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Roles of GDF9 and BMP15 in equine follicular development: in vivo content and in vitro effects of IGF1 and cortisol on granulosa cells

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Abstract

Background In horses, the mechanisms behind ovarian follicle growth and oocyte maturation remain largely unknown. In other species, oocyte-secreted factors growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) have been related to the acquisition of developmental competence and to interaction with granulosa cells for the regulation of follicle development. This study assessed the expression and localization of GDF9 in the equine ovary, and its possible relationship with granulosa cell function.

Results Using custom-made antibodies, GDF9 protein was localized in oocytes from the primary follicle stage onwards. Together with BMP15, its intrafollicular concentration was higher in small antral follicles compared to larger ones ($P < 0.05$). Negative correlations were observed between intrafollicular BMP15 concentration and estradiol sulfate (E2S) ($r = -0.36$, $P = 0.048$), as well as between BMP15 and E2S/P4 ratio ($r = -0.37$, $P = 0.046$). In vivo, equine granulosa cells showed increasing mRNA expression of genes involved in steroidogenesis (*STAR* and *HSD3B2*) and cell proliferation (*KI67*) with increasing follicle size, while expression of *GDF9* and of apoptosis-related genes (*BCL2* and *CASP3*) were not affected by follicle size. Simultaneous stimulation of granulosa cells in vitro with IGF1 and cortisol significantly increased *HSD3B2* and *CYP19A1* transcriptional levels, as well as E2 concentration in culture media, while IGF1-induced P4 secretion was suppressed in the presence of cortisol. Blocking the stimulatory effect of IGF1 on E2, E2S and P4 by H89 was associated with increased *GDF9* mRNA levels and reduced *STAR*, *PCNA*, *KI67* and *BCL2* mRNA expression. Significant negative correlations of *GDF9* with *STAR and PCNA* mRNA, respectively, were seen in vivo and in vitro.

Conclusions Together, our results show GDF9 localization and expression in the equine ovary and a temporal relationship with steroidogenesis and cell proliferation within the surrounding granulosa cells. Moreover, results of the in vitro study suggest a supporting role of cortisol during follicle maturation. Our study sheds light on possible mechanisms for the regulation of ovarian function in horses using GDF9.

Keywords GDF9, BMP15, Oocyte-derived factors, Cortisol, Steroidogenesis, Cell proliferation, Apoptosis

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Background

A healthy intrafollicular environment is crucial for the acquisition of oocyte developmental competence [1]. The oocyte is protected in vivo against harmful conditions by the combined action of granulosa cells and follicular fluid [2]. In antral follicles, oocyte development is regulated by transzonal projections of corona radiata cells [3], as well as through paracrine signalling [4]. Furthermore, granulosa cells are responsible for synthesizing activin and inhibin, both of which belong to the transforming growth factor-β (TGF-β) superfamily [5]. These proteins play a crucial role in regulating the secretion of follicle-stimulating hormone (FSH) from the pituitary gland, ultimately promoting oocyte growth [4]. Another vital function of granulosa cells is to synthesize steroid hormones in response to pituitary gonadotropins, utilizing cholesterol as a substrate [6]. Pregnenolone (P5) is further metabolized to progesterone (P4) by 3beta-hydroxysteroid dehydrogenase enzymes (HSD3B). In addition to 17β-estradiol (E2), the main steroidogenic product, granulosa cells produce other important steroids, including 11-deoxycorticosterone and 11-deoxycortisol catalyzed by 21-hydroxylase (CYP21A2) [7]. Besides granulosa cells, the adrenal is also known to contribute a significant amount of steroids and cortisol to the blood [8]. These products are then released into the follicular fluid where they act both directly (via genomic and non-genomic actions) and indirectly (via somatic cells within the follicle) to influence follicular growth and acquisition of developmental potential by the oocyte [9]. Accordingly, a higher abundance of transcripts involved in ovarian steroidogenesis has recently been demonstrated in granulosa cells of sows with high quality cumulus-oocyte complexes (COCs) [10].

In mammalian ovaries, insulin-like growth factor 1 (IGF1) plays a pivotal role in regulating steroidogenesis and follicular growth [11]. In addition to employing the cAMP/PKA pathway [12], IGF1 enhances the expression of steroidogenic regulatory genes in granulosa cells by interacting with the IGF1 receptor, triggering the PI3K/AKT pathway [13]. In mares, IGF1 is produced dynamically by granulosa cells, with the highest plasma levels being observed after follicle deviation before returning to lower levels as ovulation approaches [14]. In parallel, glucocorticoids, traditionally associated with stress adaptation, also play significant roles in regulating follicular development and ovulation. Plasma concentrations of cortisol, a prominent glucocorticoid found in equine follicular fluid [15], decline after follicle deviation and remain at consistently low levels until ovulation [16]. However, intrafollicular cortisol levels peak after follicle deviation in mares without any apparent change in plasma concentration, suggesting that the systemic concentration and intracompartmental activity of glucocorticoids are not necessarily correlated [17]. Furthermore, equine follicular fluid reveals a positive correlation between cortisol and P4, consistent with findings in humans and cattle [18, 19]. Cortisol has also been shown to modulate apoptosis in porcine, mice and human granulosa cells [20–22], but such mechanisms have not yet been investigated in the mare. Nevertheless, the addition of cortisol to the maturation media did not affect equine oocyte maturation [17]. This contrasts with observations in pigs, where a high proportion of high-quality oocytes is associated with lower intrafollicular cortisol levels [10]. While the precise mechanisms are not yet fully elucidated, these findings suggest a potential role for cortisol in the regulation of granulosa cell function during follicular maturation.

Adding complexity to this interaction, Growth Differentiation Factor 9 (GDF9) and Bone Morphogenetic Protein 15 (BMP15), belonging to the TGF-β superfamily, are key oocyte-secreted factors that interact with granulosa cells to regulate ovarian follicle development and ovulation rate in farm animals such as sheep, cattle and horses [23–25], but also in rodents and humans [26]. In mice, GDF9 is detected in oocytes from the primary follicle stage onwards, and it regulates oocyte growth and acquisition of meiotic competence [27, 28]. Moreover, oocyte-derived GDF9 stimulates granulosa cell factors which are essential for theca cell formation [29]. Furthermore, a block in follicular development beyond the primary follicle stage is observed in GDF9-deficient mice, leading to infertility [30]. In human ovaries, GDF9 is expressed in oocytes from the primordial follicle stage, suggesting its involvement in early folliculogenesis [31]. Interestingly, GDF9 expression is higher in calf oocytes than in adults [32], most likely due to different developmental competence between calf and adult oocytes. Both GDF9 and BMP15 can be detected in equine ovarian tissue before and after in vitro culture [33] and their expression is associated with oocyte maturation in horses [17]. Also, active immunization of mares against BMP15 reduced the number of ovulations, while GDF9 vaccination only reduced follicle size without affecting ovulation rate [23]. Recently, it has become possible to determine the intrafollicular concentration of GDF9 using immunoassays and its levels were linked to oocyte quality and maternal age in humans [34], highlighting its significance in the context of ovarian function and reproductive success. Nevertheless, GDF9 protein expression extends beyond the oocyte in several species with its presence in granulosa cells in humans [31], goats [35], pigs [31], and cows [32], adding new potential targets for its activity. In developing ovarian follicles, GDF9 can affect reproductive success. Thus, in the absence of GDF9 in mice, follicular development is disrupted and the oocytes have unusual organelle aggregation, altered Golgi complexes,

and a lack of cortical granules [28]. Furthermore, GDF9, in collaboration with BMP15, impedes apoptosis and promotes proliferation in rat cumulus cells [36], ultimately leading to cumulus cell expansion by upregulating the gene expression of hyaluronic acid synthase 2 (HAS2) and cyclooxygenase 2 (COX2/PTGS2) [37, 38]. The interaction between GDF9 and BMP15 also induces cholesterol biosynthesis in mouse cumulus cells [39]. As primarily an oocyte-secreted mitogen [40], GDF9 not only exerts effects on the cumulus-oocyte complex, but also regulates granulosa cell function via SMAD-dependent and -independent pathways in humans [41]. In bovine granulosa cells, GDF9 promotes cell proliferation and inhibits steroid hormone production induced by IGF1 and FSH [42]. In humans, GDF9 enhances activin A-induced inhibin B secretion in luteinized granulosa cells [43]. The synergistic effect of GDF9 and BMP15 was also shown to increase Anti-Müllerian hormone (AMH) expression through the PI3K/Akt and Smad2/3 pathways [44]. In horses, *BMP15* and *GDF9* mRNA expression has been identified in granulosa cells and in corpus luteum [45], but further research is needed to understand the specific role of GDF9 in the regulation of follicle development and maturation.

Here, to test the expression and localization of GDF9 in the ovary, we first generated a polyclonal antibody for immunohistochemistry that recognizes equine GDF9 and then used commercially available anti-human GDF9 and BMP15 ELISA assays to measure their intrafollicular concentrations during follicular development. Considering its dynamic presence in developing follicles, we hypothesized that *GDF9* expression in granulosa cells is correlated to that of *STAR*, *HSD3B2*, *CYP19A1*, *PCNA*, *KI67*, *BCL2* and *CASP3*, genes involved in essential processes such as steroidogenesis, cell proliferation and apoptosis. These processes were chosen based on their relevance during follicle maturation and based on observations made regarding GDF9 activity in other species. Moreover, we investigated the effects of cortisol on granulosa cell function in vitro following the induction of steroidogenesis with IGF1, to resemble follicle maturation, or in response to the inhibition of PKA activity by a specific blocker (H89). Testing these hypotheses will give a better understanding of how equine granulosa cells act during follicle growth and maturation.

Materials and methods

Sample collection and preservation

Ovaries from 15 nonpregnant, clinically healthy mares with normal reproductive tracts upon macroscopic examination were collected from a slaughterhouse. After collection, ovaries were cooled on ice and transported to the laboratory for six hours. Then the ovaries were washed three times with saline solution (NaCl 0.9%) and blotted dry. Follicle size was determined using a conventional caliper, and follicular fluid and granulosa cells were collected from small (< 10 mm), medium (10–30 mm) and large (> 30 mm) follicles, respectively. Follicular fluid was aspirated using 20G needles and 10 ml syringes. In the case of small follicles, follicular fluid from several follicles from the same ovary was pooled into a single sample to provide sufficient volume for further analyses. Samples were only collected from healthy, growing follicles, identified based on their translucency, vascularization of the follicle wall and the integrity of the granulosa cell layer, as previously described for sheep follicles [46]. Moreover, corpus luteum (CL) samples were also collected and categorized as early or late/regressing CL based on macroscopic appearance. Follicular fluid samples were then centrifuged to remove granulosa and blood cells (1500 g for 10 min at 4 °C), followed by storage of the supernatant at – 80 °C until further laboratory analysis. Granulosa cell samples were snap-frozen and stored at – 80 °C until further analyses. In addition, ovarian tissue fragments from different regions of the same ovaries were collected for histological analysis and fixed in 10% phosphate-buffered formalin for 24 h, washed with phosphate-buffered saline (PBS) for 7 consecutive days, dehydrated in an ethanol series, transferred into xylene, and embedded in paraffin.

In a second step, granulosa cells were collected by ovum pick-up (OPU) during the months of September until November from eight ($n = 8$) cyclic mares aged 10 to 20 years and located at the AgroVet Strickhof research facility in Lindau, Switzerland for the in vitro part of the study. Mares were undergoing normal estrous cycles, as determined by the fact that in all mares a collapsed antral follicle and subsequent development of a CL were present after the last sample collection. Animal experiments were carried out in accordance with animal welfare legislation and approved by the Cantonal Veterinary Authority Canton Zurich under the license 33582/ZH059/2021 from 27.7.2021. For OPU procedures, mares were restrained in a palpation chute to restrict movement. The mare´s tail was wrapped with tape to minimize contamination. Feces were removed from the rectum and the perineal area was washed with octenidine solution (Octenisan, Schülke&Mayr GmbH, Norderstedt, Germany). Epidural anesthesia with 20 mg lidocainhydrochlorid (Lidocain 2%; G. Streuli & Co. AG, Switzerland) was used for every mare. Approximately 5 min before the start of each procedure, sedation and analgesia were induced with a combination of butorphanol (0.05 mg/kg, Morphasol, Graeub, Switzerland) and detomidine (0.02 mg/kg, Medesedan, Virbac, Switzerland). Additionally, 1.1 mg/kg of the anti-inflammatory drug flunixin (Vetaflumex Neo, Vet-Agro, Lublin, Poland) was given to each mare. All follicles smaller than 25 mm in diameter

(e.g. before deviation) were punctured using a 12G double lumen needle (Minitube, Tiefenbach, Germany) connected to an aspiration pump and vigorously flushed 12 times with EquiPlus OPU Recovery Medium (Minitube) prewarmed to 38 °C. The recovered fluid was first filtered through the EmSafe system (Minitube) to isolate cumulus-oocyte complexes (COCs) and then through a 40 µm cell strainer. The 40 µm cell strainer was backwashed with EquiPlus OPU Recovery Medium into a petri dish and the granulosa cell suspension then transferred to a 50 ml Falcon tube.

Immunofluorescence staining

In the absence of a commercially available species-specific or cross-reacting antibody for the equine species, a polyclonal custom-made antibody anti-GDF9 was successfully developed (Eurogentec, Seraing, Belgium) as previously described [47, 48]. For this, two guinea pigs were immunized using two peptide sequences each: C + HYKRRPSQGPDQKRD (aa 281–295) and C + AYATKEGIPKSNRGH (aa 95–109), and the immune serum from the animal showing the highest titer and the best signal-to-noise ratio was used for further analysis. Keyhole limpet hemocyanin (KLH) was used as protein carrier and quality control was performed using mass spectrometry and ELISA. Immunofluorescence staining was done following the previously published protocol [49, 50]. In brief, sections (3 µm) of formalin-fixed paraffin-embedded ovarian tissue were cut with a rotary microtome and mounted onto SuperFrost microscope slides (Menzel-Glaeser, Braunschweig, Germany), deparaffinized in xylene, rehydrated in a graded ethanol series and washed under running tap water for 3 min. For antigen retrieval, slides were placed in a plastic cuvette containing 10 mM citrate buffer pH 6.0 at 20–22°C for 5 min and subsequently heated in a microwave oven at 600 W for 15 min. Afterwards, slides were allowed to cool at 20–22°C for 20 min. After washing under running tap water for 5 min, sections were incubated in 10% goat serum for 30 min at 20–22°C to block non-specific binding. Then, sections were incubated overnight at + 4°C with the immune serum at 1:500 dilution. For negative controls, guinea pig pre-immune serum was used in place of primary antibody. Finally, after one wash with PBST (PBS + 0.3% Triton X-100) to remove unbound primary antibody, slides were incubated with a goat anti-guinea pig Alexa 594 secondary antibody (A11076, Thermo Fisher Scientific AG, Reinach, Switzerland) at 1:500 dilution supplemented with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) at a final concentration of 1:1000 for 60 min at 20–22 °C. Slides were mounted using Mowiol and subjected to microscopy analysis using a Leica DMI6000B fluorescence microscope equipped with a Leica K5 camera (Leica Microsystems, CMS GmbH, Breisgau, Germany).

Hormone assays

For the quantification of GDF9 and BMP15 intrafollicular concentrations, human GDF9 and BMP15 ELISA assays (AL-176 and AL-179, ANSH Labs, Webster, TX, USA) were used. Serial dilutions of equine follicular fluid showed good parallelism to the standard curves (deviation within 10% of expected values). According to manufacturer`s recommendations, 50 µl undiluted follicular fluid were used for the determinations. In case of GDF9, the intra- and inter-assay coefficients of variation were 6.2% and 3.6%, respectively, and the minimal detectable concentration was 20 pg/mL. In case of BMP15, the intra- and inter-assay coefficients of variation were 8.3% and 5.1%, respectively, and the minimal detectable concentration was 50 pg/mL. In both cases, the determinations were performed in a single experiment (on a single ELISA plate).

The radioimmunological measurement of progesterone (P4) concentrations was carried out using the method described by [51]. Before radioimmunological determination, 100 µl of follicular fluid or 20 µl cell culture medium were extracted twice with hexane. The pooled extracts were dried in a vacuum evaporator (Micro-Dancer, Hettich AG, Baech, Switzerland) and redissolved in assay buffer. The lower limit of detection was 0.5 ng/ml in follicular fluid and 0.1 ng/ml in cell culture medium. The intra- and inter-assay variation coefficients were 8.8 and 8.9%, respectively.

Due to the very different concentration ranges in follicular fluid and cell culture medium, two different methods were used for the determinations of E2 and E2 sulfate (E2S). The determinations of E2 and E2S in follicular fluid followed exactly the previously described procedure for the determination of free and conjugated estrone in equine blood plasma [51]. Their separate determination was achieved by extracting 0.1 ml follicular fluid twice with toluene and measuring the free form in the first step. In the second step, the aqueous sample residue was enzymatically hydrolyzed (β-glucuronidase/arylsulfatase from Helix pomatia, Roche Diagnostics GmbH, Mannheim, Germany), extracted again, and the conjugated form was subsequently determined. Different from the cited work [51], the measurements were performed on an E2 standard curve and a highly specific antiserum against E2 was used. The lower limit of detection was 0.1 ng/ml and the inter-assay variation coefficient was 5.8%.

The method for the determination of E2/E2S in cell culture medium followed basically the principle described above for follicular fluid. However, instead of the equilibrium assay, a much more sensitive sequence assay [52] was used for the radioimmunological determination of

E2 concentrations in the re-dissolved sample extracts, as described previously [53]. The lower limit of detection was 5 pg/ml. The interassay and intraassay coefficients of variation were 7.1% and 12.0%, respectively.

All hormone measurements were performed as duplicate determinations. For concentrations above the standard curve, the measurement was repeated with a reduced sample volume.

Cell culture and in vitro experiments

The 50 ml Falcon tube containing the granulosa cells underwent centrifugation (300 g for 15 min), then the supernatant was removed and the pellet resuspended in Dulbecco's Modified Eagle Medium (DMEM)/F-12 (1:1, Thermo Fisher Scientific AG) containing 0.5 mg/ml DNase I (Apollo Scientific, Stockport, Cheshire, UK), 1.25 mg/ml collagenase (Nordmark Pharma GmbH, Uetersen, Germany), 1% L-glutamine, 1% gentamycin and sodium bicarbonate (1.125 g/L, Sigma-Aldrich Chemie GmbH) and incubated for 20 min at 37 °C under a humidified 20% O_2, 5% CO_2 atmosphere as previously shown [14]. After another centrifugation, the supernatant was discarded and the pellet resuspended in the maintenance medium, i.e., DMEM/F-12 containing 10% FBS, 1% L-glutamine, 1% gentamycin and sodium bicarbonate (1.125 g/L). Cells were seeded at a density of 50,000–80,000 cells/ml in a 12-well plate and incubated for 48 h at 37 °C under a humidified 20% O_2, 5% CO_2 atmosphere.

At the conclusion of this incubation, the cells had reached at least 70% confluence. They were then washed once with 1% PBS and stimulated for 24 h in culture media without FBS (stimulation medium). Stimulation was done with 100 ng/ml IGF1 (Thermo Fisher Scientific AG) as shown in previous studies [54] in the presence or absence of cortisol (H6909, Sigma-Aldrich Chemie GmbH). Different concentrations of cortisol (50 ng/ml, 100 ng/ml, and 150 ng/ml) were added to the stimulation medium to mimic the conditions observed in vivo in small and large antral follicles [17]. In blocking experiments, cells were preincubated with 20 µM H89, a protein kinase A (PKA) inhibitor, for 40 min before stimulation, and subsequently stimulated in the presence of H89, based on previous studies [55, 56]. At the end of the stimulation experiment, granulosa cells were washed with 1% ice-cold PBS, collected in 500 µl of TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and stored at − 80 °C until assessment. Four independent experiments were conducted (biological replicates) using pooled granulosa cells obtained from the ovaries of four to six mares for each experiment.

Total RNA extraction, reverse transcription and semi-quantitative real-time TaqMan qPCR

For the granulosa cells collected in vivo, each sample was placed in 2 ml tubes in the presence of metallic beads and 500 µl TRIzol. Mechanical homogenization was performed on the TissueLyser LT instrument (Qiagen, Hilden, Germany) at 50 Hz for 60 s followed by cooling on ice for 1 min. If necessary, the homogenization step was repeated once. Total RNA was isolated with TRIzol reagent from in vivo and in vitro samples according to the manufacturer's instructions. Concentration and purity of RNA was measured on a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific AG). Then, 1.3 µg of total RNA from each sample was treated with DNase using the RQ1 RNase-free DNase kit (Promega, Duebendorf, Switzerland). The MultiScribe Reverse Transcriptase using random hexamers (Applied Biosystems by Thermo Fisher, Foster City, CA, USA) was applied to synthesize cDNA. Using 5 µl of the obtained cDNA corresponding to 100 ng RNA per sample and FastStart Universal Probe Master (ROX, Roche Diagnostics), semi-quantitative real time (TaqMan) PCR was performed in duplicate in 96-well optical plates. Autoclaved water was used as negative control and the routine inclusion of the minus RT control confirmed the absence of genomic DNA contamination. All TaqMan systems used were commercially available and were ordered from Applied Biosystems by Thermo Fisher. The detailed information regarding the assays used is listed in Table 1. The expression of the target genes was quantified with the comparative Ct method (ΔΔCt) and normalized to the expression

Table 1 List of symbols, corresponding gene names and TaqMan systems used for semi-quantitative real-time TaqMan PCR

Gene symbol	Gene name	Product number	GenBank access number
STAR	Steroidogenic acute regulatory protein	Ec03467762_m1	NM_001081800
HSD3B2	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2	Ec03468359_m1	NM_001081911
CYP19A1	Cytochrome P450 family 19 subfamily A member 1 (P450 aromatase)	Ec03467802_m1	AF031520.1
KI67	Marker of proliferation Ki-67	Ec07039069_g1	XM_005602153
PCNA	Proliferating cell nuclear antigen	Ec06974312_m1	XM_001494721
BCL2	B-cell lymphoma 2	Ec07005801_g1	XM_001490436
CASP3	Caspase 3	Ec03470391_m1	NM_001163961
GDF9	Growth differentiation factor 9	Ec07021047_s1	XM_001504427
PSMB4	Proteasome 20 S subunit beta 4	Ec07043334_g1	Ec07043334_g1
SNRPD3	Small nuclear ribonucleoprotein D3 polypeptide	Ec07002201_g1	XM_001489060

of the reference genes PMSB4 and SNRPD3, shown to be stably expressed in the equine ovary [57].

Statistical analysis

The computer software SPSS version 27 (IBM-SPSS, Armonck, NY, USA) was used for all analyses. To evaluate differences in follicular fluid concentrations and granulosa cells gene expression among different follicle size groups, a nonparametric one-way ANOVA was applied and, if the *P* value was less than 0.05, was followed pairwise comparisons after Bonferroni correction. In cases where the determined intrafollicular concentrations of BMP15 or GDF9 were below the reliable detection limit of the assay, the minimal detection limit was used for the statistics. For PCR results of the in vitro study and hormone concentrations in the cell culture media, a multivariate GLM was applied to test for the effects of IGF1 (no, yes, yes but preceded by H89), cortisol (0, 50, 100, 150 ng/ml), as well as their interaction. In case of significant effects, pairwise comparisons after Bonferroni correction were performed. Furthermore, Pearson test was used to test for correlations between hormone concentrations, gene expression in granulosa cells, as well as their combination. For relative quantification of gene expression, values generated after logarithmic transformation were used. All PCR results are presented as geometric mean (Xg) ± geometric standard deviation (SD), while hormone concentrations are presented as mean ± SD.

Results

Immunolocalization of GDF9 in the adult equine ovary

Immunofluorescence staining demonstrated the presence of GDF9 protein in the cytoplasm of the oocyte in primary, secondary, and tertiary follicles, but not in primordial follicles (Fig. 1). This staining pattern was observed independent of the presence/absence of a CL and no apparent difference in the staining intensity was observed throughout follicular development.

Concentrations of BMP15, GDF9 and steroids in follicular fluid throughout follicular development

The concentrations of BMP15 and GDF9 were determined, for the first time, in equine follicular fluid using human-specific ELISA kits. Measurements were performed in follicular fluid samples from 11 small (< 10 mm) and 11 medium (10–30 mm) follicles, as well as in nine samples from large follicles (> 30 mm). Concentrations of BMP15 were detectable in 9/11 (82%) samples from small follicles, 11/11 (100%) samples from medium follicles and 7/9 (78%) samples from large follicles. Concentrations of GDF9 were detectable in 10/11 (91%) samples from small follicles, 2/11 (18%) samples from medium follicles and 0/9 (0%) samples from large follicles. Overall, the concentrations of both BMP15 and GDF9 decreased significantly during follicle development ($P < 0.05$; Fig. 2). BMP15 concentration was significantly lower in large follicles than in medium follicles ($P < 0.01$), but did not differ from that in small follicles ($P > 0.05$, Fig. 2A). In the case of GDF9, its concentration was significantly lower in medium and large size follicles compared to small follicles ($P < 0.01$ in both cases, Fig. 2B).

Follicle size did not affect intrafollicular concentrations of P4 ($P > 0.05$, Fig. 2C), but affected E2 and E2S concentrations, as well as E2/P4 and E2S/P4 ratios, respectively ($P < 0.05$ in all cases). In large follicles, E2 concentration was higher than in small and medium follicles, respectively ($P < 0.05$ in both cases, Fig. 2D). Also, E2S concentration was higher ($P < 0.01$) in large follicles compared to small and medium follicles, respectively ($P < 0.05$ in both cases, Fig. 2E). A similar pattern was observed in case of E2/P4 and E2S/P4 ratios (Fig. 2F, G). There was a negative correlation between intrafollicular BMP15 concentration and E2S (r = -0.41, $P = 0.023$), as well as between BMP15 and E2S/P4 ratio (r = -0.43, $P = 0.015$). No correlation between GDF9 concentration and any other investigated hormone was found in the follicular fluid.

Gene expression in granulosa cells and corpus luteum in vivo

Relative mRNA levels of genes involved in steroidogenesis, proliferation, and apoptosis, as well as the oocyte-secreted factor, GDF9, were determined in granulosa cell samples from small (< 10 mm, $n = 14$), medium (10–30 mm, $n = 12$), and large (> 30 mm, $n = 5$) follicles, as well as in early ($n = 2$) and late ($n = 2$) CL samples. Apart from the steroidogenesis-related *STAR*, *HSD3B2* and *CYP19A1* analyses, all CL samples were analyzed together because the SD between stages was low in these cases.

Transcriptional levels of *STAR* significantly increased from the small to large follicle stage ($P < 0.05$, Fig. 3A). There was no difference in *STAR* mRNA between early and late CL ($P > 0.05$). Expression of *HSD3B2* significantly increased from small to large follicles ($P < 0.001$) and was at its highest levels in the early CL, while the late CL had very low *HSD3B2* levels (Fig. 3B). A similar expression pattern was observed for *CYP19A1*, although no significant differences were observed due to high standard deviation (Fig. 3C). There was a positive correlation in the gene expression of *STAR* and *HSD3B2* ($r = 0.34$, $P = 0.044$) but also in *HSD3B2* and *CYP19A1* ($r = 0.53$, $P = 0.001$). Also, transcriptional levels of *HSD3B2* were positively correlated with intrafollicular P4 ($r = 0.47$, $P = 0.017$), E2 ($r = 0.55$, $P = 0.004$) and E2S ($r = 0.40$, $P = 0.048$) concentrations, respectively. Similarly, transcriptional levels of *STAR* were positively correlated with intrafollicular P4 ($r = 0.41$, $P = 0.045$) and E2 ($r = 0.41$, $P = 0.040$) concentrations, respectively.

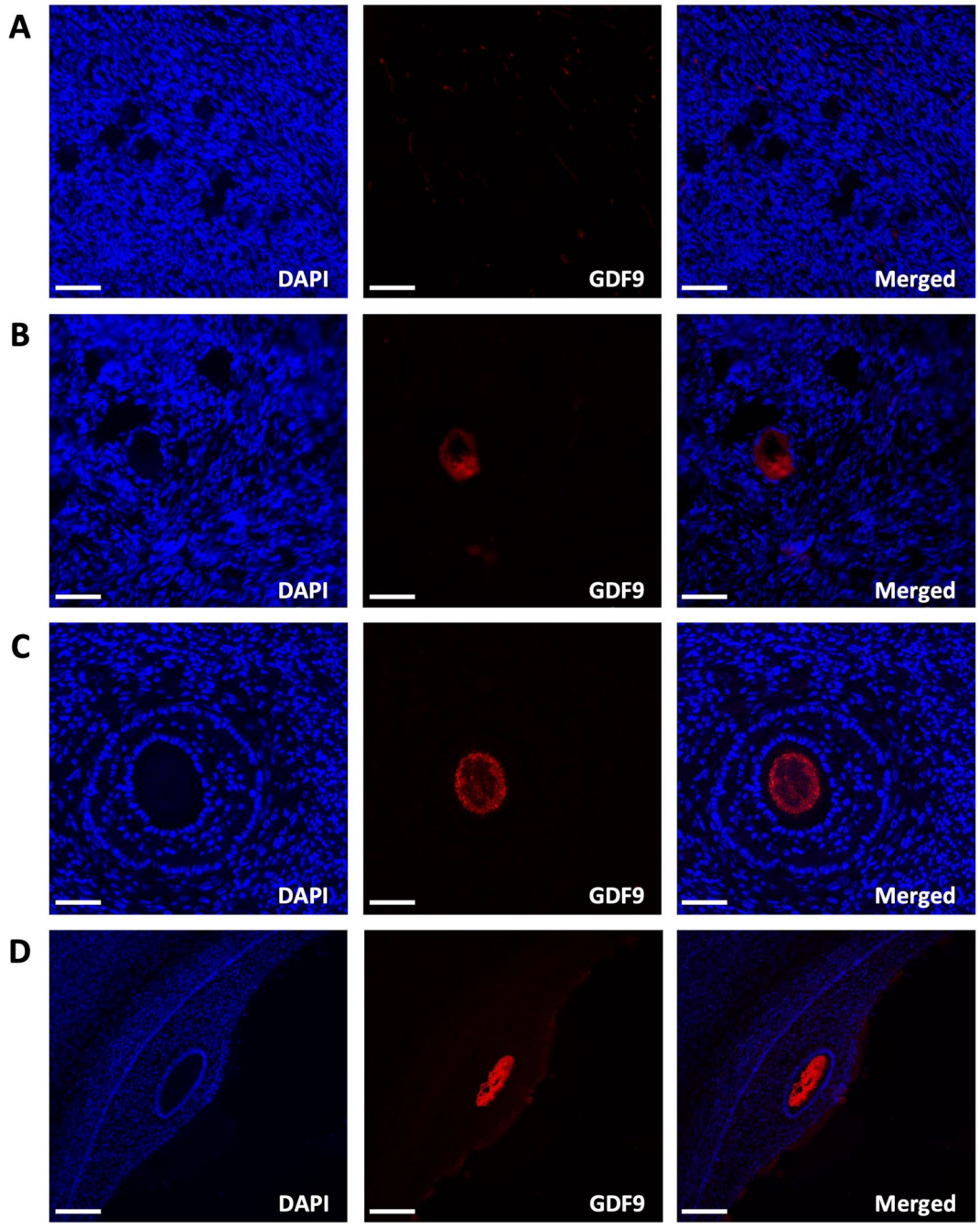

Fig. 1 Expression of Growth Differentiation Factor 9 (GDF9 – red color) in equine ovarian tissue. (**A**) Representative immunofluorescence staining of GDF9 in primordial follicles. (**B**) Representative immunofluorescence staining of GDF9 in primary follicle. (**C**) Representative immunofluorescence staining of GDF9 in secondary follicle. (**D**) Representative immunofluorescence staining of GDF9 in tertiary follicle. Nuclei were counterstained with DAPI (blue color). Scale bar represents 50 µm

Gene expression of *GDF9* was detectable in all but one sample in the small follicle group. Due to the high SD in the small and medium size follicles, there were no statistical differences between groups ($P > 0.05$, Fig. 3D). There was a negative correlation between *GDF9* and *STAR* expression (r = -0.46, $P = 0.006$), as well as between *GDF9* and *PCNA* (r = -0.35, $P = 0.042$).

Looking at the proliferation related genes, there was no difference ($P > 0.05$) in *PCNA* mRNA levels in granulosa cells among different size follicles or compared to the CL (Fig. 3E). However, the expression of *KI67* increased with the progression of follicular growth and was higher in granulosa cells obtained from medium sized follicles compared to small follicles and the CL ($P < 0.05$ in

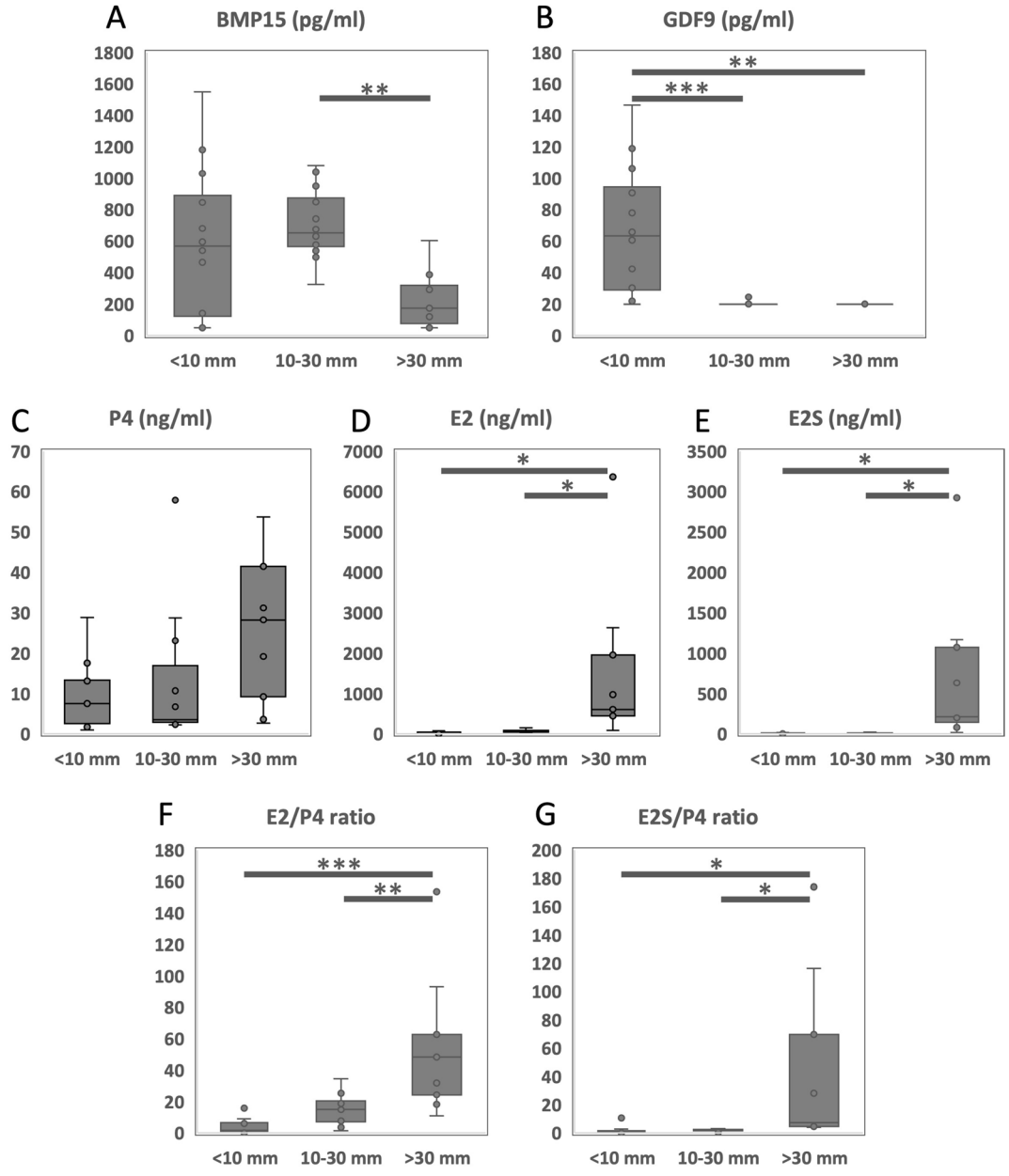

Fig. 2 Box and whisker chart presenting intrafollicular concentrations of (**A**) Bone Morphogenetic Protein 15 (BMP15), (**B**) Growth Differentiation Factor 9 (GDF9), (**C**) progesterone (P4), (**D**) free estrogens (E2), (**E**) conjugated estrogens (E2S), as well as (**F**) E2/P4 and (**G**) E2S/P4 ratios in small (< 10 mm, $n = 11$), medium (10–30 mm, $n = 11$) and large (> 30 mm, $n = 9$) equine ovarian follicles Bars with different asterisks differ at: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

all cases), but was similar between medium and large sized follicles ($P > 0.05$, Fig. 3F). A positive correlation was observed between *PCNA* and *HSD3B2* ($r = 0.34$, $P = 0.043$).

Regarding factors involved in apoptosis, no differences were observed in the *BCL2* (Fig. 3G) or *CASP3* (Fig. 3H) mRNA levels in any of the analyzed groups ($P > 0.05$). Gene expression of *BCL2* and *CASP3* was positively correlated ($r = 0.65$, $P < 0.001$) and both were negatively correlated with *CYP19A1* ($P < 0.001$ in both cases). Positive correlations were also observed between *BCL2* and *KI67* ($r = 0.37$, $P = 0.029$), and between *CASP3* and *PCNA* ($r = 0.41$, $P = 0.015$), respectively.

Effects of cortisol addition on granulosa cell gene expression after in vitro culture

Granulosa cells collected via transvaginal aspiration and cultured in vitro ($n = 4$ independent experiments) were stimulated with 100 ng/ml IGF1 for 24 h, in the presence or absence of cortisol in increasing concentrations: 50, 100 and 150 ng/ml. These concentrations were previously observed in antral follicles before deviation (50 ng/ml)

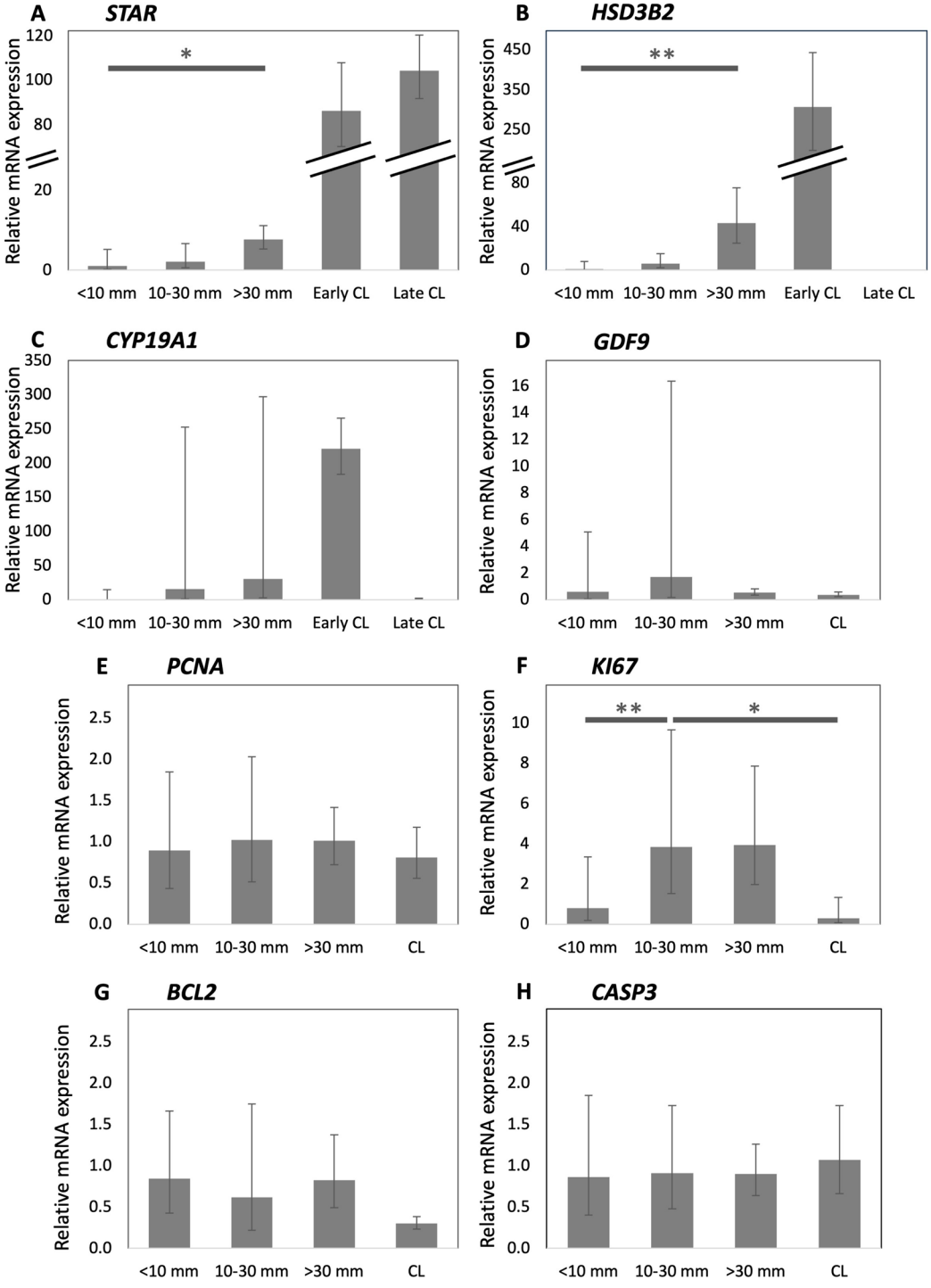

Fig. 3 Relative gene expression of *STAR*, *HSD3B2*, *CYP19A1*, *GDF9*, *PCNA*, *KI67*, *BCL2*, and *CASP3* in equine granulosa cells from small (< 10 mm, $n = 14$), medium (10–30 mm, $n = 12$) and large (> 30 mm, $n = 5$) equine ovarian follicles and corpus luteum ($n = 4$) during different stages of development. To evaluate the effect of the stage, one-way non-parametric ANOVA was applied, and when $P < 0.05$, the analysis was followed by multiple comparisons following Bonferroni correction. Relative gene expression is presented as determined by semi-quantitative real time (TaqMan) PCR (Xg ± SD). Bars with asterisks differ at: * $P < 0.05$, ** $P < 0.01$

and after deviation (120 ng/ml) [17]. In blocking experiments, cells were preincubated with 20 µM H89.

Expression of *STAR* was affected by IGF1 ($P < 0.001$), but not by cortisol or the interaction between IGF1 and cortisol ($P > 0.05$). It was increased in response to IGF1 compared to control ($P = 0.004$) and H89 ($P < 0.001$), but also in control versus H89 ($P = 0.001$, Fig. 4A). This increase was most evident in the presence of 100 ng/ml cortisol. Transcriptional levels of *HSD3B2* responded positively and were higher when IGF1 and cortisol were used simultaneously ($P < 0.001$ in all cases, Fig. 4B). The rise in *HSD3B2* levels was independent of cortisol dosage. A similar expression pattern as in the case of *HSD3B2* was observed also for *CYP19A1* (Fig. 4C). Gene expression of *GDF9* was affected by IGF1 treatment ($P = 0.001$), but also by cortisol and the interaction between IGF1 and cortisol ($P < 0.05$). Suppressing the PKA activity by H89 resulted in increased expression of *GDF9* compared to control ($P = 0.004$) and IGF1 ($P = 0.002$) and this effect was suppressed in the presence of cortisol (Fig. 4D).

There was no effect of cortisol treatment or the interaction between cortisol and IGF1 on *PCNA* levels ($P > 0.05$), but the *PCNA* mRNA expression was significantly increased by IGF1 compared to control or H89 treatment ($P < 0.001$ in both cases, Fig. 5A). Similarly, the gene expression of *KI67* marker was significantly increased in response to IGF1 compared to control or H89 treatment ($P < 0.001$ in both cases), but not affected by cortisol (Fig. 5B). Expression of BCL2 was highest in the absence of IGF1 ($P < 0.001$), independent of cortisol concentration, and it was further inhibited by H89 ($P < 0.05$, Fig. 5C). Neither IGF1, nor cortisol treatment or their interaction influenced *CASP3* levels (Fig. 5D).

In the cell culture experiments, expression of *STAR* was negatively correlated with *GDF9* expression (r = -0.45, $P = 0.002$) and positively correlated with *HSD3B2* ($r = 0.40$, $P = 0.005$), *CYP19A1* ($r = 0.39$, $P = 0.015$) and *KI67* ($r = 0.49$, $P < 0.001$). Moreover, *HSD3B2* transcriptional levels were positively correlated with *CYP19A1* ($r = 0.88$, $P < 0.001$), *KI67* ($r = 0.71$, $P < 0.001$), *PCNA* ($r = 0.59$, $P < 0.001$) and *CASP3* ($r = 0.34$, $P = 0.019$) levels, respectively. The *PCNA* mRNA expression was positively correlated with *KI67* ($r = 0.70$, $P < 0.001$) and *CASP3* ($r = 0.39$, $P = 0.007$) expression. A positive correlation was also observed between *BCL2* and *CASP3* levels ($r = 0.59$, $P < 0.001$).

Effects of cortisol addition on steroid production by granulosa cells after in vitro culture

Production of P4, E2 and E2S by granulosa cells in vitro was influenced by IGF1 treatment ($P = 0.001$ in all cases), whereas cortisol effects were only observed for P4 and E2 ($P < 0.05$ in both cases, Fig. 6). Treatment of granulosa cells with IGF1 increased P4, E2 and E2S secretion compared to control ($P < 0.001$), whereas H89 treatment reversed the IGF1-induced increase in E2 and E2S ($P < 0.001$, Fig. 6). Cortisol addition at 150 ng/ml increased this IGF1-induced E2 secretion, while the IGF1-induced increase in P4 was suppressed in the presence of 50 ng/ml cortisol.

Discussion

The present study identified the mRNA and protein expression of GDF9 in equine ovarian tissue at different stages of follicular development. Using a newly generated equine-specific custom-made antibody against GDF9, we were able to show that the protein is localized solely in the cytoplasm of oocytes from the primary follicle stage onwards, and is lacking in the oocytes of dormant, primordial follicles. Furthermore, the concentrations of intrafollicular GDF9 and BMP15 were determined for the first time in antral equine follicles using ELISA assays that were successfully validated for this species. We observed the highest concentrations of GDF9 in small antral follicles (< 10 mm) and of BMP15 in growing small and medium antral follicles (10–30 mm) with a significant decrease during the preovulatory stage. Moreover, a negative correlation between intrafollicular E2S concentration as well as E2S/P4 ratio and BMP15 concentration was noticed. As part of our investigation of possible paracrine effects of GDF9, we confirmed its presence in granulosa cells. Although GDF9 expression did not differ in these samples, it was clearly negatively correlated with that of genes involved in steroidogenesis and cell proliferation, implicating a possible functional relationship. In our in vitro experiments, increased steroidogenic activity of granulosa cells was observed in response to IGF1 and cortisol, as assessed by gene expression analyses and steroid hormone production, while suppressing the stimulatory effect of IGF1 on E2, E2S and P4 by H89 increased expression of GDF9 and decreased expression of apoptosis-related genes, respectively. Also, *GDF9* transcriptional levels were negatively correlated with *STAR* levels, similar to the observations in vivo.

Localization of GDF9 protein in the equine ovary is similar to that in mice, where GDF9 becomes detectable in oocytes from the primary follicle stage onward [31]. However, in other species such as humans, pigs, and goats, GDF9 is detected from the primordial follicle stage [31, 35]. Additionally, in pigs, the GDF9 protein increases in oocytes during follicular growth [58], but remains unchanged during maturation [59]. We did not detect any changes in the GDF9 staining pattern relative to the stage of equine follicular development. Interestingly, GDF9 protein is also present in the cumulus or mural granulosa cells in some species [32, 35], but this was obviously not the case in the equine ovary. While protein localization in follicular cellular components has

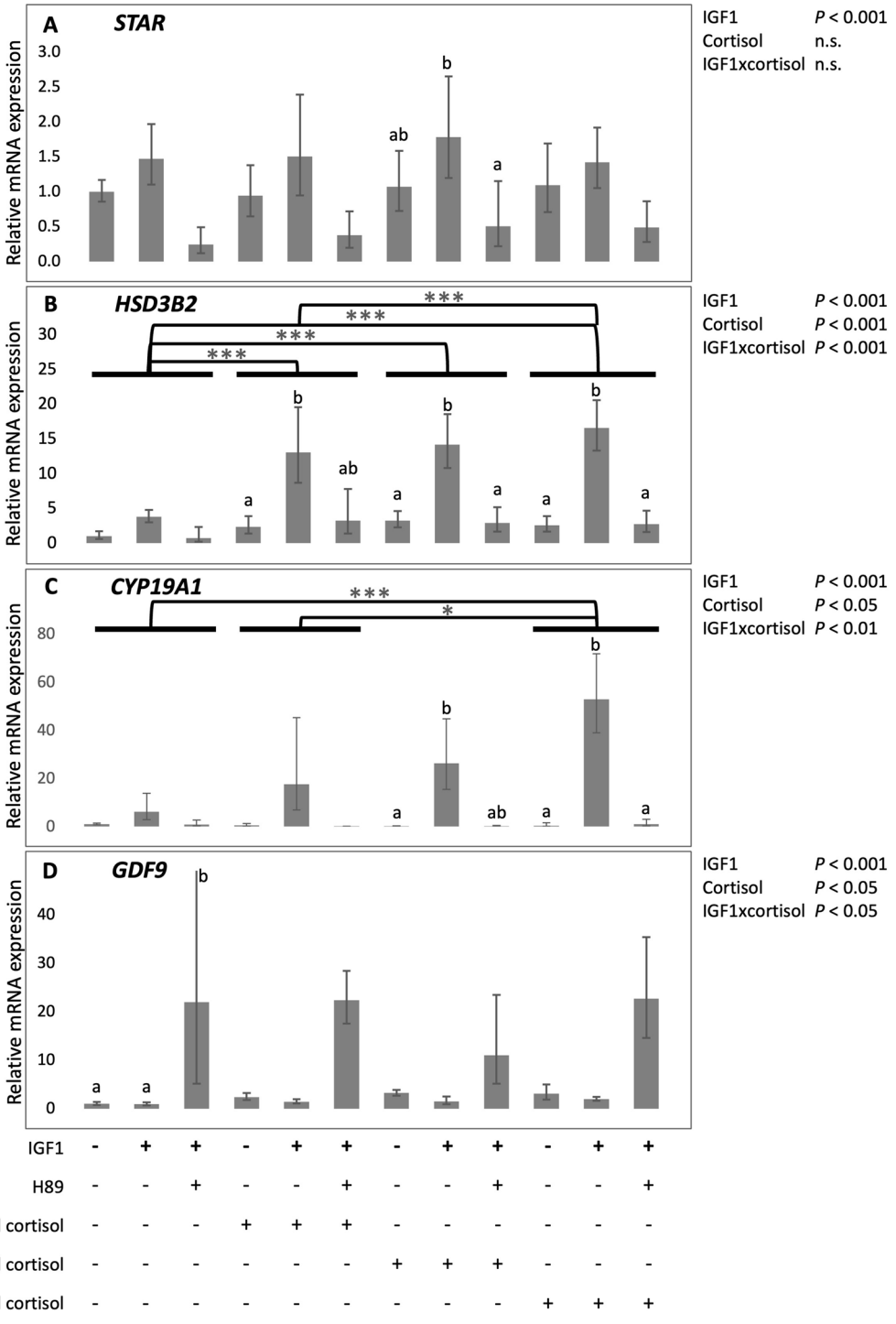

Fig. 4 Relative gene expression of *STAR*, *HSD3B2*, *CYP19A1* and *GDF9* in equine granulosa cells in vitro. Equine granulosa cells collected by OPU were treated for 24 h either with 100 ng/ml IGF1 or with 20 µM H89 (PKA blocker) in the absence or presence of cortisol in the following dosages: 50 ng/ml, 100 ng/ml and 150 ng/ml, respectively. Experiments ($n = 4$ biological replicates) were conducted using pooled granulosa cells obtained from the ovaries of four to six mares for each experiment. A multivariate GLM was applied to test for the effects of IGF1, cortisol, as well as their interaction. In case of significant effects, pairwise comparisons after Bonferroni correction were performed. Relative gene expression is presented as determined by semi-quantitative real time (TaqMan) PCR (Xg ± SD). Significant differences and the respective *P* values are presented in each graph. Different superscripts indicate significant differences ($P < 0.05$) within each cortisol concentration, whereas bars indicate significant differences among different cortisol concentrations. *$P < 0.05$, ***$P < 0.001$

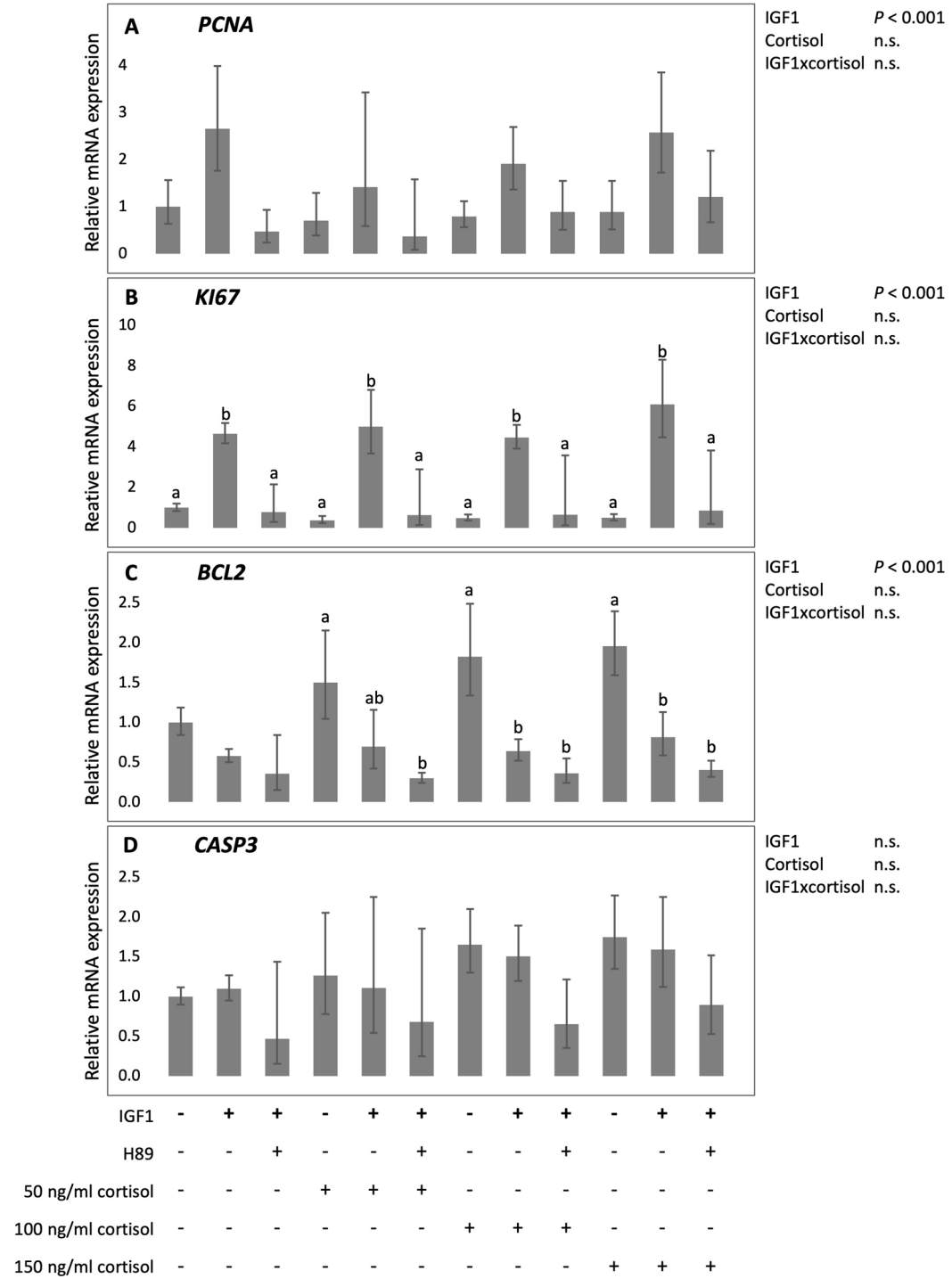

Fig. 5 Relative gene expression of *PCNA*, *KI67*, *BCL2* and *CAPS3* in equine granulosa cells in vitro. Equine granulosa cells collected by OPU were treated for 24 h either with 100 ng/ml IGF1 or with 20 µM H89 (PKA blocker) in the absence or presence of cortisol in the following dosages: 50 ng/ml, 100 ng/ml and 150 ng/ml, respectively. Experiments ($n = 4$ biological replicates) were conducted using pooled granulosa cells obtained from the ovaries of four to six mares for each experiment. A multivariate GLM was applied to test for the effects of IGF1, cortisol, as well as their interaction. In case of significant effects, pairwise comparisons after Bonferroni correction were performed. Relative gene expression is presented as determined by semi-quantitative real time (TaqMan) PCR (Xg ± SD). Significant differences and the respective *P* values are presented in each graph. Different superscripts indicate significant differences ($P < 0.05$) within each cortisol concentration

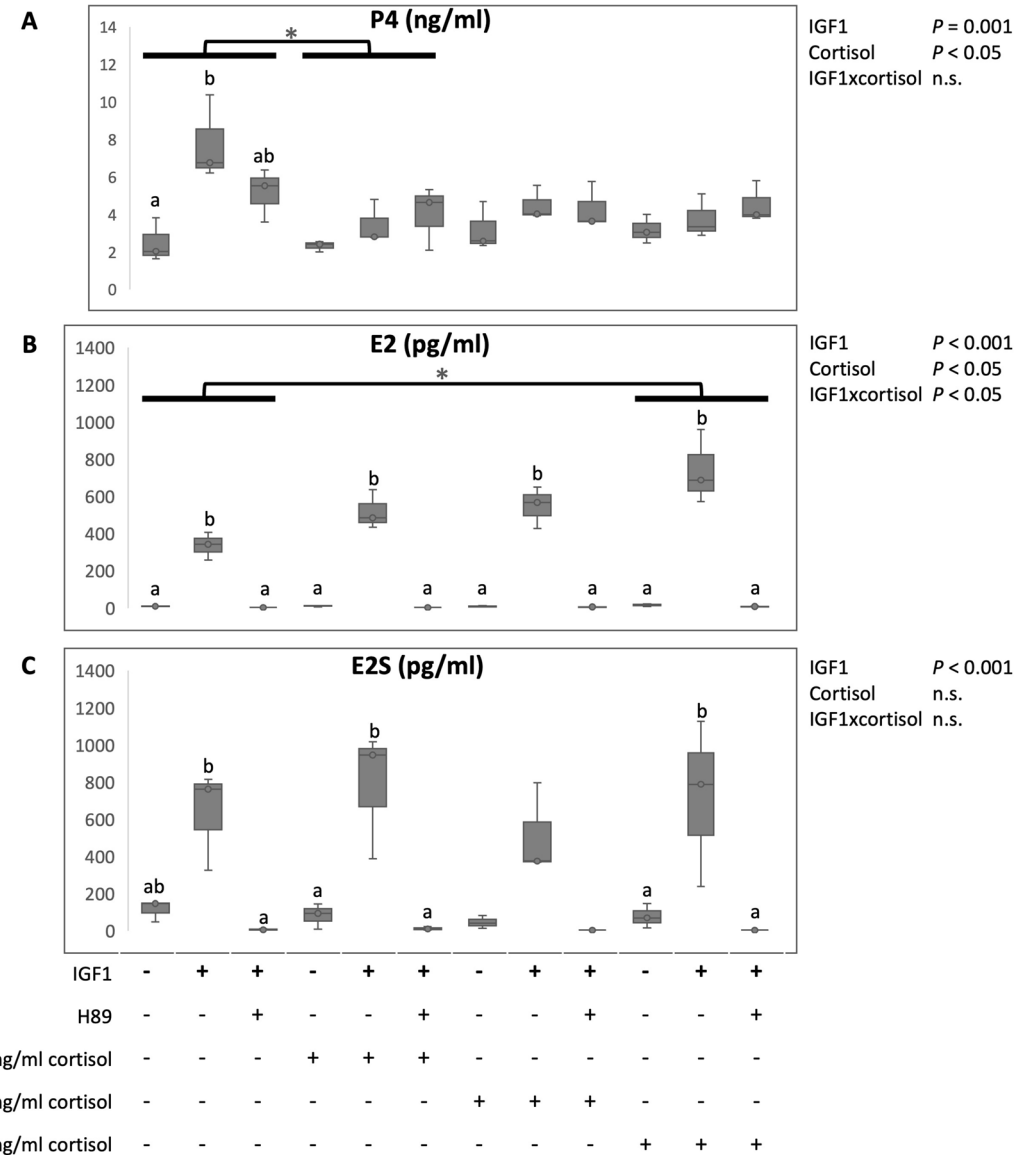

Fig. 6 Box and whisker chart presenting concentrations of (**A**) progesterone (P4), (**B**) free estrogens (E2), and (**C**) conjugated estrogens (E2S) in cell culture media from equine granulosa cells collected by OPU and treated for 24 h either with 100 ng/ml IGF1 or with 20 µM H89 (PKA blocker) in the absence or presence of cortisol in the following dosages: 50 ng/ml, 100 ng/ml and 150 ng/ml, respectively. Experiments ($n = 4$ biological replicates) were conducted using pooled granulosa cells obtained from the ovaries of four to six mares for each experiment. Significant differences and the respective *P* values are presented in each graph. Different superscripts indicate significant differences ($P < 0.05$) within each cortisol concentration, whereas bars indicate significant differences among different cortisol concentrations. *$P < 0.05$

already been studied extensively, the characterization of GDF9 and BMP15 concentrations in follicular fluid is not as well-defined due to limited assay availability. In humans, the concentrations of GDF9 and BMP15 in follicular fluid have been reported to be ten times higher in small antral follicles compared to preovulatory follicles [60, 61]. Furthermore, both GDF9 and BMP15 are mostly undetectable in follicular fluid samples from large antral follicles in humans [60]. Using the same ELISA assays as in these previous studies, we demonstrated similar concentration patterns for GDF9 and BMP15 in equine antral follicles as seen in humans. Based on the concentrations in relation to the time of follicle deviation, we suggest differences in temporal regulation of the two proteins in the horse. In general, these two factors act either as biologically active heterodimers or as homodimers in a synergistic cooperation [62]. In humans, an inverse correlation between the mature form of GDF9 and P4 levels in follicular fluid was highlighted [63]. In our study, no correlation was observed between GDF9 and steroid hormones, but there was a negative correlation between BMP15 and E2S as well as between BMP15 and E2S/P4

ratio. It should be considered that our assay did not distinguish between the proforms and mature forms of the oocyte-derived factors. Interestingly, the horse is particular among domestic animal species, as granulosa cells of preovulatory follicles are able to produce high amounts of E2S [64]. Adding our findings from the equine species, it seems obvious that GDF9 and BMP15 are released into the follicular fluid where they exert paracrine effects on the surrounding granulosa cells.

Several studies have demonstrated a relationship between GDF9 and steroidogenesis. Thus, e.g., in granulosa cells of rats and cows, GDF9 regulates steroidogenesis and gonadotropin-induced differentiation of granulosa cells, regardless of the follicle size [42, 65]. In our study, *GDF9* transcripts could be determined in granulosa cells, as well as in the CL, confirming previous observations [45]. Although the protein was not detected in these cells, the detection of mRNA – a method far more sensitive than protein localization by immunohistochemistry and Western blotting - is not surprising, as an active selective transfer of molecules by a synapse-like vesicular trafficking connection is known to exist between granulosa cells and oocytes [3]. Moreover, *GDF9* mRNA synthesis by bovine and canine granulosa cells has been suggested and a greater abundance was observed in small follicles than in large follicles [66, 67]. Interestingly, *GDF9* transcriptional levels appeared to be similar in early versus late CL in our study, whereas in the earlier study [45] the stage of the CL was not determined. Due to the absence of commercially available TaqMan assays and antibodies for BMP15, we decided not to analyze this factor in our study. While the role of GDF9 in the progression of follicle development has received some attention in the past, particularly in women [62], it is not clear whether there might be also an involvement in CL formation and function. However, the negative correlation between *GDF9* and *STAR* mRNA expression suggests regulation of granulosa cell steroidogenesis by GDF9 in horses. Based on the correlation between *GDF9* and *STAR* and because P4 is rather low during the preovulatory period in the mare [68], we can conclude that GDF9 regulation is also related to E2 production. Indeed, an inhibitory effect of GDF9 on E2 concentration could be demonstrated using cultured bovine granulosa cells, independent of the size of the follicle [42]. Confirming the relationship between GDF9 and steroidogenesis in equine granulosa cells, we observed a pronounced increase in GDF9 expression paralleled by decreased E2 production in our in vitro study in response to blocking STAR expression by suppressing PKA activity. On the other hand, the presence of cortisol, a prominent glucocorticoid found in equine follicular fluid [17], induced an increase in *HSD3B2* and *CYP19A1* expression and E2 production. This observation underscores a potential positive influence of cortisol on the regulation of steroidogenesis in response to stimulatory factors such as IGF1.

In our examination of equine granulosa cells, we observed an increase in the mRNA availability of *STAR* and *HSD3B2* as follicles progressed from small to large, confirming previous reports in horses [69, 70]. Notably, *HSD3B2*, encoding for an enzyme involved in the synthetic provision of steroidogenic substrates for the synthesis of P4 and cortisol from pregnenolone, and *CYP19A1*, which is responsible for the aromatization of androgens into estrogens, both peaked during the early CL phase, while *STAR* expression was similar between early and late CL. Furthermore, a positive correlation was observed between *HSD3B2* transcriptional levels in granulosa cells and the intrafollicular concentrations of P4, E2 and E2S in the respective follicles. This suggests that the availability of HSD3B2 could be the rate limiting factor for P4 production, as its expression decreases in late CL, while STAR remains high. So far, only one isoform of the equine HSD3B has been cloned and characterized [71] and is referred to as type 2 based on its gonadal localization, analogous to humans [72]. Interestingly, transcriptional levels of *HSD3B2* and *CYP19A1* were significantly increased in vitro when IGF1 and cortisol were introduced simultaneously compared to when they were administered separately, suggesting a synergistic effect of these factors. Conversely, transcriptional availability of *STAR* was not affected by cortisol in cell culture experiments. In bovine species, cortisol appears to have a negligible effect on IGF1-induced P4 secretion and aromatase activity in granulosa cells [54]. In our study, cortisol inhibited IGF1-induced P4 production by granulosa cells but stimulated IGF1-induced E2 secretion and *CYP19A1* gene expression, as observed in human granulosa cells [73]. In contrast, cortisol inhibits E2 production by bovine granulosa cells, even in the presence of testosterone as precursor [74]. It is important to note that E2 secretion by granulosa cells in the present study was not optimized because no androgen precursor was added to the medium. However, E2 synthesis was apparently active, as reflected by *CYP19A1* levels mirroring those of E2. Also, it should be considered that our steroid data is presented as ng/ml and was not normalized to the amount of protein in the well, as not enough material was available for this. To our knowledge, this is the first report showing E2S by equine granulosa cells in culture. Luteinization of granulosa cells involves a significant shift in the expression of genes involved in cortisol synthesis [75]. Granulosa cells primarily express HSD11B2 before LH exposure, while HSD11B1 is amplified after LH exposure [76], suggesting that luteinizing granulosa cells predominantly exhibit reductive metabolism, converting cortisone to cortisol. The presence of high concentrations of cortisol in follicular fluid after the LH surge, together

with the increased transcriptional levels of glucocorticoid receptor in granulosa cells of dominant follicles, further underscores the involvement of cortisol in the ovulatory process [19, 75].

Besides being related to steroidogenesis, GDF9 was also shown to promote cell proliferation in bovine granulosa cells [42]. Moreover, mouse granulosa cells strongly express KI67 during follicle activation while lacking SMAD2/3 [77], which is the primary signalling pathway for GDF9. We observed the highest mRNA availability of *KI67* in granulosa cells from medium and large follicles, which are also characterized by reduced GDF9 levels in the follicular fluid. Furthermore, we observed a negative correlation between *GDF9* and *PCNA* mRNA expression in the in vivo study, suggesting a role for GDF9 in modelling granulosa cell proliferation in horses. Interestingly, GDF9 enhances follicular survival during the transition to the antral stage by inhibiting granulosa cells apoptosis in rats [78]. Nevertheless, H89-mediated suppression of PKA activity reduced IGF1-induced *PCNA* and *KI67* expression in our in vitro investigations. As mitogenic effects of IGF1 on granulosa cell proliferation are well-established in several species [79–81], this suggests an involvement of the cAMP/PKA system in mediating cell proliferation and secretory activity, in line with previous studies in rabbits [12]. Cortisol addition can further amplify IGF1-induced proliferation in bovine granulosa cells based on cell count by about 20% [54, 74], but it did not affect *PCNA* or *KI67* transcriptional levels in equine granulosa cells in our study. This suggests species-specific differences in the response of granulosa cells to cortisol, as the reliability of *PCNA* and *KI67* mRNA expression determination for assessing cell proliferation has been demonstrated before [82, 83].

Apoptosis plays a critical role in influencing oocyte maturation by disrupting communication between granulosa cells and oocytes [84]. In our in vivo experiments, the mRNA expression of *BCL2*, a well-known anti-apoptotic factor, and of *CASP3*, a pro-apoptotic factor, in granulosa cells did not differ significantly during follicular development, indicating a basic availability of pro-/anti-apoptotic factors. In another study of mRNA availability of these genes in equine follicles, follicular maturation was characterized by increased *BCL2* and decreased *CASP3* transcriptional levels, respectively [85]. As caspases remain dormant within cells until cleaved and activated by specific signals that trigger cell death [86], their mRNA availability should be interpreted with caution. Our in vivo results are supported by the concomitant in vitro observations, where the interaction between IGF1 and cortisol did not significantly affect *BCL2* and *CASP3* expression. However, it is important to highlight that apoptosis within granulosa cells is an essential continuous process during normal follicle development [84]. Although based only on their mRNA availability, the positive correlation between *CASP3* and *BCL2*, but also with proliferation markers *KI67* and *PCNA*, could implicate a well-defined intrafollicular mechanism controlling the number of cells within the equine ovarian follicle.

Conclusions

This study sheds new light on the involvement of GDF9 and BMP15 in equine ovarian follicle development and maturation. Building on observations from other species that show a close interplay between oocyte-secreted factors and granulosa cell function, here we have demonstrated the presence of GDF9 in oocytes of antral follicles, together with dynamic intrafollicular concentrations, underscoring its possible involvement in follicle development. Moreover, a correlation between GDF9 and mRNA availability of genes involved in granulosa cell steroidogenesis and proliferation was observed in both our in vivo and in vitro studies. While no causality was proven in this study, a regulating role of GDF9 in granulosa cell function seems plausible, therefore confirming our hypothesis. Furthermore, the study demonstrates that IGF1 and cortisol interact to regulate steroid secretion by granulosa cells. Specifically, cortisol stimulates IGF1-induced E2 production by upregulating *HSD3B2* and *CYP19A1* mRNA, which seems to be required for physiological ovulation to occur. Taken together, these findings extend our knowledge of the intricate interplay between essential ovarian processes and GDF9, with implications for mare reproduction and fertility. Further studies should elucidate the effects of GDF9 on ovarian function in the mare and if modulating GDF9 expression can improve fertility.

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Author contributions

KAS: investigation, formal analysis, writing - original draft. MPK: methodology, resources, writing – review & editing. GS: investigation, methodology, writing – review & editing. GDAG: investigation, methodology, writing – review & editing. HB: methodology, resources, writing – review & editing. DS: conceptualization, investigation, formal analysis, supervision, writing - original draft, writing – review & editing.

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Data availability

All data generated or analyzed during this study are included in this published article. Further inquiries can be directed to the corresponding author.

Declarations

Competing interests
The authors declare no competing interests.

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