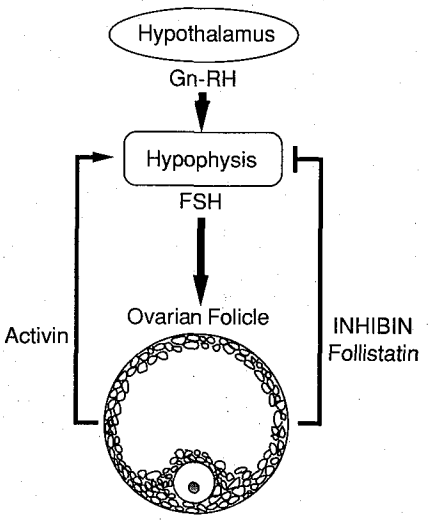



Figure 5: Inhibin, activin, and follistatin as endocrine regulators

***Inhibin and activin***: An administraion of steroid hormones to gonad induces the secretion of water-soluble proteins, known as inhibin and activin, which regulate the hypothalamo-hypophysial system. Inhibin has an activity to suppress the synthesis and secretion of FSH $\beta$-subunit selectively at pituitary [135-138] (Fig. 5). The growth and proliferation of granulosa cells are thus regulated through such feedback mechanism by FSH and inhibin.

Two forms of inhibins have been purified from porcine follicular fluid [139]; each form is a heterodimer comprised of common $\alpha$-subunit and a distinct $\beta$-subunit, that is, $\beta$A or $\beta$

B-subunit. The former one, $\alpha\beta$A, is called inhibin A and the latter one, $\alpha\beta$B, is called inhibin B. $\beta$A and $\beta$B subunits share 70% similarity each other in their primary structures and much less similarity is present between $\alpha$ and $\beta$[140].

In the ovary, the $\alpha$-subunit mRNA is expressed always much more abundantly than the $\beta$-subunit mRNA [143,144], because the inhibin $\alpha$-subunit is expressed not only in the granulosa cells but in thecal cells and ovarian stroma, while the inhibin $\beta$A-subunit is expressed only in granulosa cells in maturating follicles [145]. The cellular content of mRNA coding for each subunit is regulated by variety of factors. PMSG was reported to increase the expression of the inhibin $\alpha$ and $\beta$ subunit 141) and FSH, LH and TGF-$\beta$1 were found to have the same activities in cultured rat granulosa cells [127,142] EGF, bFGF and GnRH, on the other hand, which are known to suppress granulosa cell maturation (LH receptor expression steroidogenesis, and so on) repress the expression of the $\alpha$ and $\beta$A subunits [127]. IGF-I and PRL have no significant effect on inhibin expression. Forskolin can mimic FSH and increases the inhibin subunit mRNA level [127], indicating the involvement of signaling pathway via cAMP dependent protein kinase(s).

The dimerization of the inhibin $\beta$A- and $\beta$B-subunits gives rise to the three formes of activin: A ($\beta$A-$\beta$A), B($\beta$B-$\beta$B), and AB ($\beta$A-$\beta$B) [139,146-148]. Although activin A [147] and AB [148] have been purified, activin B has not been isolated nor fully characterized yet. Interestingly, despite of sharing common $\beta$-subunit, biological activities of activin are opposite to that of inhibin. As this $\beta$-subunit has similarity with TGF-$\beta$ [149], which also possesses FSH-releasing activity [150], activin and inhibin are classified as members of TGF-$\beta$ family.

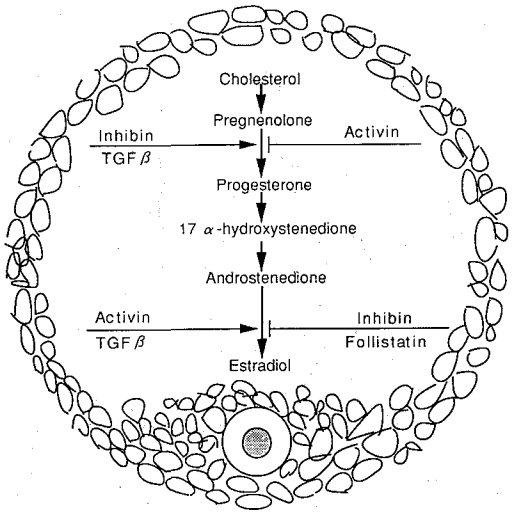



Figure 6: Inhibin, activin, and TGF-$\beta$, as autocrine/paracrine regulators

Both inhibin and activin function mostly in endocrine fashion, but it is also known that these factors exert their effects in autocrine or paracrine manner as well. Inhibin, for example, has been reported to repress the biosynthesis of estradiol in granulosa cells through the inhibition of P450aromatase activity [151] and to augment progesterone secretion in cultured

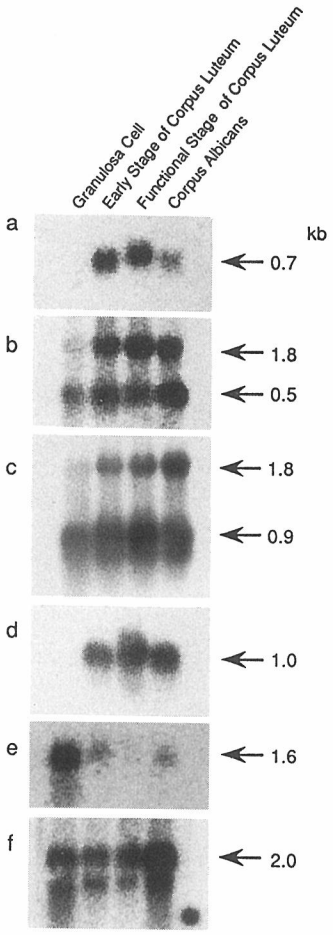
granulosa cells [152,146], whereas activin has been shown to enhance P450aromatse activity and inhibit progesterone synthesis [146,151,152]. On the other hand, TGF-$\beta$ has dual activities; it enhances both P450aromatse activity and progesterone synthesis [151-153]. Futhermore. activin increases both basal and FSH-stimulated expression of inhibin in cultured rat granulosa cells, while inhibin has no effect on activin expression [154]. These relationships are summarized schematically in Figure 6.

Proteins capable of binding to inhibin and activin have been identified in serum and ovarian follicular fluid. Follistatin in follicular fluid is one of such proteins [155,156] and follistatin inhibits pituitary release of FSH [146,156]. Follistatin is reported to be expressed in the same tissue where activin and inhibin are expressed [157-160]. Another inhibin/activin binding protein, $\alpha$ 2M, has been identified in human serum. Although $\alpha$ 2M can bind to both activin and inhibin, it has no effect on the biological activities of inhibin and activin as far as tested [156].

### 5. Seaching for Stage-Specific Genes

As described above, a variety of growth factors or substances have been revealed to play important roles in the process of development and maturation of ovarian follicles. It is getting clearer that these ligands exert their signals ultimately by regulating the expression of essential genes either positively or negatively. Therefore, an identification of a group of such genes may be crucial to understand the follicular development and maturaion at the molecular level. Tanaka *et al.* constructed cDNA libraries from porcine granulosa cells as well as corpus luteum, and identified a group of stage- and tissue-specific genes by differential screening. Among clones so far characterized, the clone TS543, which was found to be expressed preferentially at early and functional stages of the corpus luteum by Northern blot analysis [161] (Fig.7A), turned out to encode a counterpart of human collagenase inhibitor [162] by DNA sequence analysis. The product was localized in the capsule of corpus luteum as well as in the connective tissue around blood vessels (Fig. 7B). Tissue inhibitor of metalloproteinase (TIMP), including the collagenase inhibitor, are proposed to be induced by LH or steroid hormone [163,164] and the enzyme activities or mRNA can be detected in fully matured follicular fluid 164) or in corpus luteum [165]. Taken together, it seems likely that TIMP including collagenase inhibitor regulates metalloproteinae activity upon luteinization, ovulation, or corpus luteum formation. In fact, it is proposed that the apical collagenous matrix of the preovulatory follicle may decrease partly due to proteolytic enzymes such as the metalloproteinase [163,166,167]. In addition, plasminogen activator is reported to be synthesized in granulosa cells [111,168] and to play a role in ovulation [169].

Three other cDNA clones specific to the corpus luteum have been characterized so far (Fig. 7A, panel b, c, d, and e), and two of them were found to encode mitochondrial genes (to be published elsewhere). Since mitochondria play essential roles in the process of luteinization and in the maintenance of corpus luteum as described above, characterization of these gene products will reveal important aspects of luteinization.




Firure 7A: Northern blot analysis for isolated cDNA clones. The right number shows predicted size of each signal from ribosomal RNA. Used probes are as follows. a:TS543 cDNA clone, b:TS537 cDNA clone, c:TS049 cDNA clone, d:TS507 cDNA clone, e:TS052 cDNA clone, f:control ($\beta$-actin)

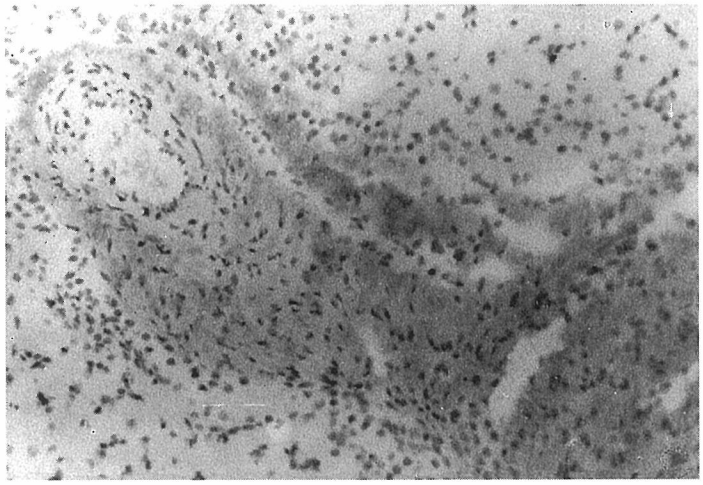


Figure 7B: In situ hybridization for collagenase inhibitor. An ovary at functional stage was used.

### 6. Conclusions

Many investigators have used granulosa cells as a model system to understand cellular transformation as well as steroidogenesis. However, there still exist many interesting questions to be resolved in this field; e.g., the way of oocyte maturation is regulated by granulosa cells in a manner of intercellular responses, the identification of genes that trigger luteinization and meiotic resumption and so on. Making use of established granulosa cell lines may be useful to this end [170,171] (T. Tanaka, unpublished).

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