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REGULATION OF AMH BY OOCYTE SPECIFIC GROWTH FACTORS IN HUMAN PRIMARY CUMULUS CELLS

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Abstract

The regulation of AMH production by follicular cells is poorly understood. The purpose of this study was to determine the role of the oocyte-secreted factors, growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15), on AMH production in primary human cumulus cells. Cumulus cells from IVF patients were cultured with a combination of GDF9, BMP15, recombinant FSH, and specific signaling inhibitors. Stimulation with GDF9 or BMP15 separately had no significant effect on AMH mRNA levels. In contrast, simultaneous stimulation with GDF9 and BMP15 (G+B) resulted in a significant increase in AMH mRNA expression. Increasing concentration of G+B (0.6, 2.5, 5 and 10 ng/ml) stimulated AMH in a dose-dependent manner, showing a maximal effect at 5 ng/ml. Western blot analyses revealed an average 16-fold increase in AMH protein levels in cells treated with G+B when compared to controls. FSH co-treatment decreased the stimulation of AMH expression by G+B. The stimulatory effect of G+B on the expression of AMH was significantly decreased by inhibitors of the SMAD2/3 signaling pathway. These findings show for the first time that AMH production is regulated by oocyte-secreted factors in primary human cumulus cells. Moreover, our novel findings establish that the combination of GDF9 + BMP15 potently stimulates AMH expression.

Introduction

Ovulation results from a lengthy process known as folliculogenesis which comprises primordial follicle recruitment, followed by granulosa cell (GC) proliferation and differentiation leading to antral/preovulatory follicle formation. Throughout folliculogenesis, bidirectional communication between the oocyte and the GCs is essential to create the microenvironment necessary for normal follicle growth. Despite its importance, most, if not all, reported work on oocyte-GC interactions is based on studies in rodents. Therefore,

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Declaration of Interest

The authors declare no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

further research is essential to understand the nature of the dialogue between the oocyte and the GCs in humans.

Growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) are two of the major oocyte-secreted factors involved in the regulation of GC function. GDF9 and BMP15 cooperate to regulate GC proliferation and gonadotropin-induced differentiation (McNatty *et al.* 2005b, McNatty *et al.* 2005a, Mottershead *et al.* 2012). In mice and sheep, GDF9 is essential not only for the stimulation of early follicular growth but also for cumulus expansion, ovulation, and fertility (Elvin *et al.* 1999, Hayashi *et al.* 1999). In rodents, BMP15 promotes GC proliferation and inhibits FSH-induced progesterone synthesis (Otsuka *et al.* 2000). However, BMP15-knockout female mice exhibit normal folliculogenesis with slightly dysfunctional ovulation resulting in subfertility (Yan *et al.* 2001, Yoshino *et al.* 2006). In contrast, in humans and sheep, homozygous mutations of the BMP15 gene cause infertility (Galloway *et al.* 2000, Di Pasquale *et al.* 2004). This evidence led to the notion that BMP15 has species-specific functions; being more important in mono-ovulatory mammals but largely superfluous in mice (Su *et al.* 2004, Al-Musawi *et al.* 2013, Monestier *et al.* 2014). GDF9 and BMP15 are present in the oocyte on primordial human follicles onward suggesting they are also involved in the control of human folliculogenesis (Sun *et al.* 2010). Indeed, GDF9 and BMP15 have been shown to play a role in human fertility as demonstrated by the association between mutations in these genes and premature ovarian failure (Di Pasquale *et al.* 2006, Dixit *et al.* 2006, Kovanci *et al.* 2007, Zhao *et al.* 2007). Moreover, the signaling between the oocyte and the GCs is impaired in patients with polycystic ovary syndrome a condition whose main characteristics are folliculogenesis disruption and subfertility (Teixeira Filho *et al.* 2002, Dumesic & Richards 2013). However, the specific reciprocal interactions between the oocyte and GCs that are mediated by GDF9 and BMP15 in humans remain unexplored.

AMH was initially identified as a testicular factor involved in the regression of Müllerian ducts during male sex differentiation (Blanchard & Josso 1974). However, AMH is also expressed in the GCs of primary follicles, the first stage of follicular development (Dumont *et al.* 2015). Thereafter, AMH expression increases in growing follicles until they reach the antral stage, from which point AMH expression decreases and is undetectable in large preovulatory follicles in rodents and humans (Dewailly *et al.* 2014). In humans, in particular, several studies demonstrated that AMH remains highly expressed until follicles reach a diameter of approximately 8 mm (Weenen *et al.* 2004, Andersen *et al.* 2010, Jeppesen *et al.* 2013). Accordingly, in women, AMH levels in follicular fluid from small antral follicles are 2–3 orders of magnitude higher than in the fluid from preovulatory follicles (Andersen & Byskov 2006). Serum AMH levels also decrease with age and eventually become undetectable at menopause (Dolleman *et al.* 2014). Moreover, AMH declines prematurely due to events associated with ovarian aging (de Vet *et al.* 2002), dysfunction such as premature ovarian failure (Meduri *et al.* 2007), or after gonadotoxic chemotherapy (Dunlop & Anderson 2015). Based on this particular pattern of expression, it has been proposed that AMH levels can be used to determine the size of the ovarian follicular reserve (Visser *et al.* 2012, Pankhurst 2017). Despite the importance of AMH as a clinical marker of ovarian reserve, the regulatory network controlling AMH expression in the ovary is poorly understood especially in humans.

In cultured mouse GCs, AMH increases after the addition of oocytes to the culture media; although, the specific factors involved are unknown (Salmon *et al.* 2004). This suggests that oocyte-secreted factors may participate in the regulation of AMH. Here, we studied the regulation of AMH expression in primary human cumulus cells, which is the sub-population of GCs surrounding the oocyte. Previous reports from our laboratory demonstrated that cumulus cells obtained from IVF patients respond to gonadotropins and growth factors and can be used as a proxy of undifferentiated preantral GCs (Baumgarten *et al.* 2014, Baumgarten *et al.* 2015, Stocco *et al.* 2017). Thus, the aim of this investigation was to determine the role of GDF9, BMP15, and FSH on the regulation of AMH in human cumulus cells. In addition, recent findings demonstrated that GDF9 and BMP15 form heterodimers (GDF9:BMP15), which are significantly more active than their respective homodimers (Peng *et al.* 2013, Mottershead *et al.* 2015). Therefore, the effect of the combined treatment with GDF9 and BMP15 on the expression of AMH was also examined.

Material and Methods

Patients and Human Cumulus Cell Culture

Cumulus cells were collected from the follicular aspirates of women undergoing in vitro fertilization treatment at the University of Illinois at Chicago Fertility Center, under Institutional Review Board approval. All participants gave written informed consent. Only patients with male, uterine, or tubal factor infertility were included. After controlled ovarian hyperstimulation with gonadotropins, patients underwent transvaginal oocyte retrieval, follicular aspirates were collected, and CCs were mechanically separated from the oocytes. Isolated CCs were transported immediately to the laboratory where they were dispersed by hyaluronidase digestion (8 IU/ μ l) and then centrifuged at $500 \times g$ for 5 minutes. Cells were incubated at room temperature in red blood cell lysis buffer for 2 minutes to eliminate contaminating erythrocytes, centrifuged again at $500 \times g$ for 5 minutes, and suspended in 0.5 ml of serum-free DMEM/F12–0.25% BSA media containing antibiotics. To investigate mRNA expression or promoter activity, cells were plated at a density of 30,000 cells/well in 24-well plates coated with Matrigel (DB Biosciences). To investigate protein expression, cells were plated at a density of 200,000 cells/well in 6-well Matrigel-coated plates. Cumulus cells were treated with human recombinant GDF9 (R&D Systems), BMP15 (R&D Systems), and/or FSH (Serono) with or without specific inhibitors of intracellular signaling; H89, GF109203X (EMD Biosciences-Calbiochem), MK2206, LDN-193189 (Selleck Chemicals), U0126 (Millipore), or SB431542 (Tocris). Each data point reflects a result obtained from an individual patient. Cells from each patient were cultured separately. GDF9 and BMP15 from R&D systems are produced in HEK293 cells, therefore, these ligands are expected to be glycosylated and mimic the effect of the natural ligands as previously shown by Peng *et al.* (Peng *et al.* 2013).

RNA extraction and quantitative real-time PCR (qPCR)

Total RNA was isolated from CCs using the TRIzol reagent (Invitrogen) as stated in the manufacturer's protocol. The isolated RNA was reverse transcribed using anchored oligo-dT primers (IDT, Coralville, IA) and Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen) at 42°C for 1 hour. The resulting cDNA was diluted with water to a final

volume of 100µl and 5µl of diluted cDNA was used for each qPCR reaction. For each sample, *Amh* expression was adjusted to the expression of ribosomal protein L19 mRNA (*Rpl19*) as an internal control. Primer sequences are: *Amh*; gctgcttgccctctctac and gaacctcagcgagggtgtt, *Rpl19*; tgttttccggcatcgagccc and gctgtggcaagaagaaggctctgg.

Promoter Reporter Assays

AMH reporters were generated by cloning the -521, -1440, or -2222 upstream region of the human AMH promoter, followed by the firefly luciferase cDNA, into pTRIP plasmid (Stove *et al.* 2006). Lentiviruses containing this construct were generated as previously described (Zhou *et al.* 2013). All constructs contain in addition an expression cassette for green fluorescence protein (GFP), which allows the determination of infection efficiency. In all experiments, the infection efficiency was higher than 90 percent as determined by the number of cells expressing GFP. Empty pTRIP plasmids were used as controls (Luc). To analyze promoter activity, cells were infected with lentiviral constructs for 48 hours followed by 6-hour treatments with G+B. Luciferase activity was determined in 50 µl of cell lysate as previously described (Zhou *et al.* 2013).

Western Blot

Cultured cumulus cells were harvested in ice-cold RIPA lysis buffer supplemented with protease inhibitor cocktail (Sigma). Protein concentration and Western blotting of cell lysate were performed as previously described (Bennett *et al.* 2013). Antibodies against AMH (1:500; R&D Systems), and ACTB (1:1000; Proteintech) were used to detect total protein expression by Western blot as previously described (Baumgarten *et al.* 2014).

Statistical Analysis

Graphed data represents the mean value ± SEM. For qPCR and Western blot, statistical analysis was performed using *t*-test or one-way Analysis of Variance (ANOVA). Differences between groups were statistically significant as indicated in the figure legend.

Results

Stimulatory effect of the GDF9 and BMP15 combination on *AMH* mRNA expression

To determine the effect of oocyte-derived factors on AMH regulation in human GCs, we used a recently developed primary culture of human cumulus cells from IVF patients (Stocco *et al.* 2017). Human cumulus cells were treated with recombinant human GDF9 or recombinant human BMP15 at 2.5 and 10 ng/ml. In addition, cells were treated with a combination of GDF9 plus BMP15 (G+B) at 0.1, 0.6, 5, or 10 ng/ml of each factor. Treatment with GDF9 alone had no effect on the expression of *AMH* at any of the concentrations used. Treatment with BMP15 alone tended to stimulate *AMH* expression, particularly at the 2.5 ng/ml concentration, although this increase did not reach statistical significance when compared using one-way ANOVA. In contrast, treatment with G+B stimulated *AMH* mRNA accumulation in a dose-dependent manner (Figure 1). Cumulus cells exposed to 0.6 ng/ml of G+B showed some increase in *AMH* expression, whereas a significant increase in the expression of *AMH* was detected with 5 or 10 ng/ml of G+B (Figure 1). It is noteworthy that although the GDF9 and BMP15 co-treatment at 5 and 10

ng/ml consistently stimulated *AMH* in all patients examined, we observed high variability in the response of cumulus cells to 10 ng/ml. For this reason, we used 5 ng/ml of each GDF9 and BMP15 in all further experiments.

The combination of GDF9 and BMP15 strongly stimulates AMH protein levels

Next, we investigated whether the stimulation of *AMH* mRNA levels by GDF9 and BMP15 translated into an increase in AMH protein production. Cumulus cells obtained from three different patients were cultured in the presence or absence G+B (5 ng/ml each). For each patient, AMH protein expression was readily detectable in cells treated with G+B (Figure 2A). In contrast, AMH was barely detectable in control conditions. An increase in AMH protein levels was observed in cells treated with GDF9 and BMP15 when compared to controls. The ratio of the densitometry analysis of AMH and β -actin signal indicated that the relative expression of AMH protein in G+B treated cells is higher than in vehicle treated cells (Figure 2A). Additionally, a dose-dependent experiment was conducted with cells from a fourth patient. For this experiment, AMH was undetectable in untreated cells. A small increase in AMH was observed with G+B at 2.5 ng/ml each. Protein expression increased further in cells treated with 5 ng/ml of G+B (Figure 2B). Interestingly, although a concentration of 20 ng/ml of G+B also stimulated the AMH protein expression, the stimulation obtained with this concentration was lower than the one obtained with 5 ng/ml. Thus, in good agreement with our findings at the mRNA level, a concentration of 5 ng/ml of G+B most effectively stimulates AMH protein expression.

GDF9 and BMP15 have no major effect on the stimulation of *AMH* promoter activity

Different lengths of the promoter region of the *AMH* gene were cloned into a reporter vector. These reporters were transfected into cumulus cells using a lentivirus system, which provides the highest infection efficiency usually more than 90% as determined by the expression of GFP (see material and methods). Forty-eight hours after transfection, cells were treated with vehicle or the combination of GDF9 and BMP15 at 5 ng/ml of each. Luciferase activity was quantified 6 and 24 hours after the initiation of the treatments. Treatment with GDF9 and BMP15 had no effect on the activity of reporter constructs carrying 521, 1440, or 2222 bps of the *AMH* promoter most adjacent to the transcription start site (Supplemental Figure 1).

FSH diminishes the stimulatory effect of GDF9 and BMP15 on AMH

It has been recognized that the expression of AMH decreases as GCs differentiate to the preovulatory stage, a process largely controlled by FSH. Therefore, next, we examined whether co-treatment with FSH had any effect on the stimulation of *AMH* mRNA by G+B. For this purpose, cumulus cells were treated with GDF9 and BMP15 in the presence or absence of FSH for 48 hours. An additional group of cells was treated with FSH alone. As shown in Figure 3, treatment with FSH alone had no effect on *AMH* mRNA levels. As expected, all groups treated with G+B showed increased *AMH* expression. However, this increase was blunted in the presence of FSH suggesting that FSH inhibits the stimulation of *AMH* by GDF9 and BMP15.

Role of SMAD on AMH regulation in human granulosa cells

The signaling used by BMP15 and GDF9 in human granulosa cells has not been examined. It has been shown that BMP15 activates the SMAD1/5/8 signaling pathway whereas GDF9 signals through SMAD2/3 (Moore *et al.* 2003, Mazerbourg *et al.* 2004). To elucidate whether these pathways mediate G+B actions on AMH, cumulus cells were treated with inhibitors of SMAD2/3 (SB431542) or SMAD1/5/8 (LDN-193189) for one hour before the addition of GDF9 and BMP15 to the media. Forty-eight hours later, cells were harvested for *Amh* mRNA determination. The results indicated that inhibition of SMAD1/5/8 had no major effects on the induction of *Amh* levels by G+B (Figure 4). However, a strong inhibition was observed with SB431542 (Figure 4), which selectively inhibits SMAD2/3 (Inman *et al.* 2002).

Discussion

In mice, AMH knockdown increases the entry rate of primordial follicles into the growing follicle pool (Durlinger *et al.* 1999), indicating that AMH is involved in regulating primordial follicle recruitment. AMH also acts in antral follicles where it blocks FSH induction of aromatase expression and estradiol production, two markers of preovulatory GC differentiation (Durlinger *et al.* 2001). In humans, a similar relationship appears to exist, as follicular AMH and estradiol levels are inversely correlated in small antral follicles (Andersen & Byskov 2006). These studies indicate that appropriate regulation of AMH expression is critical throughout folliculogenesis and highlight the urgent need to investigate the molecular mechanisms controlling this important reproductive hormone, especially in humans. Our results have established that AMH expression increases more than 10-fold in primary human cumulus cells co-treated with the oocyte-secreted factors GDF9 and BMP15. This observation suggests that by modulating AMH, the oocyte may play a role in the control of follicle recruitment and dominant follicle selection in humans.

Co-treatment of cumulus cells with low concentrations of GDF9 and BMP15 induced AMH expression. However, treatment with either GDF9 alone had no effect; whereas treatment with BMP15 stimulates AMH to levels that are significantly lower than those induced by GDF9 + BMP15. These results suggest that GDF9 and BMP15 interact to control AMH expression in human GCs. The strong synergism between GDF9 and BMP15 on AMH expression is likely to be attributed to the formation of GDF9:BMP15 heterodimers (Yan *et al.* 2001, Liao *et al.* 2003, Peng *et al.* 2013), named “cumulin” (Mottershead *et al.* 2015). This last report showed that cumulin forms after the simple co-addition of GDF9 and BMP15 to the culture medium and that the effect of the cotreatment with GDF9 and BMP15 cannot be mimicked by the co-addition of GDF9 and BMP15 stable homodimers (Mottershead *et al.* 2015). Of note, previous reports have used highly sensitive mouse GCs isolated from preantral follicles to study the effects of purified GDF9:BMP15 heterodimers (Peng *et al.* 2013, Mottershead *et al.* 2015). The fact that the combination of GDF9 and BMP15 behave similarly in primary human cumulus cells is remarkable and provides further support for the use of cumulus cells to study follicular cell differentiation and the role of oocyte-secreted factors in the regulation of GC function.

In this regard, as discussed in the introduction, we used in this report a novel experimental approach to study AMH regulation, which consists of cumulus cells from IVF patients. We have recently fully characterized this model and extensively discussed the fact that human cumulus cells from IVF do not luteinize and respond to gonadotropins, and IGFs as cells from preantral follicles do (Stocco *et al.* 2017). This model was also used and validated in two recent publications (Baumgarten *et al.* 2014, Baumgarten *et al.* 2015). It must also be highlighted that cumulus cells are the natural and physiological targets of oocyte-secreted factors (GDF9 and BMP15). Therefore, this model is not only novel but also provides a more physiological approach to study AMH regulation. However, since the regulation of AMH expression may differ significantly between undifferentiated granulosa cells, mural granulosa cells, and cumulus cells, the effect of GDF9 and BMP15 on AMH in other types of follicular cells requires further studies.

We based our dose-response experiments on published data from two independent groups (Peng *et al.* 2013, Mottershead *et al.* 2015). So far, there are few reports in the literature on the levels of GDF9 and BMP15 in normal follicular fluid; whereas there are no publications reporting levels of the heterodimer. In women with primary or secondary infertility for diminished ovarian reserve, BMP15 and GDF9 levels in the follicular fluid at oocyte retrieval were reported to be 0.81 (± 0.25) ng/ml and 6.82 (± 3.77) ng/ml, respectively (Zhang *et al.* 2014). Also in the follicular fluid at oocyte retrieval of women undergoing in vitro fertilization, BMP15 levels were reported at 278 (± 12) ng/ml (Ilhan *et al.* 2017); whereas another independent study reported GDF9 levels of 6.8 (± 0.9) ng/ml in the follicular fluid of women younger than 35 years old (Han *et al.* 2011). It is difficult to extrapolate the in vitro concentrations of GDF9 and BMP15 used in the literature and in this report to GDF9 and BMP15 levels in the follicular fluid, especially considering that these factors are locally produced, and therefore they may reach high local concentrations. Despite this limitation, the concentrations we have used are in the low range of the follicular fluid concentrations reported for these factors. GDF9 and BMP15 are detectable in human follicular fluid using Western blot and mature GDF9 levels in the follicular fluid are significantly correlated with oocyte nuclear maturation and embryo quality (Gode *et al.* 2011). The development of sensitive assays for GDF9 and BMP15 that can distinguish the most biologically potent GDF9:BMP15 heterodimer will be a great tool to better understand the role of these factors in human ovarian function.

It has been previously demonstrated that GDF9 slightly enhances the stimulatory effect of BMP15 alone on AMH expression in ovine GCs obtained from preantral follicles (Pierre *et al.* 2016). In contrast, we observed a strong synergism between GDF9 and BMP15 on the expression of AMH in humans, whereas either factor has negligible effects on AMH. Species differences may account for this discrepancy although the concentration of GDF9 and BMP15 used in each study should also be considered. For instance, we observed that concentrations higher than 10 ng/ml are less efficient at inducing AMH expression at both the mRNA and protein levels. In the above-mentioned report, ovine GCs were treated with 50 ng/ml of each factor. This suggests that at higher concentrations the formation of GDF9:BMP15 heterodimers may be impaired. However, it is also possible that the receptors used by the heterodimers are not expressed in ovine GCs or that the heterodimers are more active in human than in ovine GCs. An example of this possibility is that in ovine GCs,

Pierre et al. (Pierre *et al.* 2016) could show an increase in AMH promoter activity after treatment with GDF9 and BMP15. In contrast, we were not able to do so, suggesting species-specific differences. Further studies are needed to determine the species-specific effects of GDF9:BMP15 heterodimers. Finally, in ovine GCs, Pierre et al. only reported mRNA levels for AMH, which is not enough to conclude that the AMH protein is also stimulated by GDF9 and BMP15. In contrast, we have correlated mRNA levels with protein expression using dose-response experiments. Thus, our report demonstrates for the first time a stimulatory effect of GDF9 and BMP15 on AMH expression in primary human cumulus cells.

Pierre et al. (Pierre *et al.* 2016) also used mural luteinized granulosa cells from IVF patients. However, in contrast to our findings, this report showed that there are no significant differences between BMP15 and BMP15+GDF9 treatments on *AMH* mRNA levels in human luteinized granulosa cells (See figure 1D on Pierre *et al.* 2016). These findings show that mural cells are not the ideal model to study AMH regulation. This idea is supported by the AMH regulation in GCs. For instance, Campbell et al. (Campbell *et al.* 2012) demonstrated that AMH remains expressed in cumulus cells while it becomes very reduced in the mural GCs of large follicles. Moreover, after the administration of human chorionic gonadotropin (hCG) to IVF patients, mural cells undergo terminal differentiation towards luteinization and therefore shut down AMH expression (Grondahl *et al.* 2011, Kedem-Dickman *et al.* 2012, Merhi *et al.* 2013). Thus, *AMH* mRNA is absent in preovulatory follicles and corpora lutea (Baarends *et al.* 1995). Therefore, our results in cumulus cells are not only novel but also entirely different to those obtained by Pierre et al. using luteinized granulosa cells.

The stimulatory effect of GDF9 plus BMP15 on the activity of AMH promoter reporter constructs was minimal when compared to the effect of this combination on AMH mRNA and protein levels. This finding suggests that mechanisms other than the activation of gene expression may be involved in the regulation of AMH by GDF9 and BMP15 in primary human cumulus cells. Also, it is possible that additional elements beyond the 2.2 Kb promoter region used in this report are involved in AMH regulation in primary human cumulus cells. Despite this controversy, our findings prove that the SMAD2/3 signaling pathway plays a crucial role in the induction of AMH in these cells. In COV434 cells, an ovarian granulosa tumor cell line, GDF9:BMP15 heterodimers signal through a unique receptorsome formed by BMP receptor type 2 (BMP2) and a type 1 receptor complex composed by ALK4 and ALK6 (a.k.a. ACVR1B and BMP1B) (Peng *et al.* 2013). Although we have not determined which receptors mediate the effect of GDF9 plus BMP15 in primary human cumulus cells, it is possible that ALK4 and ALK6 may be involved since these receptors are known to activate SMAD2/3. Finally, our finding agrees with previous reports demonstrating that GDF9 and BMP15 cotreatment stimulates thymidine incorporation in rat granulosa cells in an SMAD2/3 dependent manner (Reader *et al.* 2011). Together, the evidence points to a crucial role of SMAD2/3 on the regulation of AMH by GDF9 and BMP15 in human primary GCs.

FSH blunted the effect of GDF9 and BMP15 in human CCs, a finding that resembles the physiological regulation of AMH during folliculogenesis (see introduction). This

observation is consistent with previous *in vivo* findings in rats showing that FSH decreases AMH expression in GCs (Baarends *et al.* 1995). Evidence obtained in a mouse cell line suggests that estrogen receptor β mediates the inhibitory effect of FSH on AMH (Grynberg *et al.* 2012). The distal promoter of the AMH gene contains estrogen receptor binding sites (Picard *et al.* 1986), suggesting a direct effect of estrogen on AMH. Despite this evidence, the mechanisms involved in the downregulation of AMH by FSH in human cumulus cells are currently unknown. Moreover, since FSH and cAMP increase AMH expression in Sertoli cells (Lukas-Croisier *et al.* 2003), in human granulosa-luteal cells, and in the mouse KK1 granulosa cell line (Taieb *et al.* 2011), it is possible that the pathways by which FSH controls AMH may be not only cell but also species specific. In Sertoli cells, the transcription factors SF1, SOX9, WT1, and GATA4 are involved in AMH upregulation (Giuli *et al.* 1997, Lukas-Croisier *et al.* 2003, Lasala *et al.* 2004, Lasala *et al.* 2011). Excluding SOX9, which is not expressed in the ovary, these factors are present in GCs (Salmon *et al.* 2005) and have binding sites in the AMH promoter. However, whether they mediate the stimulatory effect of GDF9 and BMP15 or the inhibitory effect of FSH remains to be determined.

In conclusion, these results demonstrate for the first time that the cotreatment with GDF9 and BMP15 stimulates AMH expression in human primary cumulus cells. The induction of AMH expression by low concentrations of GDF9 and BMP15 and the fact that the treatment with GDF9 or BMP15 alone has no effects suggest that GDF9:BMP15 heterodimers are involved in the regulation of AMH. Elucidation of AMH regulation in humans will provide a better understanding of the inter-follicular inhibition of follicle recruitment and will help to more accurately interpret circulating levels of AMH in clinical applications where it is widely used as a marker of ovarian reserve.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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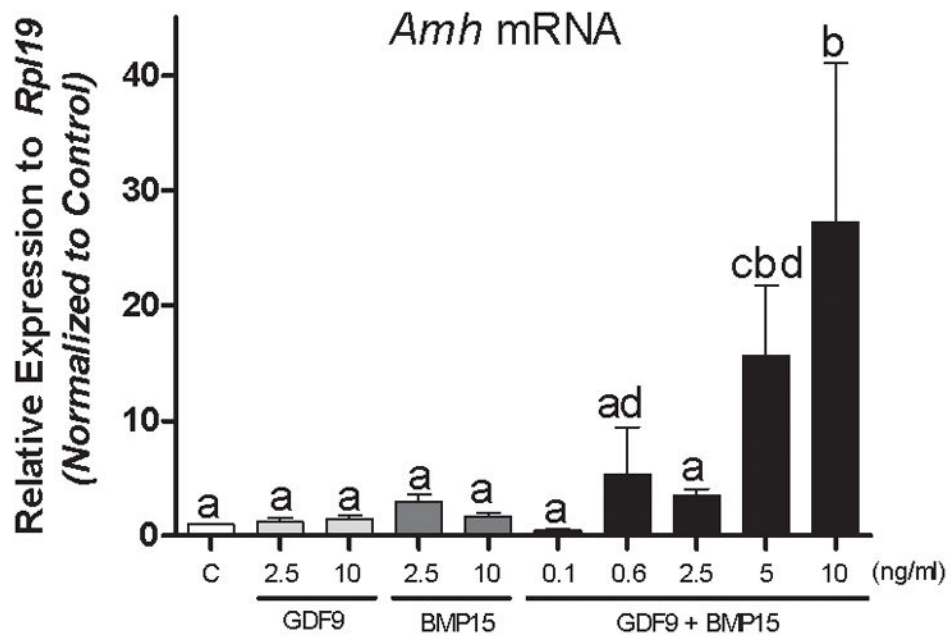


Figure 1. The combination of GDF9 and BMP15 stimulate *Amh* mRNA expression in human cumulus cells

Cumulus cells were treated for 48 hours with vehicle (C), GDF9 (2.5, 10ng/ml), BMP15 (2.5, 10ng/ml), or the combination of GDF9 and BMP15 (0.1, 0.6, 2.5, 5, or 10ng/ml of each). *Amh* mRNA levels were determined by qPCR and expressed as relative to *Rpl19*. Columns represent the mean \pm SEM, Columns with different letters differ significantly a–b $P < 0.05$, a–c $P < .001$ vs controls, one-way ANOVA, Bonferroni test, n=15.

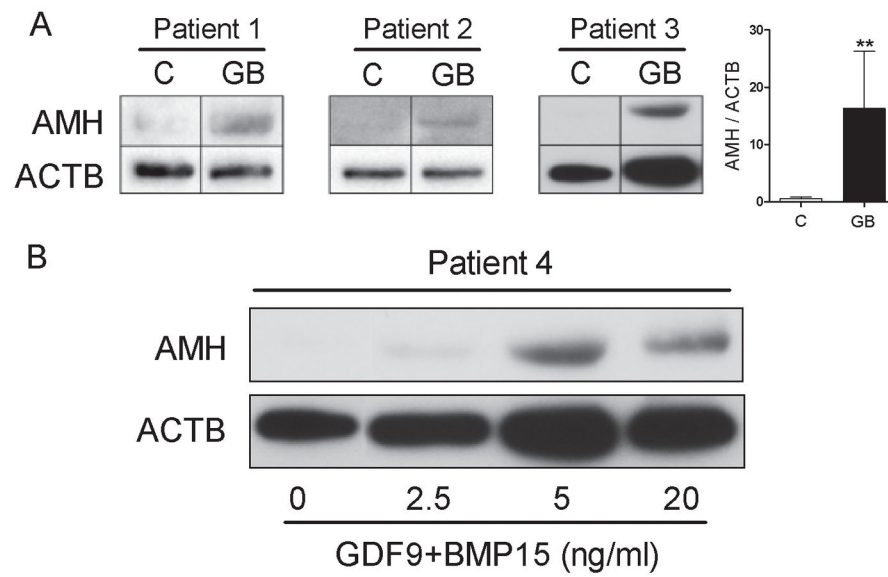


Figure 2. AMH protein levels in human cumulus cells treated with GDF9 and BMP15

A) Cumulus cells from three different patients (Patient 1, 2, and 3) were treated with 5 ng/ml of both GDF9 and BMP15 (GB) for 48 hours. AMH β -actin (ACTB) protein levels were determined by Western blotting. On the left, the average (\pm SEM) of the relative optical density units of AMH to ACTB is shown (** P < 0.001, t -test, $n=3$). B) Cumulus cells from Patient 4 were treated with 2.5, 5, or 20 ng/ml of both GDF9 and BMP15. AMH protein levels were determined as in A. Expression of ACTB is shown as a loading control.

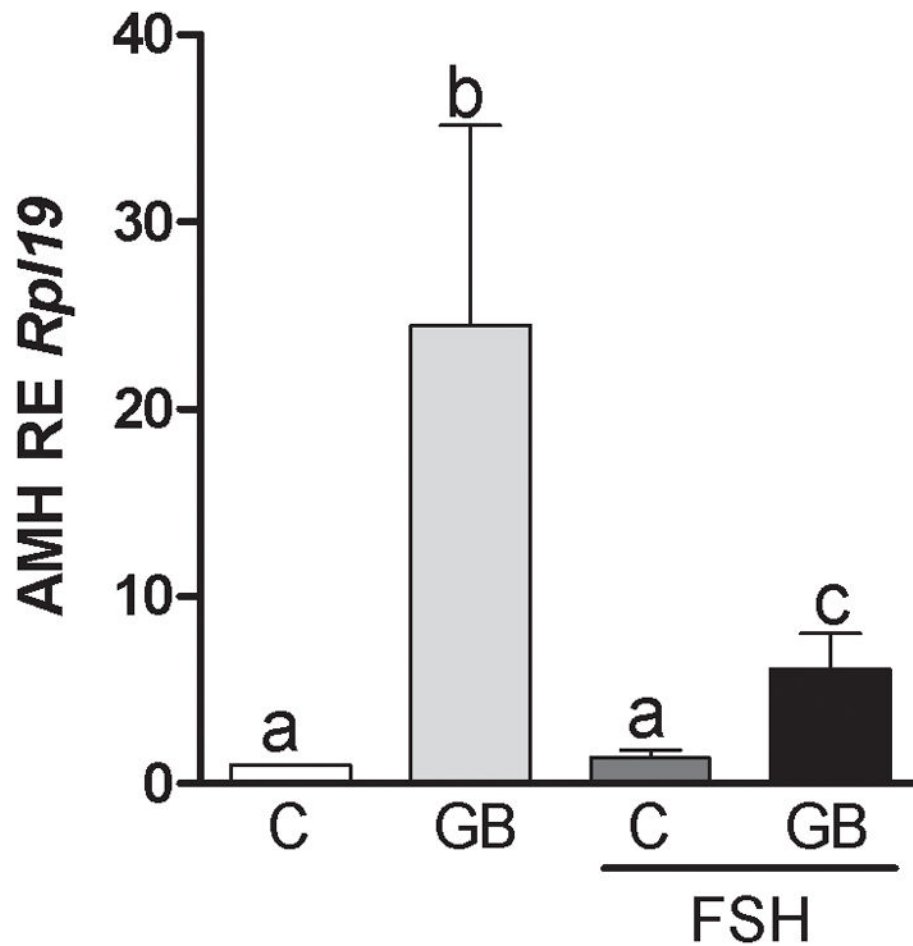


Figure 3. FSH inhibits the stimulation of GDF9 and BMP15 on *Amh* mRNA expression
 Cumulus cells were treated for 48 hours with GDF9 and BMP15 (G+B; 5 ng/ml of each) in the presence or absence of FSH (50 ng/ml). *Amh* mRNA levels were determined by qPCR and expressed relative to *Rpl19*. Columns with different letters differ significantly a–b $P < 0.001$, a–c $P < 0.05$, b–c $P < 0.01$, one-way ANOVA, Bonferroni test, $n = 11$.

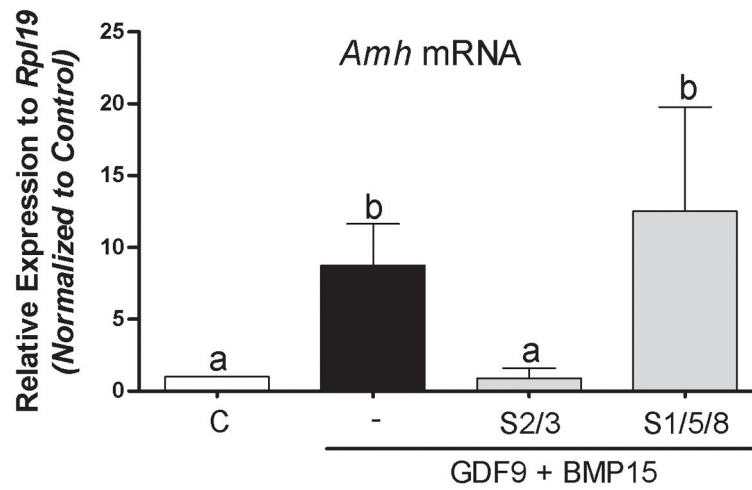


Figure 4. Effect of cell signaling inhibition on GDF9 and BMP15 induced *Amh* mRNA levels Cumulus cells were treated for 1 hour with the following specific SB431542 (SMAD2/3; 10 μ M), or LDN-193189 (SMAD1/5/8; 100 nM) followed by a 48-hour co-treatment with GDF9 and BMP15 (5 ng/ml of each). *Amh* mRNA levels were determined 48 h after by qPCR and expressed relative to *Rpl19*. Columns with different letters differ significantly a–b $P < 0.001$, one-way ANOVA, Bonferroni test, $n = 9$.