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Molecular and cellular mechanisms for the regulation of ovarian follicular function in cows

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Abstract. Ovary is an important organ that houses the oocytes (reproductive cell). Oocyte growth depends on the function of follicular cells such as the granulosa and theca cells. Two-cell two gonadotropin systems are associated with oocyte growth and follicular cell functions. In addition to these systems, it is also known that several growth factors regulate oocyte growth and follicular cell functions. Vascular endothelial growth factor (VEGF) is involved in thecal vasculature during follicular development and the suppression of granulosa cell apoptosis. Metabolic factors such as insulin, growth hormone (GH) and insulin-like growth factor 1 (IGF-1) also play critical roles in the process of follicular development and growth. These factors are associated not only with follicular development, but also with follicular cell function. Steroid hormones (estrogens, androgens, and progestins) that are secreted from follicular cells influence the function of the female genital tract and its affect the susceptibility to bacterial infection. This review covers our current understanding of the mechanisms by which gonadotrophins and/or steroid hormones regulate the growth factors in the follicular cells of the bovine ovary. In addition, this review describes the effect of endotoxin on the function of follicular cells.

Key words: Endotoxin, Metabolic factors, Ovarian follicle, Steroid hormone, Vascular endothelial growth factor (VEGF)

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The mammalian ovary has two functional roles, steroidogenesis and gametogenesis. These functions depend on the activity of ovarian follicles that consist of follicular cells and oocytes. The activity and development of ovarian follicles are regulated by gonadotropins secreted from the hypothalamus-pituitary axis. During follicular development, follicular cells such as granulosa and theca cells differentiate into endocrine cells and secrete estrogens and progestins. Follicle-stimulating hormone (FSH) from the pituitary stimulates ovarian follicular development and promotes estradiol production by granulosa cells in coordination with luteinizing hormone (LH). During the estrous cycle, the ovary contains primordial, primary, secondary, and tertiary follicles, but follicle development that is dependent on gonadotropins occurs from the secondary follicle stage to the ovulatory phase. Some secondary follicles enter to gonadotrophin-dependent development upon FSH stimulation. This process is called follicular recruitment. The recruited secondary follicles develop into tertiary follicles that have the follicular antrum. Of these tertiary follicles, one follicle develops to reach the ovulatory phase and the others undergo atresia. In addition to gonadotropins, growth factors and cytokines are also associated with such follicular development.

In cattle, follicular development begins with the primary follicles that have a layer of 11–20 cuboidal granulosa cells around the oocyte [1, 2]. At the secondary follicle stage, the follicles gain a second layer of granulosa cells [3], and become responsive to gonadotropins. In fact, mRNA of the *FSH* receptor mRNA is expressed in the granulosa cells of the secondary follicles in cattle [4, 5]. At the tertiary follicle stage, the follicular cells such as the granulosa and theca cells proliferate and differentiate into endocrine cells. These follicles form an antral cavity that is filled with the fluid [3], and the *LH* receptor gene is expressed in the theca cells [4].

The study of follicular dynamics in cattle has gained momentum in the last two decades through the utilization of instruments that have allowed serial, non-invasive inspection. Transrectal and transvaginal ultrasonography have led to the examination of the character of follicular deviation by providing information regarding individual ovarian follicles in dairy cattle. In the bovine estrous cycle, there are either two or three follicular waves [6, 7]. Follicle deviation is an important system of follicle selection in dairy cattle, and the largest follicle apparently has dominance before the subordinate follicle reaches a similar diameter [6, 8, 9]. Generally, it is thought that gonadotropins and growth factors are related to follicle deviation in cattle [6, 8]. Since the activity and function of follicular cells (granulosa and theca) is associated with the deviation process, several factors that activate these cells determine the fate of the follicle.

Estradiol and progesterone secreted from follicular cells both have counter and supplementary effects on the female genital tract. Estradiol stimulates epithelialization and vascularization of the endometrium [10]. Progesterone supports the differentiation of endometrial glands and stimulates uterine gland secretions, decreases cervical mucus production, and disturbs uterine contractility [11]. Cattle are resistant to uterine infections when the plasma concentration of progesterone is low, whereas they are susceptible when the plasma concentration

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of progesterone increases [12]. In cattle, postpartum uterine infection do not usually develop after the formation of the first corpus luteum, although bacterial infection can be sufficient to induce the onset of puerperal metritis when the progesterone plasma concentration is at a basal level [13, 14].

The purpose of this review is to highlight the effect of gonadotropins and steroids on growth factors (angiogenic and metabolic factors) that are associated with follicular development and growth, and to provide information on follicle function in cow with uterine inflammatory disease.

Physiological Functions of the Ovarian Follicle

Vascular endothelial growth factor (VEGF) and follicular functions

The female reproductive organs undergo cyclic changes that are associated with intense angiogenesis [15]. Of these reproductive organs, the process of cyclic vascular formation in the ovary is well-studied [15–18]. In addition, the ovary was the first organ wherein VEGF, the most important angiogenic factor such as VEGF was first characterized [19, 20]. The VEGF family comprises of five members in mammals (Table 1): VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PIGF). Several isoforms of VEGF-A are generated by alternative splicing of a single of mRNA which transcribes the 8-exon the *VEGFA* gene (Fig. 1). Moreover, alternate splicing of exon 6 and 7 changes the heparin-binding affinity and amino acid number of VEGF-A (in humans: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$; the rodent and bovine orthologs of these proteins contain one fewer amino acid).

VEGF mRNA is detected in the preovulatory follicles of monkeys and rats [19, 21]. Increased expression of the *VEGF* gene is observed in granulosa cells of the tertiary follicles [22]. In contrast, in atretic follicles show reduced expression of VEGF in granulosa cells and undetectable levels in the theca cells [22]. The rodent and bovine ovary show the expression of *VEGF 120* and *VEGF 164* [23, 24], which are associated with follicular vasculature during follicular development [25, 26]. *In vivo* injection of the *VEGF* gene or protein induces the appearance of a large number of preovulatory and antral follicles [26–28]. These results indicate that VEGF is an important factor that promotes follicular development in the ovary.

The endocrine environment influences the cyclic processes in the ovary. Estrogens induce vascular formation *in vivo* [29, 30], and anti-estrogens exert angio-inhibitory activity [30]. Estradiol induces the expression of *VEGF 120* gene but not of *VEGF 164* gene in bovine granulosa cells *in vitro* [31]. On the other hand, progesterone stimulates the expression of *VEGF 120* gene and inhibits *VEGF 164* gene expression in cultured bovine granulosa cells [31]. These results suggest that VEGF isoforms are differentially expressed during follicular development in dairy cows. The expression of *VEGF 120* and *VEGF 164* is enhanced in the granulosa cells of follicles from eCG-treated porcine ovaries [25]. FSH induces mRNA expressions of *VEGF120*, *VEGF164,* and *Flk-1* in the granulosa cells *in vitro* [31]. Interestingly, the expression of the *VEGF164* was induced by low concentration of FSH (1 ng/ml), whereas the expression of *VEGF 120* was induced by high FSH concentration (10 ng/ml). These results suggest that FSH may influence the abundance of VEGF isoforms in granulosa cells. However, the mechanism of FSH-mediated regulation of VEGF isoforms in granulosa cells is still unknown.

In the mammalian ovary, apoptosis of granulosa cells is associated with induction of follicular atresia [32, 33]. VEGF reduces the apoptosis of bovine granulosa cells *in vitro* [34, 35]. VEGF suppresses apoptosis of vascular endothelial cells by regulating the Bcl-2 family [36, 37]. The members of the Bcl-2 family can be classified as anti-apoptotic factors (such as Bcl-2 and Bcl-xL) and pro-apoptotic factors (such as Bax). These factors control the permeability of the mitochondrial membrane by each interaction, and regulate the release of apoptosis inducers (such as cytochrome c, and Smac/Diablo) from the mitochondria to the cytosol. Release of mitochondrial apoptotic inducers into the cytosol can result in the activation of the caspase cascade, resulting in the apoptosis. VEGF suppresses the apoptosis of granulosa cells by inhibiting the release of caspase activated DNase (CAD) without being associated with mitochondrial pathway [38]. In addition, VEGF does not induce and suppress the *bcl-xL* and *bax*, respectively, in the granulosa cells, and does not inhibit the expression of active caspase-3, which is inhibited by FSH. These results suggest that VEGF may not only be associated directly with vascular formation but may also participate in granulosa cell function during follicle development.

Metabolic factors and follicular functions

Metabolic factors such as insulin-like growth factor (IGF) and growth hormone (GH) have crucial role in follicle development and in the process of follicular atresia [39–42]. GH influences several organs, directly and/or indirectly, in cooperation with IGF-1, with mediates the indirect actions of GH. Thus, GH, as well as systemic and locally produced IGF-1, can regulate follicular development and growth in the ovary.

IGF-1: The expressions of IGF-1 gene and protein are observed in bovine granulosa and theca cells, and this expression shows a tendency to increase the levels during the final follicular phase [43]. The existence of follicular IGF-1 production [43–46] suggests that IGF-1 plays a crucial role in follicle development. However, other studies have reported that neither IGF-1 mRNA nor protein were observed in granulosa and theca cells of follicles at any of the developmental stages in the bovine ovary [47, 48]. In contrast, IGF-1 concentration in follicular fluid was higher in dominant follicles compared to the second large follicles [48–50], and was higher in follicular fluid from estrogen-active follicles than in follicular fluid from estrogen-inactive follicles in the bovine ovary [48, 51]. On comparing the concentration of follicular IGF-1 with that in plasma, even though follicular fluid and plasma IGF-1 concentrations are highly correlated, its values are lower in follicular fluid compared with those in circulation [51–53]. Therefore, circulating IGF-1 may contribute to the accumulation of follicular IGF-1, and influence the function of follicular cell.

IGFs mainly act through the IGF receptor type 1 (IGFR-1) and the binding of IGFs to the receptor is modulated by the IGF-binding proteins (IGFBPs). The changes in IGF-1 in the follicular fluid appear through altered expression of the IGFBPs gene [54] and proteolysis [55, 56]. High *IGFR-1* expression is observed in the granulosa cells of healthy follicles at different developmental stages in the bovine ovary [48, 57]. IGF-1 shows direct mitogenic effects on endothelial

Table 1. VEGF family

Type	Function	Receptor
VEGF-A	Angiogenesis	VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1), neuropilin-1
VEGF-B	Embryonic angiogenesis	VEGFR-1 (Flt-1)
VEGF-C	Lymphangiogenesis	VEGFR-2 (KDR/Flk-1), VEGFR-3 (Flt-4)
VEGF-D	Endothelial cell proliferation	VEGFR-2 (KDR/Flk-1), VEGFR-3 (Flt-4)
PIGF	Endothelial cell proliferation	VEGFR-1 (Flt-1)

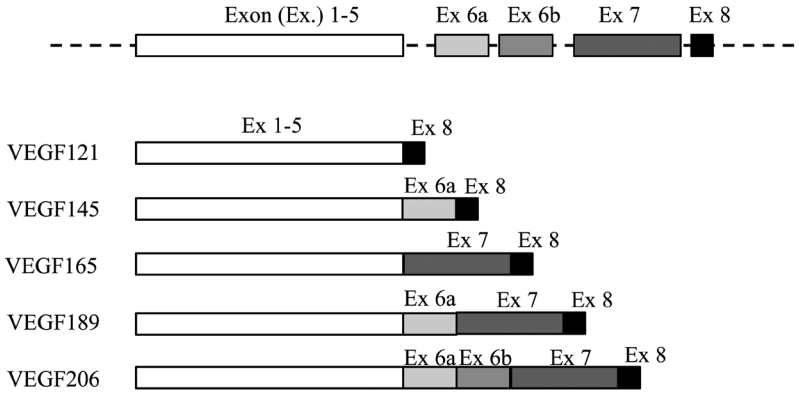

Fig. 1. Gene structure of VEGF-A. The VEGF-A gene consists of eight exons that give rise to five isoforms of 121, 145, 165, 189 and 206 amino acids through differential splicing.

cells and enhances endothelial proliferation [58, 59]. Thus, IGF-1 may enhance granulosa cell proliferation via IGFR-1 and have positive effects on follicular development in the bovine ovary.

Pregnancy-associated plasma protein-A (PAPP-A) is a 200 kDa metalloprotease identified as an IGFBP-4 protease and is an important regulator of IGF bioavailability. The expression of *PAPP-A* gene is observed in granulosa and theca cells [60–62] and the PAPP-A protein is detected in follicular fluid [60, 63, 64]. FSH induces proteolytic activity of PAPP-A in bovine follicular fluid [55] and PAPP-A-like activity appears concomitantly with increased estradiol (E2) during the follicular phase [62]. In addition, FSH induced *PAPP-A* mRNA expression in bovine granulosa cells [48]. Thus, FSH induces *PAPP-A* expression in granulosa cells, and estradiol may support the action of FSH in bovine granulosa cells. Therefore, follicle with high PAPP-A activity due to the action of FSH might be able to develop to the ovulatory phase.

GH: GH protein consists of a single-chain that possesses two sites for interaction with the GH receptor (GHR) [65]. Liver tissue expresses a large amount of GHR, but the expression of *GHR* is also observed in the ovary. GHR expression is observed in oocytes, and in granulosa and theca cells in the rat ovary [66]. In addition, the expression of *GHR* gene has been detected in rat secondary follicles [39]. In the bovine ovary, GH protein in follicular fluid is detected in estrogen-active dominant follicles and in preovulatory follicles [41]. On the other hand, the *GHR* gene was expressed in the granulosa cells, thecal cells and luteal cells of the bovine ovary [41, 67]. Cell-specific expression of *GHR* is observed during the ovarian cycle [41, 67]. These reports suggest that *GHR* expression is affected by gonadotrophin and steroid hormone. In fact, *GHR* expression in bovine granulosa cells treated with FSH alone or with E2+FSH is significantly higher than in untreated granulosa cells [41]. These results suggest that FSH may be the main regulator of *GHR* expression in granulosa cells. Thus, the expression density of *GHR* in granulosa and theca cells may be associated with follicular deviation to move the follicle toward the ovulatory phase.

Treatment with exogenous GH has noteworthy effects on follicular growth [68, 69] and the function of the corpus luteum [70] in cattle. Since GH suppresses the dominant follicular development and enhances the growth of subordinate follicles, GH may selectively induce particular follicle populations, in heifers [71]. In addition, murine secondary follicles that are cultured with bovine GH showed stimulated proliferation of theca and granulosa cells [72]. Moreover, GH stimulates steroid production in cultured granulosa cells from the antral follicles of rats [73] and cows [74]. Therefore, since GH has positive effects on follicular function, follicles having a large amount of GH in the follicular fluid might be able to develop to the ovulatory phase.

GH in follicular fluid not only influences granulosa cell functions, but also influences oocyte functions. *In vitro* maturation of bovine oocytes, using cumulus-oocyte complexes (COCs) from small sized follicles that are cultured with bovine GH accelerated the process of germinal vesicle (GV) breakdown [75]. Moreover, the number of MII oocytes was increased in oocytes that are cultured with bovine GH compared with untreated oocytes [75]. Thus, GH has positive effects on oocyte maturation. Therefore, oocyte matured within follicles that contain a large amount of GH in the follicular

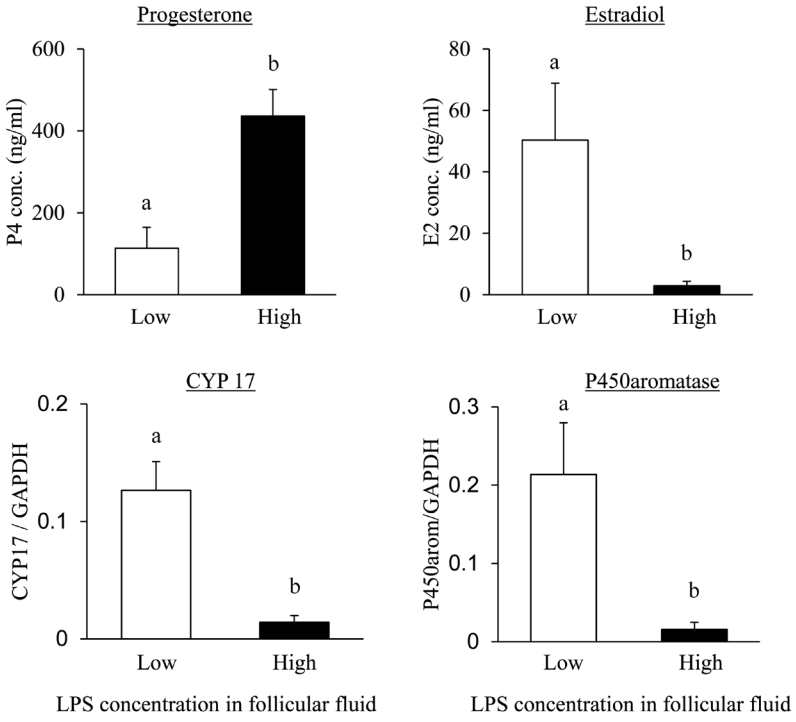

Fig. 2. Concentration of progesterone and estradiol, *CYP17* gene expression in theca cells, and *P450aromatase* gene expression in granulosa cells of large follicles with high or low LPS concentration in the follicular fluid. Cows with a follicular fluid LPS concentration of < 0.5 EU/ml were categorized as 'low' (white bar, n = 13) and those with a concentration greater than 0.5 EU/ml were categorized as 'high' (black bar, n = 13). All values are shown as mean ± SEM. Values with different letters (a, b) are different between groups ($P < 0.05$). Graphs redrawn from Magata *et al*. [81].

fluid may possess high susceptibility to fertilization after ovulation.

Pathophysiological Functions of Ovarian Follicle

Ovarian follicular functions and inflammatory uterine disease

Infection with gram-negative bacteria, such as *Escherichia coli (E. coli)*, *Salmonella* and *Pseudomonas* tend to occur frequently in farm animals. In dairy cows, uterine infection after parturition results in metritis in 40% of the animals and is associated with low fertility [76]. *E. coli* is a gram-negative bacterium that induces uterine inflammatory conditions such as metritis and endometritis. In addition, much of the tissue pathology is associated with the bacterial endotoxin, lipopolysaccharide (LPS) that is part of the bacterial cell wall.

Relationships between follicular cell functions and LPS in follicular fluid: LPS was detected in the plasma, uterine fluid [77], and follicular fluid of cows with metritis [78, 79]. In follicles with a high level of LPS, the concentration of E2 was lower and that of progesterone (P4) was higher when compared to those in follicles with a low level of LPS (Fig. 2) [79]. Moreover, the expression of *CYP17* gene in theca cells and *P450aromate* in granulosa cells was lower in follicles with a high level of LPS compared to follicles with a low level of LPS (Fig. 2) [79]. CYP17 converts P4 into androstenedione (A4), which is transferred to the granulosa cells and is then metabolized to E2 by P450aromatase. Thus, the reduction of E2 concentration in follicles with a high level of LPS may depend on two processes described below: first, the production of A4 in theca cells is suppressed due to the downregulation of the CYP17 enzyme by LPS, leading to a lack of substrate for E2 production in granulosa cells; second, E2 production in granulosa cells is disturbed due to the downregulation of *P450aromatase* by LPS (Fig. 3). In contrast to E2, the concentration of P4 was higher in follicles with high LPS level, even though the mRNA expression of steroidogenesis-related enzymes for P4 synthesis (i.e. *StAR*, *P450scc*, and *3β-HSD*) was mostly unchanged in both the theca and granulosa cells. It was speculated that the high P4 concentration in follicles with high levels of LPS was not due to an increase in P4 synthesis. Instead, decreased expression of *CYP17* in theca cells may have contributed to increased P4 concentration in the follicular fluid by impairing the conversion of P4 to A4, resulting in accumulation of P4 (Fig. 3). LH stimulates the production of P4 and A4 in theca cells, and FSH stimulates E2 production in granulosa cells through the activation of cAMP signalling, which upregulates the transcription of steroidogenic enzymes [80, 81]. Our data indicated lower mRNA expression of these gonadotropin receptors both in theca and granulosa cells of follicles with high LPS levels [79]. These results indicate that LPS may reduce the ability of follicles to respond to gonadotropins and perturb the stimulation of steroidogenesis.

Effect of LPS on follicular cell function: Toll-like receptors (TLRs), which are present on the membrane of immune cells, recognize pathogen-associated molecules [82, 83] and commitment to TLRs initiates a signalling cascade that stimulates the production

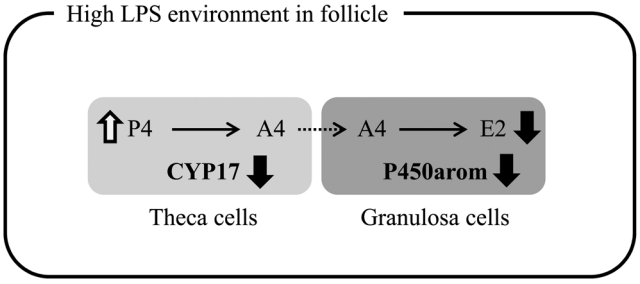

Fig. 3. Modulation of progesterone and estradiol production by LPS in large bovine follicle. LPS inhibits the expression of *CYP17* and *P450aromatase* gene in follicular cells and affects steroid production.

of cytokines which coordinate immune response [82, 84]. The main receptor for LPS recognition is TLR4 [85]. The expression of *TLR4* is observed in the granulosa and theca cells of follicles in the bovine ovary [86, 87]. Thus, granulosa and theca cells have the potential ability to recognize LPS. LPS suppressed E2 production in granulosa cells, and production of P4 and A4 in theca cells of large and small follicles [78, 79, 86]. In addition, LPS also suppressed the expression of steroidogenesis-related genes in granulosa and theca cells [86, 87]. These evidences indicate that LPS influences the functions of theca and granulosa cells: in theca cells, LPS inhibits A4 production, leading to a lack of substrate for E2 production, and in granulosa cells, E2 production is further suppressed by LPS. Thus, these results suggest that LPS might induce ovarian dysfunction through impairment of steroid production and reduce fertility in cows with postpartum uterine infection.

Concluding Remarks

In domestic animal, several infectious diseases develop after calving and affect ovarian function. The treatment of the infectious disease such as endometiritis and metritis in dairy cow should be directed towards improving fertility. However, it is difficult to acquire good physiological condition after antibiotic and/or hormonal treatments that improve these symptoms. Thus, we need to focus on improving animal health and fertility by promoting a good reproductive management rather than by relying on the widespread use of exogenous substances (antibiotics and hormones). Therefore, further studies regarding ovarian physiology and pathophysiology are necessary for the treatment of infectious diseases in dairy cows.

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