Cumulin and FSH Cooperate to Regulate Inhibin B and Activin B Production by Human Granulosa-Lutein Cells *In Vitro*

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The oocyte-secreted factors bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9) interact functionally, and it is hypothesized that this interaction may be mediated by formation of a GDF9:BMP15 heterodimer termed cumulin. GDF9 and BMP15 regulate folliculogenesis and ovulation rate and have been shown to regulate inhibin and activin, local regulators of folliculogenesis. The objective of this study was to determine whether cumulin regulates granulosa cell inhibin and activin production and whether this requires cooperation with FSH. Human granulosa-lutein (hGL) cells collected from patients undergoing *in vitro* fertilization were cultured with or without FSH with various forms of recombinant cumulin (native and cysteine mutants, with or without the prodomains), and cysteine mutant GDF9 or BMP15. Messenger RNA expression of the subunits of inhibins/activins (*INHA*, *INHBA*, *INHBB*) and secretion of inhibin A, inhibin B, and activin B were measured. Mature forms and proforms of cumulin stimulated comparable *INHBB* mRNA expression and secretion of inhibin B and activin B, whereas GDF9 or BMP15 exhibited no effect. Cumulin, but not GDF9 or BMP15, interacted synergistically with FSH to increase *INHBB* mRNA and inhibin B expression. FSH markedly stimulated *INHA*, which encodes the α subunit of inhibin A/B, and suppressed activin B. Cumulin with or without FSH did not significantly alter inhibin A. Together these data demonstrate that cumulin, but not GDF9 or BMP15, exerts paracrine control of FSH-induced regulation of inhibin B and activin B. The prodomains of cumulin may have a minimal role in its actions on granulosa cells. **(*Endocrinology* 160: 853–862, 2019)**

The pituitary gonadotropins FSH and LH stimulate folliculogenesis by promoting growth and differentiation of the granulosa cells of the ovarian follicle (1). In addition, local growth factors produced by ovarian follicular cells and the oocyte itself regulate follicle development through their direct actions on granulosa cells and also indirectly by modulating gonadotropin secretion. Belonging to the TGFβ superfamily, activins and inhibins exert opposing effects on pituitary FSH production and secretion, whereby they stimulate and perturb FSH production, respectively (2). FSH and LH actions in the follicle are also regulated by the

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Abbreviations: BMP15, bone morphogenetic protein 15; GDF9, growth differentiation factor 9; hGL, human granulosa-lutein.

oocyte-secreted growth factors growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15), and this interaction between gonadotropins and oocyte paracrine factors is thought to underpin the basic physiological mechanism regulating the species-specific ovulation rate and fecundity in mammals (3–5).

Over the past decade it has become clear that the oocyte plays a major role in regulating follicle development and modulating its own follicular microenvironment by the secretion of paracrine growth factors that act on the follicle somatic cells (6). GDF9 and BMP15 are two closely related paralogs of the TGFβ superfamily that are principally produced by the oocyte (7). These two oocyte-secreted factors act on surrounding granulosa cells to regulate their growth and differentiation, affecting folliculogenesis and oocyte development and maturation. GDF9 and BMP15 are essential for female fertility, and genetic mutations in these growth factors alter follicle development and thus reproductive potential in mammals [reviewed by Juengel and McNatty (8)]. GDF9 and BMP15 signal via BMPRII/ALK cell surface receptors to phosphorylate intracellular SMAD proteins that regulate the transcription of several genes (4).

Like all TGFβ superfamily ligands, GDF9 and BMP15 are produced as promature proteins and require proteolytic cleavage by furin-like proteases to release the *N*-terminal prodomain from the receptor binding C-terminal mature domain to become bioactive (9, 10). However, GDF9 and BMP15 are unusual TGFβ ligands in that they lack the conserved fourth cysteine residue necessary to make the intersubunit disulfide bond that links TGFβ ligand dimers covalently, and therefore they form noncovalent dimers (11). Because of their noncovalent dimer interaction, shared spatiotemporal expression pattern in the oocyte, close structural homology, and coimmunoprecipitation, it is speculated that GDF9 and BMP15 can physically interact, probably to form a GDF9:BMP15 heterodimer called cumulin (4, 12–14). Functional studies have shown potent GDF9+BMP15 synergistic responses in granulosa cell proliferation, progesterone and inhibin α subunit protein production, and SMAD activity *in vitro* (14–16), and such GDF9 +BMP15 synergism is thought to be caused by the formation of a structural heterodimer of GDF9 and BMP15 (*i.e.*, cumulin) (12). Recently, we synthesized cumulin, modeled its receptor interactions and signaling, demonstrated its high potency on granulosa cells, and hypothesized a central role for cumulin in determining the low ovulation rate phenotype in mammals such as humans (12).

Because of its recent characterization, most aspects of cumulin's molecular form and cellular and physiological functions require elucidation. Nonetheless, new evidence suggests cumulin has potential for improving the treatment of female infertility. Pro-cumulin is able to improve the quality and developmental competence of pig and human oocytes matured *in vitro* (12, 17). Interestingly, these improvements were elicited only by the pro-cumulin form and not by the highly bioactive mature form, which has no proregion (12). This finding suggests that the GDF9 and BMP15 prodomains in cumulin play an important role in its function (18, 19). Mouse pro-GDF9 induces inhibin B production by human granulosa-lutein (hGL) cells (20) and pro-GDF9 and pro-BMP15 potently synergize to promote total inhibin (including the bioinactive free α subunit monomer) production by sheep granulosa cells (15), leading us to hypothesize a role for pro-cumulin in the inhibin-activin system in granulosa cells.

The objectives of this study were to examine the role of cumulin, including the role of its prodomains, and its cooperative effects with FSH, on granulosa cell production of inhibins and activins. Various recombinant forms of cumulin dimers that were covalently or noncovalently linked, with and without their prodomain, were generated, and their effects on human granulosa-lutein cell production of inhibin A and B and activin B were examined.

Materials and Methods

hGL cell purification and culture

This study was approved by IVF Australia Ethics Committee (approval number 100). hGL cells were purified from follicular aspirates of women undergoing transvaginal oocyte retrieval after controlled ovarian stimulation for *in vitro* fertilization. Follicle aspiration was performed 36 hours after administration of recombinant human chorionic gonadotropin (Ovidrel, Merck Serono, Darmstadt, Germany) to induce final oocyte maturation. For each experiment, cells from at least four patients were pooled, and no patient samples were excluded from the study. Cells were prepared according to a modified version of the methods described by Ferrero *et al.* (21) and Chang *et al.* (22). Briefly, follicular fluid was passed through a 70-μm cell strainer (Corning, Corning, NY) that was backwashed with media to collect cell aggregates, which were recovered after centrifugation at 400*g* for 10 minutes. The cell pellet was resuspended in HEPES-buffered αMEM (Gibco, Waltham, MA) containing 0.3% BSA (CellMaxx, MP Biomedicals, Auckland, New Zealand) and hyaluronidase (100 mg/mL), followed by centrifugation at 1000*g* for 2 minutes. The pellet was then resuspended in 4 mL media and layered onto 8 mL of Ficoll-Paque Premium 1.084 (GE Healthcare, Chicago, IL) and centrifuged at 1000*g* for 25 minutes, and the interphase was collected. Cells were washed in DMEM (Gibco, Waltham, MA) containing 5% fetal bovine serum (Gibco) and 1% (v/v) Antibiotic Antimycotic Solution (Gibco) and plated in the same media at a density of 3×10^5 cells per well in 1 mL in a 24-well plate (Costar, Corning, NY). Cells were incubated for 5 days at 37°C in 5% CO_2 with media changes every second day to

rederive FSH-sensitive granulosa cells as previously described (23–25). After 120 hours, cells were washed once with 1 mL DMEM containing Antibiotic Antimycotic Solution and 0.3% BSA and treated for 24 hours with 500 μL of this media containing the various growth factors (Fig. 1) with or without 50 mIU/mL recombinant human FSH (Follitropin-β, Puregon, Organon, Oss, The Netherlands), as indicated in the text and figure legends. Cell pellets or conditioned media were then used for quantitative polymerase chain reaction or ELISA assays, respectively.

Recombinant proteins

GDF9, BMP15, and cumulin in their promature dimeric forms and mature cumulin (Fig. 1 and Table 1) were used in this study. A proform is a dimeric complex of proteolytically cleaved prodomain and mature domain forms [Fig. 1(a)–1(d)], whereas the mature form is a dimer lacking the prodomain [Fig. 1(e)]. Because GDF9 and BMP15 exist as noncovalent dimers lacking the fourth cysteine residue common in TGFβ superfamily members, Ser^{418} of GDF9 and Ser^{356} of BMP15 were modified by substituting to cysteines, as previously described, to generate stabilized covalent-linked dimers of GDF9, BMP15, and cumulin that cannot dissociate (12, 16). These covalent proteins are indicated by the yellow link on the mature dimer in Fig. 1(a)–1(c) and 1(e). All proteins were produced in house, and human protein sequences were used.

Production and purification of noncovalent pro-cumulin and covalent mature cumulin [Fig. 1(d)–1(e)] were carried out as previously described (12). Their bioactivities were assessed based on their ability to activate SMAD signaling pathways, as measured by SMAD-responsive luciferase assays (A3-luciferase and BRE-luciferase) in the COV434 human granulosa cell line, as previously described (12).

Production and purification of promature complexes containing covalently stabilized dimeric mature domains [pro-GDF9^{S418C}, pro-BMP15^{S356C}, and covalent pro-cumulin; Fig. 1(a)–1(c)] were produced similarly to that described previously (12). Differences in methods included production of recombinant proteins by transient transfection of HEK-293T cells with polyethylenimine-MAX (Polysciences, Warrington, PA). When pro-GDF9^{S418C} or pro-BMP15^{S356C} homodimers were produced, one plasmid type was transfected containing the respective cDNA. To produce the heterodimer covalent pro-cumulin, plasmids for both GDF9^{S418C} and BMP15^{S356C} were cotransfected. Protein purification was undertaken by cobalt-based immobilized metal affinity chromatography with HisPur™ Cobalt Resin (Thermo Fisher Scientific, Waltham, MA). Bound proteins were eluted from cobalt-based immobilized metal affinity chromatography resin with elution buffer (50 mM phosphate buffer, 300 mM NaCl, 200 mM imidazole, pH 7.4). Imidazole was removed by dialysis against binding buffer (50 mM phosphate buffer, 300 mM NaCl, pH 7.4) with Slide-A-Lyzer® MINI Dialysis Devices (2 mL 3.5K MW cutoff; Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's guidelines. Activation of SMAD signaling reporters, A3-luciferase and BRE-luciferase, was assessed in the COV434 human granulosa cell line as previously described (12).

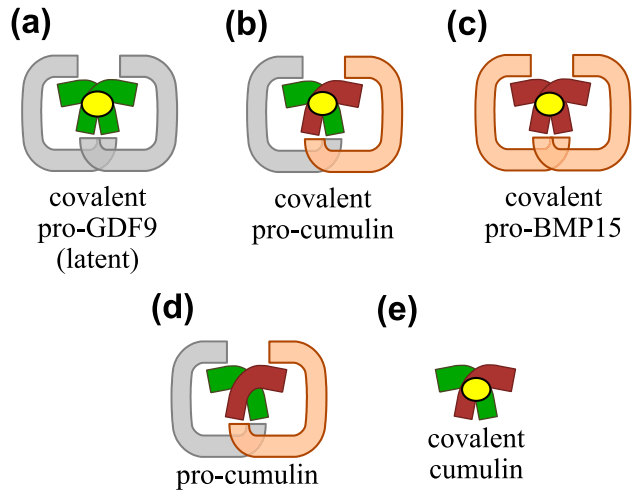


Figure 1. The forms of GDF9, BMP15, and their heterodimer cumulin examined. (a–d) Proteins were used in their respective proforms, consisting of a complex of prodomains and mature domains after proteolytic cleavage, and (e) cumulin was also used as a dimer of the mature domains lacking prodomains. (a–c, e) Covalent dimers containing an introduced intersubunit disulfide bridge are shown with a yellow link between the mature dimers. (a) Human pro-GDF9 is naturally latent.

Table 1. Description of the Purified Recombinant Growth Factors Used in This Study

Protein	Covalent	Prodomain
Cumulin$^{S418C,\ S356C}$	✓	X
Pro-cumulin	X	✓
Pro-cumulin$^{S418C,\ S356C}$	✓	✓
Pro-GDF9^{S418C}	✓	✓
Pro-BMP15^{S356C}	✓	✓

Real-time RT-PCR

Total RNA was isolated from cell pellets with the Qiagen microRNEasy kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Total RNA was measured with a NanoDrop Spectrophotometer ND-1000 (Thermo Fisher Scientific). First-strand cDNA was synthesized with Superscript III reverse transcription and random primers (Invitrogen, Waltham, MA). Real-time RT-PCR was performed in duplicate with gene primers (GeneWorks, Australia; Table 2) on a Roche LightCycler 480 (Roche, Basel, Switzerland). Controls, including the absence of cDNA template or the reverse transcription enzyme in otherwise complete reactions, showed no product amplification or genomic DNA contamination. Target gene expression was normalized to *GAPDH* expression. In the statistical program BestKeeper, *GAPDH* was determined as a stable housekeeping gene with a SD (±Ct) and coefficient of variation (%Ct) of 0.43 and 2.37, respectively, when its expression was measured across five different pools of hGL cells treated with or without pro-GDF9, pro-BMP15, and pro-cumulin, all with or without FSH. PCR data are expressed as the raw gene expression level via the $2^{-\Delta\Delta CT}$ method (26).

Inhibin A, B, and activin B ELISAs

Inhibin A and B are heterodimers of a common α-subunit bound covalently to a βA- or βB-subunit, respectively. The proforms of inhibin A and inhibin B were measured in hGL cell

Table 2. PCR Primer Sequences Used for Real-Time RT-PCR

Gene	GenBank Accession	Forward Primer (5′–3′)	Reverse Primer (5′–3′)
INHA	NM_002191.2	GTCTCCCAAGCCATCCTTTT	TGGCAGCTGACTTGTCCTC
INHBA	NM_002192.2	AGCTCAGACAGCTCTTACCAC	CAAATTCTCTTTCTGGTCCCC
INHBB	NM_002193.3	ATCAGCTTCGCCGAGACA	GCCTTCGTTGGAGATGAAGA
GAPDH	NM_002046.3	CGCCCCACTTGATTTTGG	ATGGATCCCATCACCATCTT

conditioned media via immunoassays, as detailed by Walton *et al.* (27) that use antibodies to the α-, βA- and βB-subunits of inhibin.

Activin B is a homodimer of the βB-subunit. Total activin B was measured in hGL cell conditioned media with the Ansh Labs commercial ELISA kit (Ansh Labs, Webster, TX) according to the manufacturer's instructions, or with an in-house ELISA possessing similar sensitivity and specificity for human activin B, as previously described (28). Samples that gave readings below the limit of quantification of the assay were assigned values of limit of quantification/2.

Statistical analyses

Results are presented as the mean ± SEM. The number of independent pools of granulosa cells tested for each experiment is indicated in the figure legends. Statistical analyses were performed in GraphPad Prism 7.02 software, and differences were considered to be significant at $P \leq 0.05$. Data that were not normally distributed were transformed before analysis via log transformation. One-way ANOVA or two-way ANOVA (where two independent variables were tested) with multiple comparisons tests was used.

Results

Cumulin stimulates granulosa cell inhibin B production

Covalent mature cumulin and noncovalent pro-cumulin each dose-dependently increased *INHBB* mRNA expression [Fig. 2(a)], which encodes a βB-subunit that in homodimeric form makes activin B or as a heterodimer with an α-subunit forms inhibin B. *INHBA* or *INHA* mRNA expression (encoding the βA- and α-subunits, respectively) was unaffected by treatment with either form of cumulin [Fig. 2(b) and 2(c)]. Covalent pro-cumulin stimulated a significant dose-dependent increase in *INHBB* expression relative to no treatment control; however, treatment with either of the homodimers, covalent pro-GDF9 and covalent pro-BMP15, did not affect *INHBB* mRNA expression at any dosage tested [Fig. 3(a)]. None of the proteins elicited a significant increase in *INHBA* [Fig. 3(b)] or *INHA* [Fig. 3(c)] mRNA expression, except *INHBA*, which was significantly higher than control after exposure to 12.5 ng/mL covalent pro-cumulin [Fig. 3(b)].

Cumulin and FSH exert dual control of inhibin B production

FSH, a known driver of inhibin secretion in granulosa cells (29), caused a 15-fold increase in *INHA* mRNA expression [Fig. 4(a)]. The FSH effect on *INHA* mRNA expression was significantly ($P < 0.05$) increased by cotreatment with pro-BMP15, or pro-cumulin, but not pro-GDF9. Pro-cumulin and FSH synergistically increased (*i.e.*, the effect of both factors combined was greater than the sum of either factor alone) *INHBB* mRNA and inhibin B protein production (two-way ANOVA, $P < 0.05$; interaction of main effects of FSH and growth factor), whereas pro-GDF9 with or without FSH and pro-BMP15 with or without FSH had no significant effect [Fig. 4(b) and 4(c)]. A trend in interactive effect between FSH and treatment on activin B was observed [$P = 0.07$; Fig. 4(d)]. Activin B production was upregulated by pro-cumulin, but only in the absence of FSH, whereas pro-GDF9 with or without FSH and pro-BMP15 with or without FSH had no significant effect on activin B [Fig. 4(d)].

The prodomain does not affect hGL cell responsiveness to cumulin

Because the prodomains of GDF9, BMP15, and cumulin are necessary for the stimulatory effect of these growth factors on oocyte quality, as measured by an oocyte's capacity to support development to the blastocyst stage (12, 18, 19), a comparison was made between pro-cumulin and mature cumulin. Although pro-cumulin and mature cumulin both significantly (two-way ANOVA, $P \leq 0.01$; main effects) promoted *INHBB*, inhibin B, and activin B levels, no significant difference was observed between them in terms of the extent of stimulation at the tested dosage (Fig. 5). Overall, FSH stimulated inhibin B production while suppressing activin B (two-way ANOVAs, $P < 0.01$; main effects of FSH) [Fig. 5(b) and 5(c)].

Inhibin A production is not regulated by cumulin

Pro-cumulin in the presence of FSH stimulated *INHBA* mRNA expression, whereas mature cumulin did not [Fig. 5(d)]. Inhibin A secretion in granulosa cell cultures was unaffected by FSH, by either form of cumulin, or by the interaction of FSH and cumulin [Fig. 5(e)].

Discussion

The nature of the native forms of GDF9 and BMP15, and whether they occur physiologically as the heterodimer

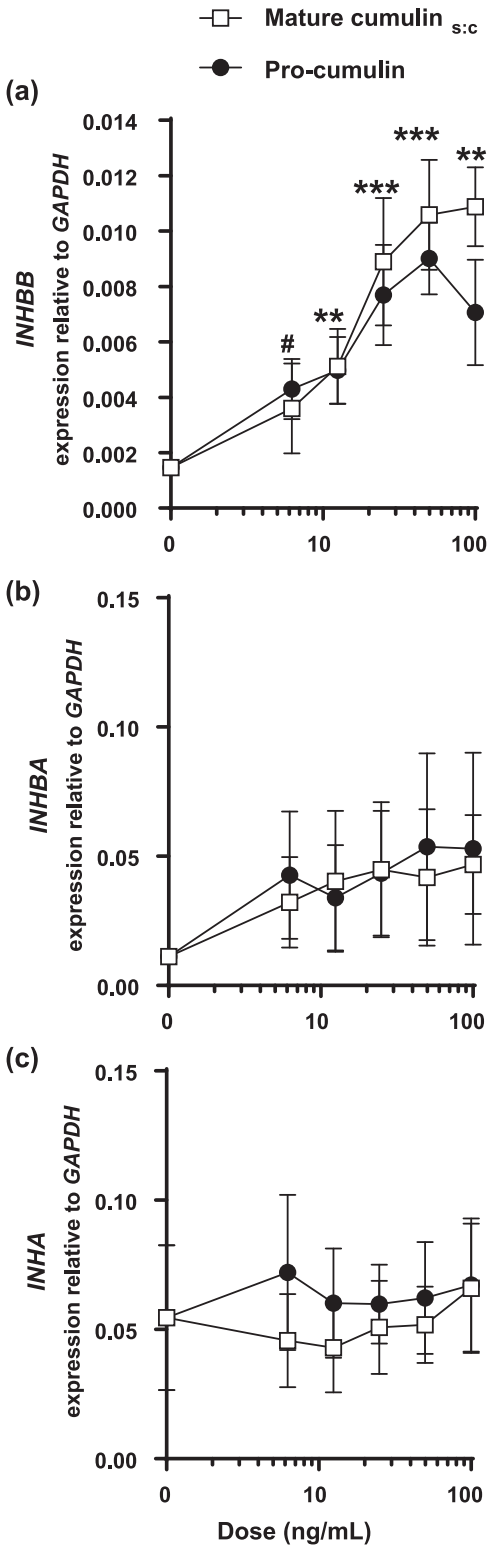


Figure 2. Dose response of pro-cumulin and mature cumulin on inhibin A and B subunit production. hGL cells were treated for 24 hours with noncovalent pro-cumulin or covalent mature cumulin ($cumulin_{s:c}$) at varying concentrations. Expression of (a) *INHBB*, (b) *INHBA*, and (c) *INHA* mRNA was measured. Data are expressed as mean ± SEM of five independent granulosa cell pools. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ vs control (0 ng/mL) for both pro-cumulin and mature cumulin treatments at the indicated dosage. $^{\#}P < 0.05$ vs control (0 ng/mL) for pro-cumulin.

cumulin, remains unclear because they are yet to be isolated from biological fluids. However, there is substantial evidence of synergistic interactions between GDF9 and BMP15, and a prominent hypothesis in the field is that such synergism is mediated by a heterodimer molecule, cumulin, consisting of at least one subunit of GDF9 and BMP15 (12). In this study, we demonstrated that cumulin regulates inhibin B and activin B production in hGL cells. We bioengineered and purified wild-type noncovalent cumulin, as well as covalent dimers containing an introduced intersubunit disulphide bridge. Pro-cumulin and mature cumulin induced comparable expression of *INHBB*, inhibin B, and activin B in hGL cells, whereas covalent pro-GDF9 or pro-BMP15 elicited no effect. This indicates that GDF9 and BMP15 linkage results in distinct activation of gene expression relative to GDF9 or BMP15 alone.

These observations are in line with a previous study showing synergistic upregulation of total inhibin (including the bioinactive free α subunit monomer) in rat granulosa cells when murine or ovine GDF9 conditioned media was added together with ovine BMP15 conditioned media, but no effect was seen when either GDF9 or BMP15 conditioned media was used in isolation (15). Moreover, others have reported a negative effect of GDF9 or BMP15 alone on inhibin production. Human BMP15 had no effect on *INHA*, *INHBA*, and *INHBB* mRNA expression in rat granulosa cells, and it decreased mRNA expression of these subunits in the presence of FSH (30). Mouse GDF9 also inhibited total inhibin production in ovine and bovine granulosa cells (31), and *Escherichia coli* expressed human GDF9 had no effect on inhibin A and B production in hGL cells (32). In contrast to these studies, GDF9 and BMP15 individually have been reported to regulate the inhibin system in granulosa cells. Pulkki *et al.* (33, 34) demonstrated that purified mature human BMP15 was a stimulator of inhibin B production in hGL cells. Roh *et al.* (35) reported upregulation of inhibin A and inhibin B in rat granulosa cells in response to mature rat GDF9, an effect that was increased by FSH. Kaivo-Oja *et al.* (20) also reported stimulation of inhibin B secretion by mature rat GDF9 in hGL cells. These discrepancies in the literature on the roles of GDF9 and BMP15 in regulating inhibin production are probably due to methodological differences such as the use of different granulosa cell types from different species at different stages of folliculogenesis and the large variability in the construct and purity of the in-house produced growth factors used. Many early studies in the field used unpurified growth factors including raw conditioned media, and this is likely to contribute to the discrepancy in reporting. Indeed, this issue persists in

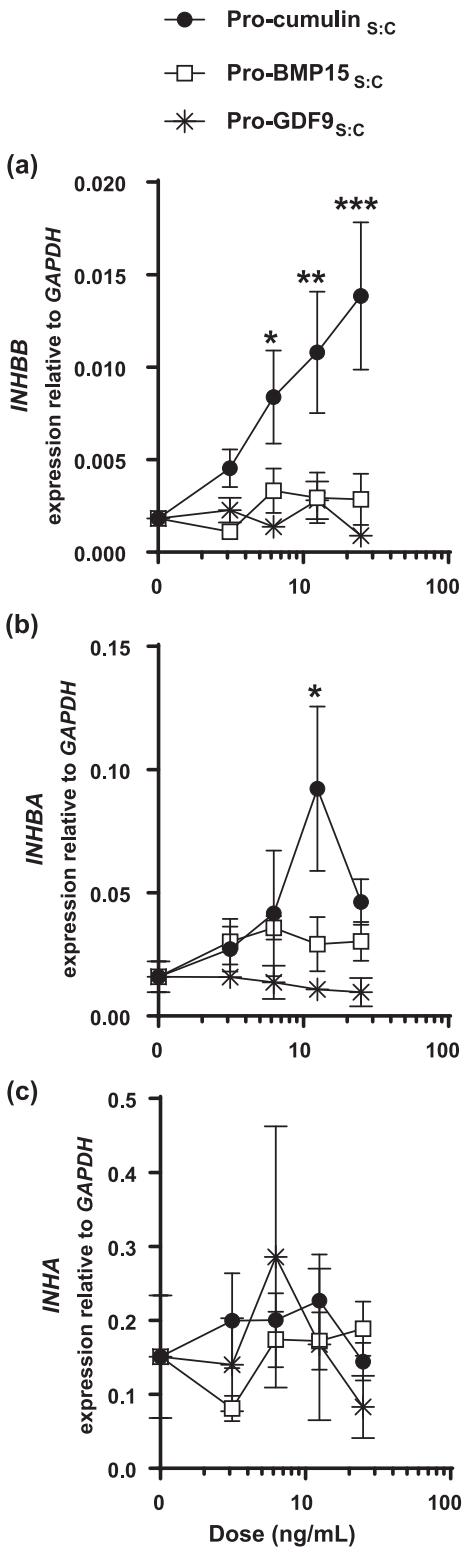
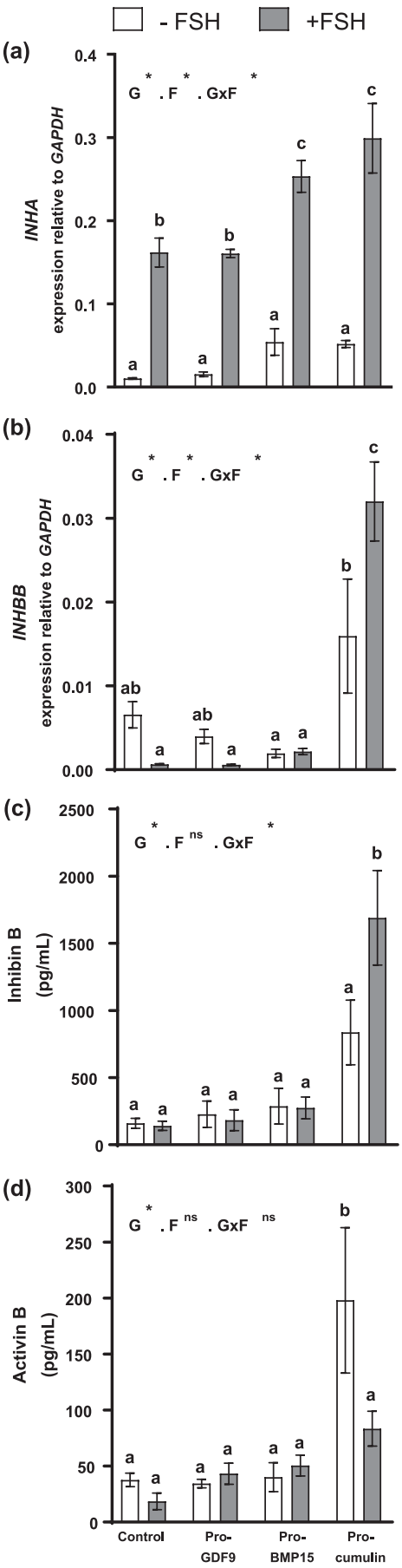


Figure 3. Comparative effect of pro-cumulin, pro-GDF9, and pro-BMP15 on inhibin subunit mRNA expression. hGL cells were treated for 24 hours with covalent forms of pro-cumulin, pro-GDF9, or pro-BMP15 at varying concentrations. Expression of (a) *INHBB*, (b) *INHBA*, and (c) *INHA* mRNA was measured. Data are expressed as mean ± SEM of seven independent granulosa cell pools. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ vs control (0 ng/mL) for pro-cumulin.

the case of cumulin because it is newly described, and there are just three publications to date (12, 13, 36). In this study we generated highly purified forms of cumulin, including variants such as covalent pro-cumulin, which has enabled further scrutiny of its role in regulating granulosa cell function. Overall, the findings of this study demonstrate that cumulin regulates granulosa cell inhibin B and activin B production, and the homodimers GDF9 or BMP15 alone do not. There was no pronounced effect of cumulin on inhibin A production despite the stimulatory effect on inhibin B. This finding is in line with previous work, where two other Smad2/3-activating members of the TGFβ superfamily, TGFβ1 and activin A, were shown to have a more marked stimulatory effect on inhibin B than on inhibin A production in rat granulosa cells (37).

FSH is a well-established stimulator of granulosa cell inhibin subunit gene expression and inhibin A and B production (37, 38). FSH markedly upregulated *INHA* expression that encodes the α subunit of inhibin A/B, and expression was further increased by BMP15 and cumulin. Moreover, a synergistic interaction was seen between FSH and covalent pro-cumulin in upregulating inhibin B. FSH also decreased activin B production. Hence, we postulate that cumulin and FSH synergistically increase inhibin B production by increasing the availability of the βB and α subunits, respectively, leading to heterodimerization of the α and βB subunits to form inhibin B. This occurs at the expense of activin B production because activin B consists of two βB subunits. No synergistic interaction was observed between FSH and either GDF9 or BMP15 alone, providing evidence to support the hypothesis that the basis for their regulation of the inhibin-activin system is through the formation of cumulin; the use of covalently linked mutant dimers of cumulin provides further evidence that they can act as a functional dimeric unit. Whether cumulin exists naturally as a free dimeric entity or GDF9 and BMP15 form a heterodimer at the receptor level remains unclear. Using Western blotting procedures, Heath *et al.* (36) recently reported that the predominant forms of GDF9 and BMP15 secreted by ovine and bovine oocytes were cleaved and uncleaved promature monomers, and they hypothesized that these monomers form a GDF9:BMP15 heterodimer at the receptor level.

Previous investigation into the effects of cumulin on oocyte quality showed that cumulin's prodomain was important for cumulin to elicit an increase in subsequent embryo yield of *in vitro* matured porcine cumulus-oocyte complexes (12). This finding supported previous findings that pro-BMP15, but not



mature BMP15 or mature GDF9, improves oocyte quality (18, 19), suggesting that the prodomain plays an important role in its function on cumulus cells. Therefore, we investigated the effect of the cumulin prodomain on the inhibin-activin system by using cumulin forms with and without the prodomain. Mature covalent cumulin and noncovalent pro-cumulin elicited comparable expression levels of *INHBB*, inhibin B, and activin B, suggesting that the disulfide link and prodomain do not affect granulosa cell responsiveness to cumulin. It is intriguing that the prodomain plays a crucial functional role on the cumulus-oocyte complex but not on granulosa cells. The current hypothesis underpinning this difference is that the prodomains of GDF9/BMP15/cumulin interact with the specialized heparin-sulfated proteoglycans of the cumulus-oocyte complex, facilitating in some manner distinct ligand-receptor signaling cascades between the two cell types (39).

There is compelling genetic evidence that paracrine signals from the oocyte in the form of GDF9 and BMP15 (and possibly cumulin) constitute key regulators of folliculogenesis, ovulation rate, and fecundity in mammals (4). This notion is bolstered by the results of the current study whereby cumulin and FSH interact to increase inhibin B production at the expense of activin B, because inhibin is a key endocrine regulator of FSH. In humans, inhibin B is the predominant form of inhibin/activin produced by granulosa cells in the early follicular phase (40). The current results suggest that oocyte paracrine signals, probably in the form of GDF9/BMP15/cumulin, cooperate with FSH to induce inhibin B during the follicular phase, which in turn feeds back to suppress pituitary FSH secretion, which limits the growth of multiple Graafian follicles and hence the ovulation quota. Oocyte paracrine signals including cumulin may have different effects on granulosa cells from other stages of folliculogenesis (*e.g.*, inhibin A or activin production may be favored). Oocyte cumulin-mediated enhancement of inhibin and corresponding antagonism of circulating

Figure 4. The interactive effects of FSH and cumulin on inhibin B and activin B production. hGL were treated for 24 hours without (control) or with 50 ng/mL covalent pro-GDF9, pro-BMP15, or pro-cumulin, all ±50 mIU/mL FSH. Cellular mRNA expression of (a) *INHA* and (b) *INHBB* and secreted (c) inhibin B and (d) activin B into conditioned media were measured. Data are expressed as mean ± SEM of five independent granulosa cell pools. Bars within a graph not sharing a common letter are statistically different ($P < 0.05$, multiple comparisons test). G*, significant main effect of growth factor by two-way ANOVA; F*, significant main effect of FSH by two-way ANOVA; GxF*, significant main effect interaction between growth factor and FSH by two-way ANOVA. ns, no significant difference.

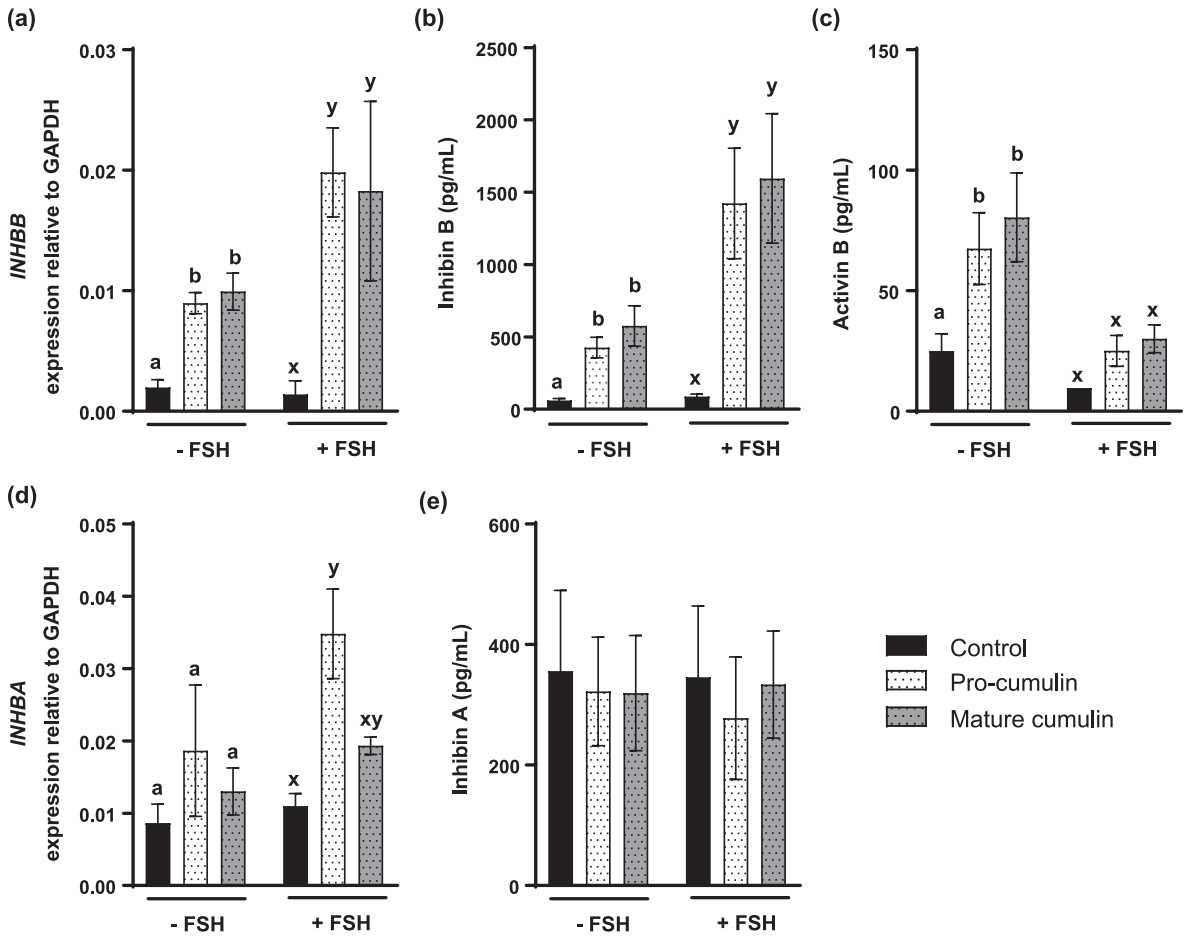


Figure 5. Effect of the cumulin prodomain on inhibin A, inhibin B, and activin B production. hGL cell (a) *INHBB* and (d) *INHBA* mRNA expression (n = 4), and (b) inhibin B (n = 3), (e) inhibin A (n = 3), and (c) activin B (n = 5) protein secretion were measured after 24-hour treatment with noncovalent pro-cumulin (50 ng/mL) or covalent mature cumulin (50 ng/mL), all with or without FSH (50 mIU/mL). Bars within a graph not sharing a common letter are statistically different ($-FSH^{a-b}$, $+FSH^{x-y}$; $P < 0.05$).

FSH may be a feature of low-ovulation phenotype mammals such as humans, because oocytes of polyovular mammals such as mice produce little BMP15 (41) and hence presumably little cumulin (12). Consequently, it can be speculated that the cooperative regulation of inhibin B by cumulin and FSH may play a role in regulating folliculogenesis and fecundity in women.

Acknowledgments

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