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Differential expression of GDF-9 and BMP- 15 during follicular development in canine ovaries evaluated by flow cytometry

Tomas Fernandez[a], Jaime Palomino[a], Victor H. Parraguez[b], Oscar A. Peralta[a], Monica De los Reyes[a,*]

[a] Laboratory of Animal Reproduction, Faculty of Veterinary Sciences, University of Chile, Casilla 2 Correo 15, Santiago, Chile
[b] Laboratory of Animal Physiology, Faculty of Veterinary Sciences, University of Chile, Casilla 2 Correo 15, Santiago, Chile

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ABSTRACT

Growth differentiation factor 9 (GDF-9) and bone morphogenetic protein 15 (BMP-15) play important functions in follicular and oocyte development in many species. This study evaluated the dynamic expression of GDF-9 and BMP-15 in canine follicles cells using flow cytometry analysis. Follicular cells were removed from three sizes of antral follicles (small, medium and large) from ovaries of bitches throughout the estrus cycle. Cells were incubated with anti-human GDF-9 polyclonal and anti-mouse BMP-15 monoclonal antibodies. A size and complexity discriminatory gate was used for the cytometryc analysis in the initial dot plot and, additionally, a CD45 marker for leukocyte and propidium iodide (PI) were used for erythrocyte and debris discrimination. The evidence corroborated the presence of both proteins in canine follicle cells, but these proteins were not expressed equally during follicular development. The results analyzed by ANOVA showed that GDF-9 expression decreased ($P < 0.05$) during follicular growth in anestrus and proestrous/estrous, but increased in diestrus ($P < 0.05$). The expression levels of BMP-15 rose ($P < 0.05$) from small to medium sizes in anestrous without changing at diestrus. Small antral follicles expressed the highest values of GDF-9 at anestrus while only BMP-15 showed higher value in small antral follicles at proestrous-estrus compared to diestrus and anestrus. Both proteins decreased in proestrous/estrous ($P < 0.05$) with increasing follicle size, registering the lowest levels in large follicles. The flow cytometric assay was able to assess GDF-9 and BMP-15 expression in canine follicular cells, showing that these proteins were differentially expressed during follicular development, possibly related to the special features of canine reproduction.

Introduction

Oocyte development and follicle growth are closely regulated by an ordered and complex series of signaling events throughout folliculogenesis. The interplay between oocytes and the somatic cells of ovarian follicles determines the developmental ability of these cells. During this process, meiotic competence of the oocyte is gained gradually (Eppig, 2001; Fair, 2003; Hussein et al., 2006) and gap

* Corresponding author at: University of Chile, Faculty of Veterinary Sciences. Laboratory of Animal Reproduction, Santa Rosa 11735, Santiago, Chile. Tel.: +56 2 29785534; fax: +56 2 29785611.
E-mail addresses: mdlreyes@uchile.cl, monica_isabel3@hotmail.com (M. De los Reyes).

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junctional communication within the developing follicle, both between oocytes and granulosa cells and among the follicular cells themselves, maintains the follicle in a functionally integrated state (Kidder and Vanderhyden, 2010; Huiyu et al., 2013). However, little is known about the physiological control of follicular growth in canines, despite being of pivotal importance in oocyte development in vivo and in vitro. The subsequent in vitro maturation (IVM) of oocytes, that remains unsuccessful in this species (De los Reyes et al., 2005, 2011; Chastant-Maillard et al., 2011), depends strongly on its prior period inside the follicles. In fact, the compromised developmental competence of IVM in canines can be attributed to the lack of knowledge of the ability of the oocyte's intrinsic requirements during follicular maturation.

The communication of oocyte and follicular cells is characterized by the secretion of several growth factors such as those members of transforming growth factor beta superfamily (TGF-β), which plays an important function in proliferation and differentiation of a variety of cell types. Many studies have shown that members of the TGF-β superfamily can regulate granulosa cell proliferation and differentiation (Sudiman et al., 2014; Cheng et al., 2015). Growth differentiation factor (GDF) 9 and bone morphogenetic protein (BMP) 15 are members of the TGF-β superfamily of proteins, and thus are similar at a structural level (Knight and Glister, 2006; Otsuka et al., 2011). Studies indicate that GDF-9 and BMP-15 are both present in follicles throughout different stages of follicular development and in many species these factors influence ovarian follicular growth. Findings in sheep, humans and rodents show that BMP-15 and GDF-9 can be considered to be new targets for fertility regulation in mammals (Gilchrist et al., 2006; Persani et al., 2014), as they participate in signaling pathways that control the development of ovarian follicles. These proteins are thought to affect granulosa cell proliferation independently or synergistically from small follicles (Fenwick et al., 2013) and the growth-promoting actions of oocytes are mediated, at least in part, by these factors (Su et al., 2004; Hussein et al., 2006), although the signals exchanged between the oocyte and the surrounding cells are far from being fully understood. Studies in mice and sheep, as well as evidences from in vitro studies in other species, have demonstrated that cooperative interactions between GDF-9 and BMP-15 occur in many functions (McNatty et al., 2005; Mottershead et al., 2012). GDF9 promotes the expansion of cumulus cells by induction of expression of Has2, Tnfaip6, Ptx3, and Ptgs2 (Varani et al., 2002); BMP-15 also is involved in this process (Gueripel et al., 2006), and both factors promote proliferation of granulosa cells (Kidder and Vanderhyden, 2010).

It has been reported that BMP-15 and GDF-9 are expressed only in oocytes in rodents (Gilchrist et al., 2008), however, these factors are expressed also in cumulus and mural granulosa cells in many other mammals (Hosoe et al., 2011; Lim et al., 2014), including canine (De los Reyes et al., 2013; Maupeu et al., 2015), demonstrating that the expression patterns and biological functions may differ among species. Therefore, many species differences have already emerged (Galloway et al., 2000; Hussein et al., 2006; Sun et al., 2008). In previous studies we suggested that oocyte-produced GDF-9 in vitro may be insufficient for promoting cumulus cell expansion in canine (De los Reyes et al., 2013), which could be related to delay for resumption of meiosis. However, the levels of GDF-9 and BMP-15 expression in canine granulosa cells during follicular development are largely unknown. As GDF-9 and BMP-15 are both possibly present in follicles throughout most stages of follicular growth, it is important to evaluate whether these paracrine factors are differentially expressed in these follicles. The knowledge of the physiology of GDF-9 and BMP-15 factors in canine could contribute to understanding the special reproductive characteristics of this species and also potentially helps to improve protocols that may to overcome the low success of IVM in canine oocytes.

Flow cytometry is a powerful analytical instrument for rapid evaluation of high numbers of cells; it detects labeling by multiple fluorochromes associated with individual cells flowing in a flowing stream (Hirshfield et al., 1988). This technique has been successfully used for granulosa cell analysis (Rao et al., 1991; De Neubourg et al., 1996; Douville and Sirard, 2014; Regan et al., 2015), providing an alternative method for assessing protein expression in follicular cells. Therefore, the experiments reported in the present study were undertaken to evaluate by flow cytometric methods the expression of GDF-9 and BMP-15 in canine follicular cells during follicular development.

2. Material and methods

This study was approved by the Bioethics Committee, Faculty of Veterinary Sciences, University of Chile and the Research Ethics Committee of the Chilean National Commission for Scientific and Technological Research (FONDECYT).

2.1. Processing of ovaries

Ovaries were collected from adult non-pregnant bitches undergoing routine ovariohysterectomy at the Veterinary Hospital El Roble, University of Chile. The selected bitches were clinically healthy of various breeds, and were 1–6 years of age. Immediately after removal, the ovaries were placed in physiological saline solution (pH 7.4, 0.9% NaCl) at 4 °C and then transported to the laboratory.

Only healthy ovaries, with no visual abnormalities, were used for experiments. The stage of estrous cycle of each donor was assessed by evaluating the presence or absence of follicles and corpus luteum (Songsasen and Wild, 2005), and by progesterone analysis from blood samples obtained during the surgery, as previously described (De los Reyes et al., 2013). Briefly, blood of each animal was collected without anticoagulant and then centrifuged at 3000 rpm for 10 min. Plasma was stored at −20 °C until use. Plasma progesterone concentrations was assessed by enzyme-linked immunosorbent assay (ELISA) (Ververidis et al., 2002), (PHomo Microplate Reader®; Autobio Labtec Instruments, Zhenghaidong, China) with a progesterone (P4) canine kit (Prog ELISA Kit, MyBioSource®; San Diego, CA, USA). Duplicate wells were used for each sample. Sen-

sitivity of the assay was 0.19 ng/mL. The mean intra-and inter assay precision was 5.1% and 6%, respectively.

2.2. Follicles and granulosa cells isolation

All canine ovaries were washed in phosphate-buffered saline (PBS) and the ovary cortex was dissected into small pieces under a stereomicroscope (Lieder MZ-730-J6 American Scientific, Portland, OR, USA). The small pieces of ovaries were incubated with 1 mg/mL Collagenase (C0130-100MG Sigma, MO, USA) in PBS for 90 min, in order to disaggregate the ovarian tissue and facilitate the isolation of follicles. Antral follicles were retrieved free of stromal tissue by using a 29 gauge needle (Nipro Corporation, Miami, FL, USA) under a stereomicroscope (Leider MZ-730-J6 American Scientific, Portland, OR, USA) and separately classified into three sizes: (a) Small ~0.25–0.39 mm; (b) Medium ~0.4–3.9 mm and (c) Large or preovulatory follicles 4–8 mm. The diameter of each individual follicle was measured using a graticule in the stereomicroscope during release of the intra-follicular contents. Fluid from each follicle was collected by flushing followed by cutting open each follicle with a small scalped blade and gently scraping with a 29-gauge needle (Nipro Corporation, Miami, FL, USA) into the same flushing fluid to recovery granulosa and theca cells. The oocyte of each follicle was discarded and cumulus cells and cells of each selected follicle were transferred into individual Eppendorf tubes and pooled in each trial within estrus cycle and follicle size. Therefore, the contents of several follicles pertaining to the same size group and reproductive phase were pooled in order to obtain enough material for analysis. Follicular cells were washed by centrifugation, 500× *g* for 10 min at 4 °C, using 1 mL of PBS, pH 8.2.

2.3. Immunofluorescence

After centrifugation, the pellet was suspended in 1 mL PBS. Cell suspensions were fixed with 100 μL of 4% paraformaldehyde in PBS and incubated at 4 °C for 20 min, followed by permeabilization in 0.1% Triton X-100 -PBS (pH 8.2) (T878-50ML Sigma, St., Louis, MO, USA) for 10 min, to allow access of the antibodies to react with the proteins. Non-specific binding sites were blocked by incubating the cells for 1 h with PBS–BSA 0.2% (A2152–10 G Sigma, St., Louis, MO, USA) supplemented with 5% goat blood serum (G9023, Sigma, St., Louis, MO, USA) followed by washing two times in PBS. After that, the cells were incubated with 500 μL of first antibodies: polyclonal anti-human GDF-9 (Ab93892 Abcam, Cambridge, MA, USA) and monoclonal anti-mouse BMP-15 (AF2925 R&D System, Minneapolis, MN, USA) both at 1/100 dilution, and incubated over night at 4 °C. Both antibodies had previously been proven to cross react with canine GDF-9 and BMP-15 respectively (Maupeu et al., 2015). After three washings of 10 min with 0.05% Tween-20 in PBS (pH 8,2) to remove excess first antibodies, the second antibodies reactive with each first antibody were added: 1/500 dilution of fluorescein-conjugated (FITC) goat anti-rabbit second antibody (Ab97050 Abcam, Cambridge, MA, USA. Emission range λ 500–550 mn) for GDF-9 and 1/500 of Chlorophyl–Peridinine conjugated mouse anti-goat second antibody (PerCP) (Sc-45091 Santa Cruz Biotechnology Inc., Dallas, TX, USA. Emission range λ 675–710 nm) for BMP-15, at room temperature (21 °C) in darkness for 1 h. The cell samples incubated with second antibodies alone were used as negative controls, as well as those incubated without antibodies to exclude emitted autofluorescence. After incubation, the samples were washed three times in PBS/Tween-20 (0.05%) (P9416-50L Sigma, St., Louis, MO, USA) for 10 min. For DNA labeling the cells were stained by propidium iodide (PI) solution (1 μg/mL, 1351916 Molecular Probes, Eugene, OR, USA. Emission range λ 562–588 nm), for 10 min and then washed again three times with PBS. The cells were also stained with 1 μg/mL anti-CD45-Phycoerithryn-texas Red (ECD) antibody (117018, Beckman Coulter, Brea, CA, USA. Emission range λ 606–635 nm) for 10 min, in order to discriminate the CD45 leukocytes from the samples. Finally, each sample was centrifuged in PBS at 800× *g* for 5 min, the supernatant fluid was discarded and the cells were suspended in 1 mL ISO-Flow (Beckman Coulter, Brea, CA, USA) and analyzed in a flow cytometer.

2.4. Flow cytometry analysis

Flow Cytometric analysis was performed on a 6 color Gallius Cytometer (Beckman Coulter, Brea, CA, USA) with standard setting, including compensation protocol for proper fluorophore discrimination.

To determine whether the different sizes of follicles contained different levels of GDF-9 and BMP-15, 1 mL suspensions of follicular cells in ISO-Flow medium were analyzed in each experimental replicate. The size gating of the follicular cell was used to eliminate debris and other cells. Thus, in each case events with very low forward scatter, representing debris, were excluded from analysis by forward and side-scatter gating.

In addition, samples stained with PI allowed separation of follicle cells expressing GDF-9 and erythrocytes in the dot plot FL1 vs FL2, respectively and GDF-9 to Leucocytes labeled with anti CD45 in FL1 vs FL3 respectively. The same for BMP-15, which were gated in the dot plot FL4 vs FL2 (PI) and FL4 vs FL3 (CD45).

Expression by follicular cells was quantified as the percent of cell expressing GDF-9 and BMP-15 on a logarithmic scale, according the proportion of labeled cells.

2.5. Statistical analysis

A total of 46 bitches was used, evaluating 522 antral follicles. Flow cytometry data were analyzed using software Gallios Cytometer 1.2 List Mode Data Acquisition & Analysis Software, 2010 (Beckman Coulter, Brea, CA, USA). More than 10,000 cells were counted for each protein throughout 3 experimental replicates.

The expression of GDF-9 and BMP-15 by follicles cells were quantified by the percent of cells expressing these proteins. Repeated measures analysis of variance (ANOVA) and Duncan's test for significance were used to compare GDF-9 and BMP-15 expression among different follicle sizes and stages of estrus cycle (InfoStat, Professional

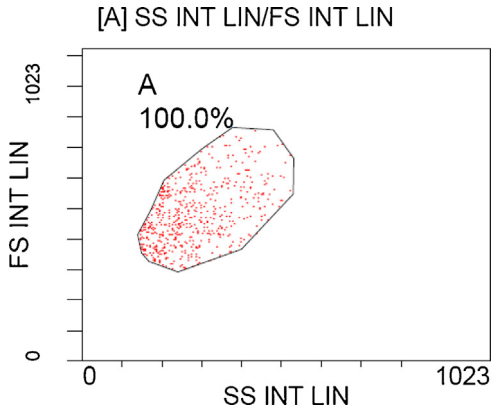

Fig. 1. Dot plot showing the discriminatory gate used forward scatter (FS: cell size) and side scatter (SS: cell complexity) to gate out debris and erythrocytes during analysis of follicular cells (circled area).

Program, National University of Córdoba, Argentina). Percentages were arcsine transformed to normalize the data prior to data analysis. The level of significance was set at $P \leq 0.05$. Data are presented as the mean ± SE.

3. Results

The ELISA test corroborated the physiological changes in progesterone concentration according the different phases of estrous cycle in accordance with the predominant ovarian structures. Individual progesterone values of bitches submitted to ovariohysterectomy were 0.01–0.1 ng/mL in anestrus phase; 0.33–14.83 ng/mL in proestrous/estrus and 17.91–31.07 ng/mL in diestrus.

All antral follicles were classified according to size and reproductive phase of the donor (Table 1).

Flow cytometry experiments were used to analyze the expression of GDF-9 and BMP-15 in follicles at different size and estrus phase. Events with low forward scatter represented particles, debris and erythrocytes, which were excluded from the analysis through a discriminatory gate (Fig. 1).

Negative control cells and cells stained by each anti-GDF-9 and BMP-15 antibodies are present in each histogram for both proteins (Fig. 2a and b respectively). No immunoreactivity was detected with negative control of autofluorescence, which was obtained within the first decade of logarithmic scale (gray curve). In the same way, the negative control without the first antibodies, for both GDF-9 and BMP-15, also remained within the first decade of measurement. In contrast, GDF-9 and BMP-15 positive cells were detected in the second decade of logarithmic (black curve).

The dot plot of FL1 (GDF-9) versus FL2 (IP) and FL4 (BMP-15) versus FL2 for gating the PI negative to debris and erythrocytes population are shown in Fig. 3a and b respectively. The same was performed for the CD45 (leukocytes) negative cells in FL3 versus FL1 (GDF-9) and versus FL4 (BMP-15), where few events represented CD45 positive cells (Fig. 3c and d respectively).

The dynamic expression of GDF-9 and BMP-15 at each follicle size was compared among across reproductive

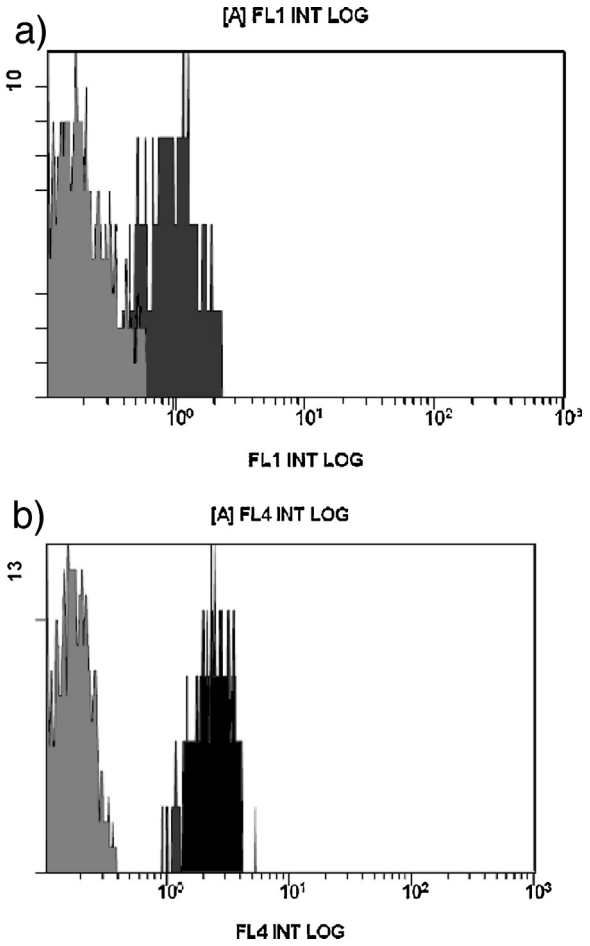

Fig. 2. Representative Histograms resulting from flow cytometry analysis of GDF-9 (a) and BMP-15 (b) positive cells. The horizontal axis of the histograms represents the signal intensity of the proteins expression (log scale) and the vertical axis represents the number of cells. Each histogram displays the populations of both negative control cells (gray curve) and cells stained by each anti-GDF-9 and BMP-15 antibodies (black curve). The comparison with the negative control of auto fluorescence was obtained within the first decade of logarithmic scale. No immunoreactivity was detected with negative control of autofluorescence, for both GDF-9 and BMP-15.

cycles (Table 2). Analysis of granulosa cells by flow cytometry showed that the frequency of granulosa cells that expressed GDF-9 and BMP-15 varied from 28.7% to 88% and 33.3% to 79.2% respectively of the cells retrieved from all follicles at different reproductive phases.

The expression levels of GDF-9 and BMP-15 changed ($P < 0.05$) from the small antral follicle to the medium or large follicles depending on reproductive cycle (Table 2). As shown, the expressions of GDF-9 in anestrus declined ($P < 0.05$) while follicular size increased from small to medium sizes. At proestrous/estrus, the expression of GDF-9 also decreased ($P < 0.05$) from small to medium and large antral follicles. At diestrus the expression of GDF-9 rose ($P < 0.05$) as follicle growth increased from small to medium size.

During proestrous/estrous BMP-15 expression differed ($P < 0.05$) among growing follicles from small or medium

Table 1
Number of antral follicles in different stages of development (sizes) obtained from different phases of the estrous cycle.

	Follicular Sizes			
Estrous Cycle	Small (~0.25–0.39 mm)	Medium (~0.4–3.9 mm)	Large (~4 –8 mm)	Total
Anestrus	96	52	–	148
Proestrus/Estrus	73	53	35	161
Diestrus	148	65	–	213
Total	317	170	35	522

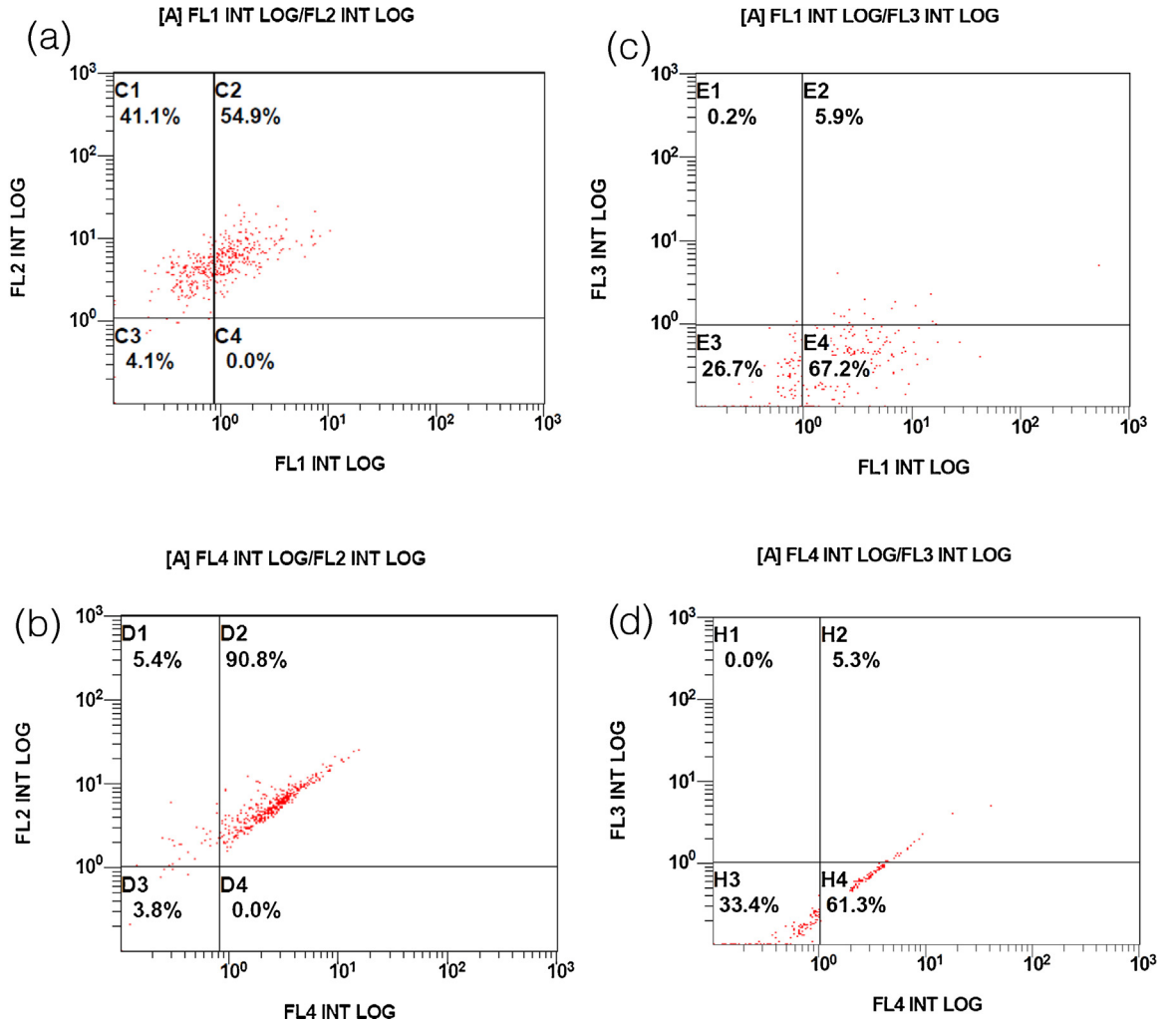

Fig. 3. Representatives Log fluorescence dot plot analysis of granulosa cells positive to GDF-9 (a, c) and BMP-15 (b, d) obtained from a group. a) The dot plot of FL1 (GDF-9) versus FL2 (IP) and b) FL4 (BMP-15) versus FL2 for gating the IP negative to debris and erythrocytes population (upper right quadrant); c) The dot plot of FL3 (CD45; leukocytes) negative cells versus FL1 (GDF-9) and d) FL3 versus FL4 (BMP-15) (down right quadrant).

Table 2
Percentage ± standard deviation (SD) of GDF-9 and BMP-15 expression in canine follicles cells at different stages of development (sizes) throughout estrus cycle evaluated by flow cytometry.

	Follicular development					
	Small antral		Medium antral		Large antral	
Estrous Cycle	GDF-9	BMP-15	GDF-9	BMP-15	GDF-9	BMP-15
Proestrous/estrous	$71.8 \pm 5.0^{A,a}$	$68.5 \pm 9.4^{A,a}$	$46.8 \pm 4.7^{A,b}$	73.8 ± 7.4^{a}	28.7 ± 1.2^{c}	33.3 ± 3.1^{b}
Diestrous	$57.6 \pm 8.2^{B,a}$	48.6 ± 2.2^{AB}	$68.1 \pm 4.7^{B,b}$	65.6 ± 1.7		
Anestrous	$88.0 \pm 5.5^{C,a}$	$34.9 \pm 3.2^{B,a}$	$79.2 \pm 5.4^{C,b}$	74.4 ± 1.9^{b}		

A,B,C: Within a column, numbers without a common superscript differed $P < 0.05$.
a,b,c: Within a row and proteins, numbers without a common superscript differed $P < 0.05$.

sizes to large size; however, there was no difference between BMP-15 expression from small to medium sizes at proestrous/estrus. At anestrus the expression level increased from small to medium follicles and at proestrous/estrus a minor expression in large follicles was observed compared with those of other sizes. At diestrus, no variation was found according to follicle size in BMP-15 level.

The incidence of GDF-9 and BMP-15 expression of each follicular cell group was also compared within the same size group at the different phases of estrus cycle. As shown in Table 2, small and medium size antral follicles showed difference ($P < 0.05$) in GDF-9 levels, expressing the highest values of GDF-9 at anestrus, while BMP-15 showed higher value only in small antral follicles at proestrous/estrus compared to diestrus and anestrus. Both proteins showed the lowest ($P < 0.05$) levels in large follicles at proestrous/estrus stage.

4. Discussion

Flow cytometry gave a fast and accurate approach to the investigation of follicular cells in the canine ovarian follicles. Antral follicles yield enough follicular cells to perform a good flow cytometry analysis, providing a visual representation of the number of cells as a function of GDF-9 and BMP-15 content and, thus, the proportion of these proteins in the follicle cells in each phase of the reproductive cycle and development. Therefore, it was possible to identify the presence of these two proteins in the different types of antral dog follicles, showing that these factors are components of the follicular environment and corroborating previous studies in canines that have demonstrated the presence of GDF-9 and BMP-15 in granulosa cells as well as the oocytes (De los Reyes et al., 2013; Maupeu et al., 2015), similar to other species including goats (Silva et al., 2005), sows (Lee et al., 2008) and cows (Hosoe et al., 2011). The analyzes in this study were performed without separating the cumulus cells from the mural granulosa or theca cells, because the aim was to evaluate the follicle environment of these two proteins influencing oocyte development at various stages of follicular sizes, no matter their cellular origin. Therefore, follicular contents were processed to recover follicular fluid, theca, granulosa and cumulus cells.

PI was rarely negative in all replicates, indicating that the initial analysis by forward and side-scatter gating efficiently excluded debris and erythrocytes. Moreover, in order to exclude the possibility of contamination with leukocytes, flow cytometric analysis was done on follicle cells also stained with anti-CD45 antibody, which is a cell surface antigen present on all leukocytes but not on follicular cells (De Neubourg et al., 1996; Jaslow et al., 2010). Thus, granulosa and theca cells were selected only as those that were CD45 negative. These discriminatory parameters were further used for all flow cytometric evaluation of granulosa cells. Possibly, the isolation of individual follicles from ovaries also helped to reduce the contamination with blood cells.

The indirect measurement by flow cytometry of protein expression on follicular cells was undertaken to evaluate the dynamic expression of these proteins during follicular growth at each reproductive stage. Thereby, the major finding from this study was that both GDF-9 and BMP-15 are expressed differentially during follicular growth in bitches.

Only small and medium sizes of antral follicles in anestrus and diestrus were compared because in canine ovaries no large follicles developat these stages of the reproductive cycle. The percentage of cells positive to GDF-9 at anestrus decreased with increasing follicular development from small to medium size. During folliculogenesis in other species, GDF-9 has been shown to promote primary follicle progression (Otsuka et al., 2000), finding this protein mainly at preantral stages (Hosoe et al., 2011). In fact, recombinant GDF-9 is potent in stimulating initial follicle growth (Vitt et al., 2000) by up-regulating theca cell androgen production (Orisaka et al., 2009). Furthermore, the decreased level of GDF-9 observed in this study confirms previous findings with in vitro maturation experiments, where the major expression of GDF-9 in both oocyte and cumulus cells in canine was present in an early stage of development (De los Reyes et at., 2013).

The major GDF-9 expression level observed in small antral follicles during anestrus as compared to small follicles at other stages might be related with the cyclic changes. At this time point in dogs a cohort of follicles begins to grow (Concannon, 2009), and GDF-9 enhanced the progression of follicular recruitment and early development from preantral to small antral stage (Lin et al., 2012; Persani et al., 2014). Hence, small antral follicles found during other more advanced stages of the estrus cycle, like those at proestrous/estrus or diestrus, could be less competent than those at anestrus.

At anestrus the expression of BMP-15 in small antral follicles was the lowest compared to the same size in other reproductive stages, increasing the levels to medium size follicles in anestrus. BMP-15 has been shown to increase the proliferation of granulosa cells from rats (Otsuka et al., 2000), humans (Di Pasquale et al., 2004) and ruminants (McNatty et al., 2005), and selectively modulates the biological effects of FSH on these cells (Persani et al., 2014). During mid and late anestrus, FSH concentrations in the bitch are elevated; often at or near preovulatory peak concentrations (Concannon, 2011), and in this stage antral follicles are selected to grow and advance to the next stage. Therefore, it is possible that the rise in BMP-15 expression from small and medium sizes follicles may be involved in follicular development and proliferation during anestrus in bitches, possibly regulating the selection of follicles at the end of anestrus.

In proestrous/estrus the expression of both GDF-9 and BMP-15 decreased from small to large preovulatory follicles. In other species these proteins have been involved in follicular maturation (Gilchrist et al., 2006; Persani et al., 2014), especially at the time of cumulus expansion (Su et al., 2008; Gueripel et al., 2006; Paulini and Melo., 2011). Studies in vitro in mouse using recombinant proteins also demonstrated that both GDF-9 and BMP-15 are crucial for cumulus expansion (Prochazka et al., 2004; Sasseville et al., 2010), influencing oocyte maturation. However, a negative correlation has been reported between the expression level of GDF-9 and cumulus expansion during in vitro maturation using canine cumulus oocytes complexes, raising the

possibility that in dogs the major expression of GDF-9 is present only in an early stage of development, possibly in a species specific manner which may be related with the delay in mucification (De los Reyes et al., 2013), because a low concentration of GDF-9 does not stimulate enzymes such as Hyaluronan synthase 2 and cyclooxygegase-2, that regulate cumulus expansion (Varani et al., 2002).

Several connexins (Cx) are expressed in ovarian follicles and Cx43 has been demonstrated to play an essential role in folliculogenesis (Lenhart et al., 1998; Nitta et al., 2010). Granulosa cells must be coupled via Cx43 gap junctions in order to respond optimally to GDF-9 (Gittens et al., 2005). In dogs, the gene encoding Cx43, was found to differ across the reproductive cycle, showing a decreased expression at estrus, rising levels during diestrus, and declining values during anestrus (Willingham-Rocky et al., 2007; Willingham et al., 2004). These differences could be in accordance with variations of GDF-9 found in this study during the follicular development at different phases of the estrous cycle, because although expression levels of GDF-9 were high in small follicles at anestrus, these levels significantly decreased with increasing follicle size during this stage.

Studies in mice have shown that in growing follicles Cx43 expression increases in response to the rising level of follicle-stimulating hormone (FSH), but it subsequently declines in response to the luteinizing hormone (LH) surge, since LH causes the closure of the channels via MAP kinase-dependent phosphorylation of Cx43 of ovarian follicle (Norris et al., 2008). In dogs during transition from proestrous to estrous, FSH and LH are increasing to the preovulatory surge of LH (Concannon, 2011); the decreased GDF-9 levels in large follicles at proestrous/estrus found herein might be coincident with the increased LH surge terminating the proestrus in the bitch. Therefore, possibly the LH peak in dogs may restrict the flow of GDF-9 into the follicle cells during estrus. This possibility would be reinforced by the subsequent increase of GDF-9 found from small to medium sizes follicles at diestrus when LH levels decrease.

The decreasing expression of BMP-15 during proestrous–estrus appears somewhat confusing because previously it was found in canine ovaries that increasing levels of this protein are expressed in both cumulus cells and oocytes during in vitro maturation experiments (De los Reyes et al., 2014). To date, nothing is known about the mechanism by which BMP-15 modulates follicular development in canines, but possibly the differences in these results may be related to different expression during the estrous cycle, and thus the reproductive phase at which the follicles and oocytes for IVM were obtained, considering the influence of the estrus cycle on cumulus–oocyte communications (Luvoni et al., 2001).

Premature luteinization is associated with high serum progesterone levels and BMP-15 suppresses progesterone production in rodent granulosa cells (Otsuka et al., 2001) and down-regulates Cx43 (Chang et al., 2014). Also, a BMP-15 inhibitory action on progesterone secretion in human granulosa cells lines has been described (Chang et al., 2013). The present data however, showed decreasing levels of BMP-15 at proestrous–estrous and lack of differences in BMP-15 expression between small to medium sizes follicle at diestrus. In the bitch, serum progesterone rises slowly during end of proestrus, reflecting luteinization of large follicles visible histologically as early as six days before the LH peak (Concannon, 2009). Therefore, estrus is concomitant with subsequently declining phase of estradiol concentration and increasing progesterone concentration (De Gier et al., 2006). This indicates that premature luteinization that is associated with high serum progesterone concentration is a normal feature in the canine estrus cycle; therefore, species variation is also possible in the role of BMP-15 in antral follicle development, and thus, the level of this protein for reproductive success may be not coincident across species. This suggest that the anti-luteinization function of BMP-15 described in other mammals could be different in dogs, or perhaps the decreasing expression of this factor observed at proestrous/estrus in large follicles, is not sufficient to avoid progressive luteinization during this period in canines.

In conclusion, the flow cytometric assay was able to assess protein expression in canine follicle cells. Although the biological significance of GDF-9 and BMP-15 expression in these cells has not been fully elucidated, the present study is the first evidence that canine follicular GDF-9 and BMP-15 are differentially expressed during development. The declining secretion of these factors in proestrous/estrus as compared to other species, might be related, at least in part, to premature luteinization which is one of the main reproductive traits of dogs.

Conflict of interest

The authors declare that there is no conflict of interest.

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References

Chang, H.M., Cheng, J.C., Klausen, C., Leung, P.C., 2013. BMP15 suppresses progesterone production by down-regulating StAR via ALK3 in human granulosa cells. Mol. Endocrinol. 27, 2093–2104.

Chang, H.M., Cheng, J.C., Taylor, E., Leung, P.C., 2014. Oocyte-derived BMP15 but not GDF9 down-regulates connexin43 expression and decreases gap junction intercellular communication activity in immortalized human granulosa cells. Mol. Hum. Reprod. 20, 373–383.

Chastant-Maillard, S., Viaris de Lesegno, C., Chebrout, M., Thoumire, S., Meylheue, T., Fontbonne, A., Chodkiewicz, M., Saint-Dizier, M., Reynaud, K., 2011. The canine oocyte: uncommon features of in vivo and in vitro maturation. Reprod. Fertil. Dev. 23, 391–402.

Cheng, J.C., Chang, H.M., Fang, L., Sun, Y.P., Leung, P.C.K., 2015. TGF-β1 Up-regulates connective tissue growth factor expression in human granulosa cells through smad and ERK1/2 signaling pathways. PLoS One 10, e0126532.

Concannon, P.W., 2009. Endocrinologic control of normal canine ovarian function. Reprod. Domest. Anim. 44 (Suppl. 2), 3–15.

Concannon, P.W., 2011. Reproductive cycles of the domestic bitch. Anim. Reprod. Sci. 124, 200–210.
De Gier, J., Kooistra, H.S., Djajadiningrat-Laanen, S.C., Dieleman, S.J., Okkens, A.C., 2006. Temporal relations between plasma concentrations of luteinizing hormone, follicle-stimulating hormone, estradiol-17β, progesterone, prolactin, and a-melanocyte-stimulating hormone during the follicular, ovulatory, and early luteal phase in the bitch. Theriogenology 65, 1346–1359.
De los Reyes, M., de Lange, J., Miranda, P., Palomino, J., Barros, C., 2005. Effect of human chorionic gonadotrophin supplementation during different culture periods on in vitro maturation of canine oocytes. Theriogenology 64, 1–11.
De los Reyes, M., Palomino, J., Parraguez, V.H., Hidalgo, M., Saffie, P., 2011. Mitochondrial distribution and meiotic progression in canine oocytes during in vivo and in vitro maturation. Theriogenology 75, 346–353.
De los Reyes, M., Parraguez, V.H., Rojas, C., Palomino, J., 2013. Expression of growth differentiation factor 9 (GDF-9) during in vitro maturation in canine oocytes. Theriogenology 80, 587–596.
De los Reyes, M., Castro, R., Palomino, J., 2014. Dynamic expression of bone morphogenetic protein 15 (BMP-15) during in vitro maturation of canine oocytes. In: Proceedings Society Study of Reproduction (SSR) 47th Annual Meeting, (abstract 295) Grand Rapids, Michigan USA.
De Neubourg, D., Robins, A., Fishel, S., Gibbon, L., 1996. Flow cytometric analysis of granulosa cells from follicular fluid after follicular stimulation. Hum. Reprod. 11, 2211–2214.
Di Pasquale, E., Beck-Peccoz, P., Persani, L., 2004. Hypergonadotropic ovarian failure associated with an inherited mutation of human bone morphogenetic protein-15 (BMP15) gene. Am. J. Hum. Genet. 75, 106–111.
Douville, G., Sirard, M.A., 2014. Changes in granulosa cells gene expression associated with growth, plateau and atretic phases in medium bovine follicles. J. Ova Res. 7, 50.
Eppig, J.J., 2001. Oocyte control of ovarian follicular development and function in mammals. Reproduction 122, 829–838.
Fair, T., 2003. Follicular oocyte growth and acquisition of developmental competence Anim. Reprod. Sci. 78, 203–216.
Fenwick, M.A., Mora, J.M., Mansour, Y.T., Baithun, C., Franks, S., Hardy, K., 2013. Investigations of TGF-beta signaling in preantral follicles of female mice reveal differential roles for bone morphogenetic protein 15. Endocrinology 154, 3423–3436.
Galloway, S.M., McNatty, K.P., Cambridge, L.M., Laitinen, M.P., Juengel, J.L., Jokiranta, T.S., McLaren, R.J., Luiro, K., Dodds, K.G., Montgomery, G.W., Davis, G.H., Beattie, A.E., Ritvos, O., 2000. Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner. Nat. Genet. 25, 279–283.
Gilchrist, R.B., Lane, M., Thompson, J.G., 2008. Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. Hum. Reprod. Update 14, 159–177.
Gilchrist, R.B., Ritter, L.J., Myllymaa, S., Kaivo-Oja, N., Dragovic, R.A., Hickey, T.E., Ritvos, O., Mottershead, D.G., 2006. Molecular basis of oocyte–paracrine signaling that promotes granulosa cell proliferation. J. Cell Sci. 119, 3811–3821.
Gittens, J.E.I., Barr, K.J., Vanderhyden, B.C., Kidder, G.M., 2005. Interplay between paracrine signaling and gap junctional communication in ovarian follicles. J. Cell Sci. 118, 113–122.
Gueripel, X., Brun, V., Gougeon, A., 2006. Oocyte bone morphogenetic protein 15 but not growth differentiation factor 9, is increased during gonadotropin-induced follicular development in the immature mouse and is associated with cumulus oophorus expansion. Biol. Reprod. 75, 836–843.
Hirshfield, A.N., Flickinger, G.L., Ben-Rafae, Z., 1988. Flow cytofluorometric analysis of granulosa cell proliferation in rats. J. Reprod. Fertil. 84, 231–238.
Hosoe, M., Kaneyam, K., Ushizawa, K., Hayashi, K., Takahashi, T., 2011. Quantitative analysis of bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9) gene expression in calf and adult bovine ovaries. Reprod. Biol. Endocrinol. 9, 33.
Huiyu, B.Z., Lib, L.X., Changhong, L.D., Lianjiang, Z.L., Xianfeng, Y.D., Zhanga, Y.J., 2013. Effects of BMP15 on porcine cumulus cell apoptosis. Cell Physiol. Biochem. 32, 264–278.
Hussein, T.S., Thompson, J.G., Gilchrist, R.B., 2006. Oocytes secreted factors enhance oocyte developmental competence. Development 296, 514–521.
Jaslow, C.R., Patterson, K.S., Cholera, S., Jennings, L.K., KeWilliam, R.W., Kutteh, H., 2010. CD9 expression by human granulosa cells and platelets as a predictor of fertilization success during IVF. Obstet. Gynecol. Int. 192461, 7.
Kidder, G.M., Vanderhyden, B.C., 2010. Bidirectional communication between oocytes and follicle cells: ensuring oocyte developmental competence Can. J. Physiol. Pharmacol. 88, 399–413.
Knight, P.G., Glister, C., 2006. Focus on TGF-β Signalling TGF-β superfamily members and ovarian follicle development. Reproduction 132, 191–206.
Lenhart, J.A., Downey, B.R., Bagnell, C.A., 1998. Connexin 43 gap junction protein expression during follicular development in the porcine ovary. Biol. Reprod. 58, 583–590.
Lee, S.G., Kim, H.S., Hwang, W.S., Hwan, S., 2008. Characterization of porcine growth differentiation factor-9 and its expression in oocyte maturation. Mol. Reprod. Dev 75, 707–714.
Lin, J.Y., Pitman-Crawford, J.L., Bibby, A.H., Hudson, N.L., Mcintosh, C.J., Juengel, J.L., McNatty, K.P., 2012. Effects of species differences on oocyte regulation of granulosa cell function. Reproduction 144, 557–567.
Lim, Z.L., Li, Y.H., Xu, Y.N., Wang, Q.L., Namgong, S., Cui, X.S., Kim, N.H., 2014. Effects of growth differentiation factor 9 and bone morphogenetic protein 15 on the in vitro maturation of porcine oocytes. Reprod. Domest. Anim. 49, 219–227.
Luvoni, G.C., Luciano, A.M., Modina, S., Gandolfi, F., 2001. Influence of different stages of the oestrous cycle on cumulus-oocyte communications in canine oocytes: effects on the efficiency of in vitro maturation. J. Reprod. Fertil. 57, 141–146.
Maupeu, D., Palomino, J., De los Reyes, M., 2015. Immunohistochemistry localization of growth differentiation factor 9 (GDF-9) and bone morphogenetic protein 15 (BMP-15) in canine follicles throughout estrus cycle. Reprod. Fertil. Dev. 27, 182.
McNatty, K.P., Juengel, J.L., Reader, K.L., Lun, S., Myllymaa, S., Lawrence, S.B., Western, A., Meerasahib, M.F., Mottershead, D.G., Groome, N.P., Ritvos, O., Laitinen, M.P., 2005. Bone morphogenetic protein 15 and growth differentiation factor 9 co-operate to regulate granulosa cell function. Reproduction 129, 473–480.
Mottershead, D.G., Lesley, J., Ritterand, L.J., Gilchrist, R.B., 2012. Signalling pathways mediating specific synergistic interactions between GDF9 and BMP15. Mol. Hum. Reprod. 18, 121–128.
Nitta, M., Yogo, K., Ohashi, M., Akiyama, M., Kunitomo, Y., Ogawa, T., Ishida-Kitagawa, N., Miyoshi, J., Sato, E., Takeya, T., 2010. Identification and expression analysis of connexin-45 and connexin-60 as major connexins in porcine oocytes. J. Anim. Sci. 88, 3269–3279.
Norris, R.P., Freudzon, M., Mehlmann, L.M., Cowan, A.E., Simon, A.M., Paul, D.L., Lampe, P.D., Jaffe, L.A., 2008. Luteinizing hormone causes MAP kinase-dependent phosphorylation and closure of connexin43 gap junctions in mouse ovarian follicles: one of two paths to meiotic resumption. Development 135, 3229–3238.
Orisaka, M., Jiang, J.Y., Orisaka, S., Kotsuji, F., Tsang, B.K., 2009. Growth differentiation factor 9 promotes rat preantral follicle growth by up-regulating follicular androgen biosynthesis. Endocrinology 150, 2740–2748.
Otsuka, F., Yao, Z., Lee, T., Yamamoto, S., Erickson, G.F., Shimasaki, S., 2000. Bone morphogenetic protein-15. Identification of target cells and biological functions. J. Biol. Chem. 275, 39523–39528.
Otsuka, F., McTavish, K.J., Shimasaki, S., 2011. Integral role of GDF-9 and BMP-15 in ovarian function. Mol. Reprod. Dev. 78, 9–21.
Otsuka, F., Yamamoto, S., Erickson, G.F., Shimasaki, S., 2001. Bone morphogenetic protein-15 inhibits follicle-stimulating hormone (FSH) action by suppressing FSH receptor expression. J. Biol. Chem. 276, 11387–11392.
Paulini, F., Melo, E.O., 2011. The role of oocyte-secreted factors GDF9 and BMP15 in follicular development and oogenesis. Reprod. Domest. Anim. 46, 354–356.
Persani, L., Rossetti, R., Di Pasquale, E., Cacciatore, C., Fabre, S., 2014. The fundamental role of bone morphogenetic protein 15 in ovarian function and its involvement in female fertility disorders. Hum. Reprod. Update 20, 869–883.
Prochazka, R., Nemcova, L., Nagyova, E., Kanka, J., 2004. Expression of growth differentiation factor 9 messenger RNA in porcine growing and preovulatory ovarian follicles. Biol. Reprod. 71, 1290–1295.
Rao, M., Allsbrook Jr., W.C., Conway, B.A., Martinez, J.E., Beck, J.R., Pantazis, C.G., Mills, T.M., Anderson, E., Mahesh, V.B., 1991. Flow cytometric analysis of granulosa cells from developing rat follicles. J. Reprod. Fertil. 91, 521–530.
Regan, S.L., McFarlane, J.R., O'Shea, T., Andronicos, N., Arfuso, F., Dharmarajan, A., Almahbobi, G., 2015. Flow cytometric quantification of receptors and apoptosis in granulosa cells and ovulation rate. Reproduction 150, 151–163.

Sasseville, M., Ritter, L.J., Nquyen, T.M., Liu, F., Mottershead, D.G., Russell, D.L., Gilchrist, R.B., 2010. Growth differentiation factor 9 signaling requires ERK1/2 activity in mouse granulose and cumulus cells. J. Cell Sci. 123, 3166–3176.

Silva, J.R., Van Den Hurk, R., Van Tol, H.T.A., Roelen, B.A., Figueiredo, J.R., 2005. Expression of growth differentiation factor 9 (GDF9), and bone morphogenetic protein 15 (BMP15), and BMP receptors in the ovaries of goats. Mol. Reprod. Dev. 70, 11–19.

Songsasen, N., Wild, D., 2005. Size of the donor follicle but not stage of reproductive cycle or seasonality, influences meiotic competency of selected domestic dog oocytes. Mol. Reprod. Dev. 72, 113–119.

Sudiman, J., Ritter, L.J., Feil, D.K., Wang, X., Chan, K., Mottershead, D.G., Robertson, D.M., Thompson, J.G., Gilchrist, R.B., 2014. Effects of differing oocyte-secreted factors during mouse in vitro maturation on subsequent embryo and fetal development. J. Assist. Reprod. Genet. 31, 295–306.

Su, Y.Q., Wu, X., O'Brien, M.L., Pendola, F.L., Denegre, J.N., Matzuk, M.M., Eppig, J.J., 2004. Synergistic roles of the oocyte-cumulus cell complex in mice: genetic evidence for an oocyte–granulosa cell regulatory loop. Dev. Biol. 276, 64–73.

Su, Y.Q., Sugiura, K., Wigglesworth, K., O'Brien, M.J., Affourtit, J.P., Pangas, S.A., Matzuk, M.M., Eppig, J.J., 2008. Oocyte regulation of metabolic cooperativity between mouse cumulus cells and oocytes: BMP15 and GDF9 control cholesterol biosynthesis in cumulus cells. Development 135, 111–121.

Sun, Y.Q., Liu, K., Kikuchi, K., 2008. Oocyte-specific knockout a novel in vivo approach for studying gene functions during folliculogenesis. oocyte maturation. fertilization, and embryogenesis. Biol. Reprod. 79, 1014–1020.

Varani, S., Elvin, J.A., Yan, C., De Mayo, J., De Mayo, F.J., Horton, H.F., Byrne, M.C., Matzuk, M.M., 2002. Knockout of pentraxin 3 a downstream target of growth differentiation factor-9, causes female subfertility. Mol. Endocrinol. 16, 1154–1167.

Ververidis, H.N., Boscos, C.M., Stefanakis, A., Krambovitis, E., 2002. Use of enzyme-immunoassay for oestradiol-17β and progesterone quantification in canine serum. Anim. Reprod. Sci. 69, 53–64.

Vitt, U.A., Hayashi, M., Klein, C., Hsueh, A.J., 2000. Growth differentiation factor-9 stimulates proliferation but suppresses the follicle-stimulating hormone-induced differentiation of cultured granulosa cells from small antral and preovulatory rat follicles. Biol. Reprod. 62, 370–377.

Willingham-Rocky, L.A., Golding, M.C., Wright, J.M., Kraemer, D.C., Westhusin, M.E., Burghardt, R.C., 2007. Cloning of GJA1 (connexin43) and its expression in canine ovarian follicles throughout the estrous cycle. Gene Expr. Patterns 7, 66–71.

Willingham, L.A., Golding, M.C., Westhusin, M.E., Kraemer, D.C., Burghardt, R.C., 2004. Characterization of connexin 43 in the canine ovarian follicle. Rep. Fert. Dev. 16, 235–236.