

SRD Outstanding Research Award 2015

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

Takashi SHIMIZU¹⁾

¹⁾Graduate School of Animal and Food Hygiene, Obihiro University of Agriculture and Veterinary Medicine, Hokkaido 080-8555, Japan

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) (J. Reprod. Dev. 62: 323–329, 2016)

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

Received: March 25, 2016

Accepted: April 4, 2016

Published online in J-STAGE: April 21, 2016

©2016 by the Society for Reproduction and Development

Correspondence: T Shimizu (e-mail: shimizut@obihiro.ac.jp)

This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License <<http://creativecommons.org/licenses/by-nc-nd/4.0/>>.

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 (PlGF). 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: VEGF₁₂₁, VEGF₁₄₅, VEGF₁₆₅, VEGF₁₈₉, VEGF₂₀₆; 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

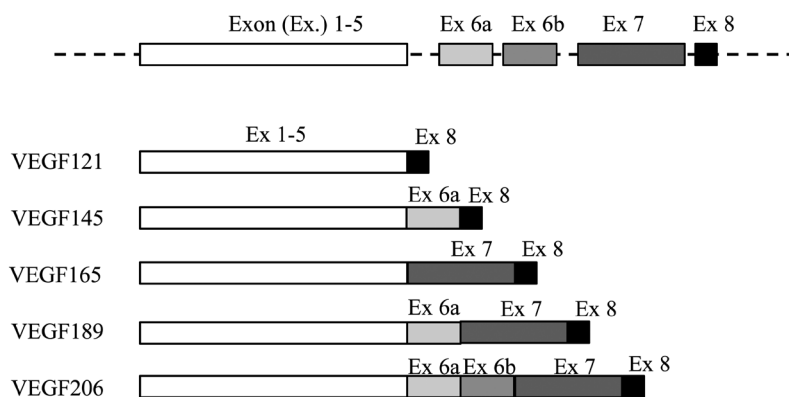
Metabolic factors such as insulin-like growth factor (IGF) and growth hormone (GH) have crucial role in follicle development and in the process of follicular atresia [39–42]. GH influences several organs, directly and/or indirectly, in cooperation with IGF-1, with mediates the indirect actions of GH.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

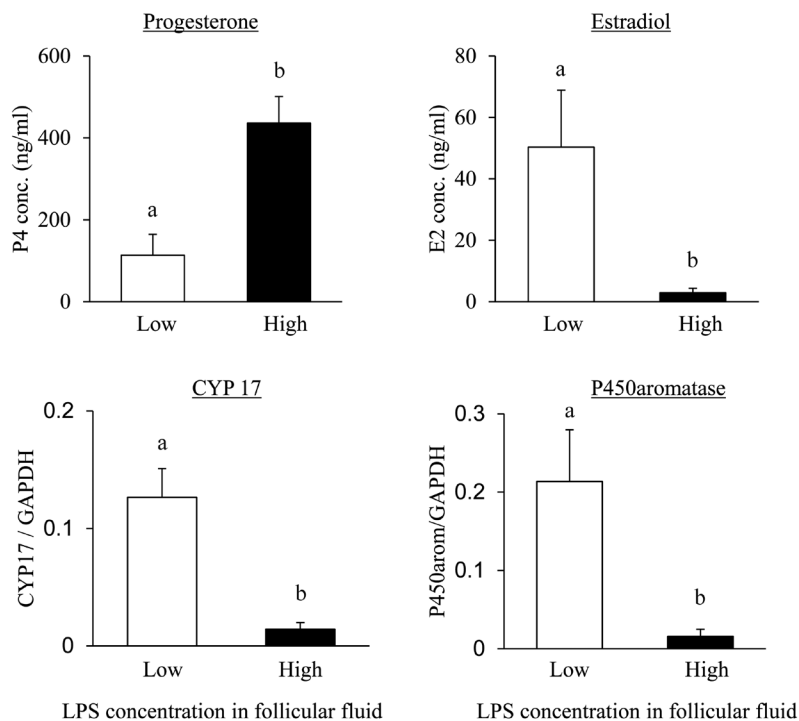


Fig. 2. Concentration of progesterone and estradiol, *CYP17* gene expression in theca cells, and *P450aromatase* gene expression in granulosa cells of large follicles with high or low LPS concentration in the follicular fluid. Cows with a follicular fluid LPS concentration of < 0.5 EU/ml were categorized 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 \pm 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 *3 β -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

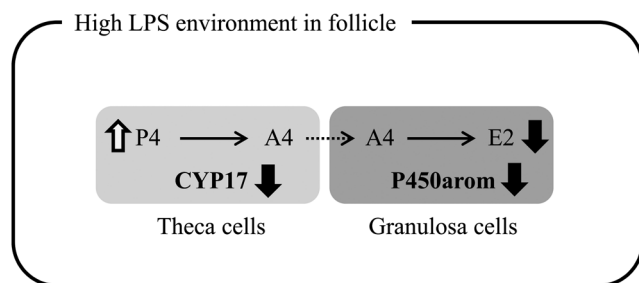


Fig. 3. Modulation of progesterone and estradiol production by LPS in large bovine follicle. LPS inhibits the expression of *CYP17* and *P450aromatase* gene in follicular cells and affects steroid production.

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 endometritis 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.

Acknowledgment

I would like to express my gratitude to the Society for Reproduction and Development (SRD) for awarding me the SRD Outstanding Research Award in 2015. I am also very grateful to Dr A Miyamoto and Dr M Matsui for their valuable suggestion and kind advice concerning my research. I am thankful to Dr M Tetsuka, S Haneda and C Kawashima for their kind cooperation. Finally, I would really like to thank all the students in our laboratory very much for their technical assistance and helpful suggestions.

References

1. Fair T, Hulshof SC, Hyttel P, Greve T, Boland M. Oocyte ultrastructure in bovine primordial to early tertiary follicles. *Anat Embryol (Berl)* 1997; **195**: 327–336. [Medline] [CrossRef]
2. Braw-Tal R, Yosefi S. Studies *in vivo* and *in vitro* on the initiation of follicle growth in the bovine ovary. *J Reprod Fertil* 1997; **109**: 165–171. [Medline] [CrossRef]
3. Driancourt MA. Follicular dynamics in sheep and cattle. *Theriogenology* 1991; **35**: 55–79. [CrossRef]
4. Xu Z, Garverick HA, Smith GW, Smith MF, Hamilton SA, Youngquist RS. Expression of follicle-stimulating hormone and luteinizing hormone receptor messenger ribonucleic acids in bovine follicles during the first follicular wave. *Biol Reprod* 1995; **53**: 951–957. [Medline] [CrossRef]
5. Bao B, Garverick HA. Expression of steroidogenic enzyme and gonadotropin receptor genes in bovine follicles during ovarian follicular waves: a review. *J Anim Sci* 1998; **76**: 1903–1921. [Medline]
6. Kulick LJ, Bergfelt DR, Kot K, Ginther OJ. Follicle selection in cattle: follicle deviation and codominance within sequential waves. *Biol Reprod* 2001; **65**: 839–846. [Medline] [CrossRef]
7. Ginther OJ, Wiltbank MC, Fricke PM, Gibbons JR, Kot K. Selection of the dominant follicle in cattle. *Biol Reprod* 1996; **55**: 1187–1194. [Medline] [CrossRef]
8. Ginther OJ, Kot K, Kulick LJ, Wiltbank MC. Emergence and deviation of follicles during the development of follicular waves in cattle. *Theriogenology* 1997; **48**: 75–87. [Medline] [CrossRef]
9. Sartori R, Fricke PM, Ferreira JCP, Ginther OJ, Wiltbank MC. Follicular deviation and acquisition of ovulatory capacity in bovine follicles. *Biol Reprod* 2001; **65**: 1403–1409. [Medline] [CrossRef]
10. Liu WJ, Hansen PJ. Effect of the progesterone-induced serpin-like proteins of the sheep endometrium on natural-killer cell activity in sheep and mice. *Biol Reprod* 1993; **49**: 1008–1014. [Medline] [CrossRef]
11. Rodriguez-Martinez H, McKenna D, Weston PG, Whitmore HL, Gustafsson BK. Uterine motility in the cow during the estrous cycle. I. Spontaneous activity. *Theriogenology* 1987; **27**: 337–348. [Medline] [CrossRef]
12. Lewis GS. Steroidal regulation of uterine resistance to bacterial infection in livestock. *Reprod Biol Endocrinol* 2003; **1**: 117–125. [Medline] [CrossRef]
13. Lewis GS. Uterine health and disorders. *J Dairy Sci* 1997; **80**: 984–994. [Medline] [CrossRef]
14. Seals RC, Matamoros I, Lewis GS. Relationship between postpartum changes in 13, 14-dihydro-15-keto-PGF₂α concentrations in Holstein cows and their susceptibility to endometritis. *J Anim Sci* 2002; **80**: 1068–1073. [Medline]
15. Augustin HG. Vascular morphogenesis in the ovary. *Best Pract Res Clin Obstet Gynaecol* 2000; **14**: 867–882. [Medline] [CrossRef]
16. Abulafia O, Sherer DM. Angiogenesis of the ovary. *Am J Obstet Gynecol* 2000; **182**: 240–246. [Medline] [CrossRef]
17. Reynolds LP, Killilea SD, Redmer DA. Angiogenesis in the female reproductive system. *FASEB J* 1992; **6**: 886–892. [Medline]
18. Fraser HM, Wulff C. Angiogenesis in the corpus luteum. *Reprod Biol Endocrinol* 2003; **1**: 88. [Medline] [CrossRef]
19. Ravindranath N, Little-Ihrig L, Phillips HS, Ferrara N, Zeleznik AJ. Vascular endothelial growth factor messenger ribonucleic acid expression in the primate ovary. *Endocrinology* 1992; **131**: 254–260. [Medline]
20. Shweiki D, Itin A, Neufeld G, Gitay-Goren H, Keshet E. Patterns of expression of vascular endothelial growth factor (VEGF) and VEGF receptors in mice suggest a role in hormonally regulated angiogenesis. *J Clin Invest* 1993; **91**: 2235–2243. [Medline] [CrossRef]
21. Koos RD. Increased expression of vascular endothelial growth/permeability factor in the rat ovary following an ovulatory gonadotropin stimulus: potential roles in follicle rupture. *Biol Reprod* 1995; **52**: 1426–1435. [Medline] [CrossRef]
22. Wulff C, Wiegand SJ, Saunders PT, Scobie GA, Fraser HM. Angiogenesis during follicular development in the primate and its inhibition by treatment with truncated Flt-1-Fc (vascular endothelial growth factor Trap(A40)). *Endocrinology* 2001; **142**: 3244–3254. [Medline]
23. Miyabayashi K, Shimizu T, Kawachi C, Sasada H, Sato E. Changes of mRNA expression of vascular endothelial growth factor (VEGF), angiopoietins and their specific receptors during the periovulatory phase in eCG/hCG-primed immature female rats. *J Exp Zool part A* 2005; **303A**: 590–597. [CrossRef]
24. Berisha B, Schams D, Kosmann M, Amselgruber W, Einspanier R. Expression and localisation of vascular endothelial growth factor and basic fibroblast growth factor during the final growth of bovine ovarian follicles. *J Endocrinol* 2000; **167**: 371–382. [Medline] [CrossRef]
25. Shimizu T, Jiang JY, Sasada H, Sato E. Changes of messenger RNA expression of an-

- giogenic factors and related receptors during follicular development in gilts. *Biol Reprod* 2002; **67**: 1846–1852. [Medline] [CrossRef]
26. Shimizu T, Jiang JY, Iijima K, Miyabayashi K, Ogawa Y, Sasada H, Sato E. Induction of follicular development by direct single injection of vascular endothelial growth factor gene fragments into the ovary of miniature gilts. *Biol Reprod* 2003; **69**: 1388–1393. [Medline] [CrossRef]
 27. Danforth DR, Arbogast LK, Ghosh S, Dickerman A, Rofagha R, Friedman CI. Vascular endothelial growth factor stimulates preantral follicle growth in the rat ovary. *Biol Reprod* 2003; **68**: 1736–1741. [Medline] [CrossRef]
 28. Quintana R, Kopcow L, Sueldo C, Marconi G, Rueda NG, Baraño RI. Direct injection of vascular endothelial growth factor into the ovary of mice promotes follicular development. *Fertil Steril* 2004; **82**(Suppl 3): 1101–1105. [Medline] [CrossRef]
 29. Schnaper HW, McGowan KA, Kim-Schulze S, Cid MC. Oestrogen and endothelial cell angiogenic activity. *Clin Exp Pharmacol Physiol* 1996; **23**: 247–250. [Medline] [CrossRef]
 30. Morales DE, McGowan KA, Grant DS, Maheshwari S, Bhartiya D, Cid MC, Kleinman HK, Schnaper HW. Estrogen promotes angiogenic activity in human umbilical vein endothelial cells *in vitro* and in a murine model. *Circulation* 1995; **91**: 755–763. [Medline] [CrossRef]
 31. Shimizu T, Jayawardana BC, Tetsuka M, Miyamoto A. Differential effect of follicle-stimulating hormone and estradiol on expressions of vascular endothelial growth factor (VEGF) 120, VEGF164 and their receptors in bovine granulosa cells. *J Reprod Dev* 2007; **53**: 105–112. [Medline] [CrossRef]
 32. Hsueh AJ, Billig H, Tsafiri A. Ovarian follicle atresia: a hormonally controlled apoptotic process. *Endocr Rev* 1994; **15**: 707–724. [Medline]
 33. Van Wezel IL, Dharmarajan AM, Lavranos TC, Rodgers RJ. Evidence for alternative pathways of granulosa cell death in healthy and slightly atretic bovine antral follicles. *Endocrinology* 1999; **140**: 2602–2612. [Medline]
 34. Quintana R, Kopcow L, Marconi G, Sueldo C, Speranza G, Baraño RI. Relationship of ovarian stimulation response with vascular endothelial growth factor and degree of granulosa cell apoptosis. *Hum Reprod* 2001; **16**: 1814–1818. [Medline] [CrossRef]
 35. Greenaway J, Connor K, Pedersen HG, Coomber BL, LaMarre J, Petrik J. Vascular endothelial growth factor and its receptor, Flk-1/KDR, are cytoprotective in the extravascular compartment of the ovarian follicle. *Endocrinology* 2004; **145**: 2896–2905. [Medline] [CrossRef]
 36. Gerber HP, Dixit V, Ferrara N. Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. *J Biol Chem* 1998; **273**: 13313–13316. [Medline] [CrossRef]
 37. Nör JE, Christensen J, Mooney DJ, Polverini PJ. Vascular endothelial growth factor (VEGF)-mediated angiogenesis is associated with enhanced endothelial cell survival and induction of Bcl-2 expression. *Am J Pathol* 1999; **154**: 375–384. [Medline] [CrossRef]
 38. Kosaka N, Sudo N, Miyamoto A, Shimizu T. Vascular endothelial growth factor (VEGF) suppresses ovarian granulosa cell apoptosis *in vitro*. *Biochem Biophys Res Commun* 2007; **363**: 733–737. [Medline] [CrossRef]
 39. Zhao J, Taverne MA, van der Weijden GC, Bevers MM, van den Hurk R. Immunohistochemical localisation of growth hormone (GH), GH receptor (GHR), insulin-like growth factor I (IGF-I) and type I IGF-I receptor, and gene expression of GH and GHR in rat pre-antral follicles. *Zygote* 2002; **10**: 85–94. [Medline] [CrossRef]
 40. Zhao J, Taverne MA, Van Der Weijden GC, Bevers MM, Van Den Hurk R. Insulin-like growth factor-I (IGF-I) stimulates the development of cultured rat pre-antral follicles. *Mol Reprod Dev* 2001; **58**: 287–296. [Medline] [CrossRef]
 41. Shimizu T, Murayama C, Sudo N, Kawashima C, Tetsuka M, Miyamoto A. Involvement of insulin and growth hormone (GH) during follicular development in the bovine ovary. *Anim Reprod Sci* 2008; **106**: 143–152. [Medline] [CrossRef]
 42. Hastie PM, Haresign W. Expression of mRNAs encoding insulin-like growth factor (IGF) ligands, IGF receptors and IGF binding proteins during follicular growth and atresia in the ovine ovary throughout the oestrous cycle. *Anim Reprod Sci* 2006; **92**: 284–299. [Medline] [CrossRef]
 43. Schams D, Berisha B, Kosmann M, Amselgruber WM. Expression and localization of IGF family members in bovine antral follicles during final growth and in luteal tissue during different stages of estrous cycle and pregnancy. *Domest Anim Endocrinol* 2002; **22**: 51–72. [Medline] [CrossRef]
 44. Spicer LJ, Alpizar E, Echterkamp SE. Effects of insulin, insulin-like growth factor I, and gonadotropins on bovine granulosa cell proliferation, progesterone production, estradiol production, and/or insulin-like growth factor I production *in vitro*. *J Anim Sci* 1993; **71**: 1232–1241. [Medline]
 45. Kirby CJ, Thatcher WW, Collier RJ, Simmen FA, Lucy MC. Effects of growth hormone and pregnancy on expression of growth hormone receptor, insulin-like growth factor-I, and insulin-like growth factor binding protein-2 and -3 genes in bovine uterus, ovary, and oviduct. *Biol Reprod* 1996; **55**: 996–1002. [Medline] [CrossRef]
 46. Yuan W, Bao B, Garverick HA, Youngquist RS, Lucy MC. Follicular dominance in cattle is associated with divergent patterns of ovarian gene expression for insulin-like growth factor (IGF)-I, IGF-II, and IGF binding protein-2 in dominant and subordinate follicles. *Domest Anim Endocrinol* 1998; **15**: 55–63. [Medline] [CrossRef]
 47. Armstrong DG, Gutierrez CG, Baxter G, Glazyrin AL, Mann GE, Woad KJ, Hogg CO, Webb R. Expression of mRNA encoding IGF-I, IGF-II and type I IGF receptor in bovine ovarian follicles. *J Endocrinol* 2000; **165**: 101–113. [Medline] [CrossRef]
 48. Sudo N, Shimizu T, Kawashima C, Kaneko E, Tetsuka M, Miyamoto A. Insulin-like growth factor-I (IGF-I) system during follicle development in the bovine ovary: relationship among IGF-I, type I IGF receptor (IGFR-1) and pregnancy-associated plasma protein-A (PAPP-A). *Mol Cell Endocrinol* 2007; **264**: 197–203. [Medline] [CrossRef]
 49. Beg MA, Bergfelt DR, Kot K, Wiltbank MC, Ginther OJ. Follicular-fluid factors and granulosa-cell gene expression associated with follicle deviation in cattle. *Biol Reprod* 2001; **64**: 432–441. [Medline] [CrossRef]
 50. Rivera GM, Fortune JE. Selection of the dominant follicle and insulin-like growth factor (IGF)-binding proteins: evidence that pregnancy-associated plasma protein A contributes to proteolysis of IGF-binding protein 5 in bovine follicular fluid. *Endocrinology* 2003; **144**: 437–446. [Medline] [CrossRef]
 51. Stanko RL, Cobick WS, Shaw DW, Harvey RW, Clemmons DR, Whitacre MD, Armstrong JD. Effect of somatotropin and/or equine chorionic gonadotropin on serum and follicular insulin-like growth factor I and insulin-like growth factor binding proteins in cattle. *Biol Reprod* 1994; **50**: 290–300. [Medline] [CrossRef]
 52. Funston RN, Seidel GE Jr, Klindt J, Roberts AJ. Insulin-like growth factor I and insulin-like growth factor-binding proteins in bovine serum and follicular fluid before and after the preovulatory surge of luteinizing hormone. *Biol Reprod* 1996; **55**: 1390–1396. [Medline] [CrossRef]
 53. Leeuwenberg BR, Hudson NL, Moore LG, Hurst PR, McNatty KP. Peripheral and ovarian IGF-I concentrations during the ovine oestrous cycle. *J Endocrinol* 1996; **148**: 281–289. [Medline] [CrossRef]
 54. Armstrong DG, Baxter G, Gutierrez CG, Hogg CO, Glazyrin AL, Campbell BK, Bramley TA, Webb R. Insulin-like growth factor binding protein -2 and -4 messenger ribonucleic acid expression in bovine ovarian follicles: effect of gonadotropins and developmental status. *Endocrinology* 1998; **139**: 2146–2154. [Medline]
 55. Rivera GM, Fortune JE. Development of codominant follicles in cattle is associated with a follicle-stimulating hormone-dependent insulin-like growth factor binding protein-4 protease. *Biol Reprod* 2001; **65**: 112–118. [Medline] [CrossRef]
 56. Spicer LJ, Chamberlain CS, Morgan GL. Proteolysis of insulin-like growth factor binding proteins during preovulatory follicular development in cattle. *Domest Anim Endocrinol* 2001; **21**: 1–15. [Medline] [CrossRef]
 57. Perks CM, Peters AR, Wathes DC. Follicular and luteal expression of insulin-like growth factors I and II and the type I IGF receptor in the bovine ovary. *J Reprod Fertil* 1999; **116**: 157–165. [Medline] [CrossRef]
 58. King GL, Buzney SM, Kahn CR, Hetu N, Buchwald S, Macdonald SG, Rand LL. Differential responsiveness to insulin of endothelial and support cells from micro- and macrovessels. *J Clin Invest* 1983; **71**: 974–979. [Medline] [CrossRef]
 59. Grant MB, Mames RN, Fitzgerald C, Ellis EA, Caballero S, Chegini N, Guy J. Insulin-like growth factor I as an angiogenic agent. *In vivo and in vitro* studies. *Ann N Y Acad Sci* 1993; **692**: 230–242. [Medline] [CrossRef]
 60. Mazerbourg S, Overgaard MT, Oxvig C, Christiansen M, Conover CA, Laurendeau I, Vidaud M, Tosser-Klopp G, Zapf J, Monget P. Pregnancy-associated plasma protein-A (PAPP-A) in ovine, bovine, porcine, and equine ovarian follicles: involvement in IGF binding protein-4 proteolytic degradation and mRNA expression during follicular development. *Endocrinology* 2001; **142**: 5243–5253. [Medline] [CrossRef]
 61. Aad PY, Voge JL, Santiago CA, Malayer JR, Spicer LJ. Real-time RT-PCR quantification of pregnancy-associated plasma protein-A mRNA abundance in bovine granulosa and theca cells: effects of hormones *in vitro*. *Domest Anim Endocrinol* 2006; **31**: 357–372. [Medline] [CrossRef]
 62. Santiago CA, Voge JL, Aad PY, Allen DT, Stein DR, Malayer JR, Spicer LJ. Pregnancy-associated plasma protein-A and insulin-like growth factor binding protein mRNAs in granulosa cells of dominant and subordinate follicles of preovulatory cattle. *Domest Anim Endocrinol* 2005; **28**: 46–63. [Medline] [CrossRef]
 63. Monget P, Mazerbourg S, Delpuech T, Maurel MC, Manière S, Zapf J, Lalmanach G, Oxvig C, Overgaard MT. Pregnancy-associated plasma protein-A is involved in insulin-like growth factor binding protein-2 (IGFBP-2) proteolytic degradation in bovine and porcine preovulatory follicles: identification of cleavage site and characterization of IGFBP-2 degradation. *Biol Reprod* 2003; **68**: 77–86. [Medline] [CrossRef]
 64. Rivera GM, Fortune JE. Proteolysis of insulin-like growth factor binding proteins -4 and -5 in bovine follicular fluid: implications for ovarian follicular selection and dominance. *Endocrinology* 2003; **144**: 2977–2987. [Medline] [CrossRef]
 65. Bramley TA, Menzies GS, McNeilly AS, Friesen HG. Receptors for lactogenic hormones in the ovine corpus luteum. I: A major discrepancy in the specific binding of radio-labelled ovine prolactin and human growth hormone. *J Endocrinol* 1987; **113**: 365–374. [Medline] [CrossRef]
 66. Carlsson B, Nilsson A, Isaksson OGP, Billig H. Growth hormone-receptor messenger

- RNA in the rat ovary: regulation and localization. *Mol Cell Endocrinol* 1993; **95**: 59–66. [Medline] [CrossRef]
67. Kölle S, Sinowatz F, Boie G, Lincoln D. Developmental changes in the expression of the growth hormone receptor messenger ribonucleic acid and protein in the bovine ovary. *Biol Reprod* 1998; **59**: 836–842. [Medline] [CrossRef]
68. Gong JG, Bramley T, Webb R. The effect of recombinant bovine somatotropin on ovarian function in heifers: follicular populations and peripheral hormones. *Biol Reprod* 1991; **45**: 941–949. [Medline] [CrossRef]
69. Gong JG, Bramley TA, Webb R. The effect of recombinant bovine somatotrophin on ovarian follicular growth and development in heifers. *J Reprod Fertil* 1993; **97**: 247–254. [Medline] [CrossRef]
70. Lucy MC, Bilby CR, Kirby CJ, Yuan W, Boyd CK. Role of growth hormone in development and maintenance of follicles and corpora lutea. *J Reprod Fertil Suppl* 1999; **54**: 49–59. [Medline]
71. Lucy MC, Byatt JC, Curran TL, Curran DF, Collier RJ. Placental lactogen and somatotropin: hormone binding to the corpus luteum and effects on the growth and functions of the ovary in heifers. *Biol Reprod* 1994; **50**: 1136–1144. [Medline] [CrossRef]
72. Kobayashi J, Mizunuma H, Kikuchi N, Liu X, Andoh K, Abe Y, Yokota H, Yamada K, Ibuki Y, Hagiwara H. Morphological assessment of the effect of growth hormone on preantral follicles from 11-day-old mice in an *in vitro* culture system. *Biochem Biophys Res Commun* 2000; **268**: 36–41. [Medline] [CrossRef]
73. Hutchinson LA, Findlay JK, Herington AC. Growth hormone and insulin-like growth factor-I accelerate PMSG-induced differentiation of granulosa cells. *Mol Cell Endocrinol* 1988; **55**: 61–69. [Medline] [CrossRef]
74. Langhout DJ, Spicer LJ, Geisert RD. Development of a culture system for bovine granulosa cells: effects of growth hormone, estradiol, and gonadotropins on cell proliferation, steroidogenesis, and protein synthesis. *J Anim Sci* 1991; **69**: 3321–3334. [Medline]
75. Izadyar F, Colenbrander B, Bevers MM. *In vitro* maturation of bovine oocytes in the presence of growth hormone accelerates nuclear maturation and promotes subsequent embryonic development. *Mol Reprod Dev* 1996; **45**: 372–377. [Medline] [CrossRef]
76. Sheldon IM, Cronin J, Goetze L, Donofrio G, Schuberth HJ. Defining postpartum uterine disease and the mechanisms of infection and immunity in the female reproductive tract in cattle. *Biol Reprod* 2009; **81**: 1025–1032. [Medline] [CrossRef]
77. Mateus L, Lopes da Costa L, Diniz P, Ziecik AJ. Relationship between endotoxin and prostaglandin (PGE2 and PGFM) concentrations and ovarian function in dairy cows with puerperal endometritis. *Anim Reprod Sci* 2003; **76**: 143–154. [Medline] [CrossRef]
78. Herath S, Williams EJ, Lilly ST, Gilbert RO, Dobson H, Bryant CE, Sheldon IM. Ovarian follicular cells have innate immune capabilities that modulate their endocrine function. *Reproduction* 2007; **134**: 683–693. [Medline] [CrossRef]
79. Magata F, Horiuchi M, Echizenya R, Miura R, Chiba S, Matsui M, Miyamoto A, Kobayashi Y, Shimizu T. Lipopolysaccharide in ovarian follicular fluid influences the steroid production in large follicles of dairy cows. *Anim Reprod Sci* 2014; **144**: 6–13. [Medline] [CrossRef]
80. Silva JM, Price CA. Effect of follicle-stimulating hormone on steroid secretion and messenger ribonucleic acids encoding cytochromes P450 aromatase and cholesterol side-chain cleavage in bovine granulosa cells *in vitro*. *Biol Reprod* 2000; **62**: 186–191. [Medline] [CrossRef]
81. Magoffin DA. Ovarian theca cell. *Int J Biochem Cell Biol* 2005; **37**: 1344–1349. [Medline] [CrossRef]
82. Beutler B. Inferences, questions and possibilities in Toll-like receptor signalling. *Nature* 2004; **430**: 257–263. [Medline] [CrossRef]
83. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006; **124**: 783–801. [Medline] [CrossRef]
84. Akira S, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol* 2004; **4**: 499–511. [Medline] [CrossRef]
85. Medzhitov R, Preston-Hurlburt P, Janeway CA Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* 1997; **388**: 394–397. [Medline] [CrossRef]
86. Shimizu T, Miyauchi K, Shirasuna K, Bollwein H, Magata F, Murayama C, Miyamoto A. Effects of lipopolysaccharide (LPS) and peptidoglycan (PGN) on estradiol production in bovine granulosa cells from small and large follicles. *Toxicol In Vitro* 2012; **26**: 1134–1142. [Medline] [CrossRef]
87. Magata F, Horiuchi M, Miyamoto A, Shimizu T. Lipopolysaccharide (LPS) inhibits steroid production in theca cells of bovine follicles *in vitro*: distinct effect of LPS on theca cell function in pre- and post-selection follicles. *J Reprod Dev* 2014; **60**: 280–287. [Medline] [CrossRef]