REPRODUCTIVE PHYSIOLOGY AND DISEASE

# Effect of Anti-Müllerian hormone (AMH) and bone morphogenetic protein 15 (BMP-15) on steroidogenesis in primary-cultured human luteinizing granulosa cells through Smad5 signalling

Ermioni Prapa[1] · Anna Vasilaki[2] · Konstantinos Dafopoulos[1] · Eleni Katsiani[1] ·
Panagiotis Georgoulias[3] · Christina I Messini[1] · George Anifandis[1] · Ioannis E Messinis[1]

**Abstract**

*Purpose* To determine if there is any effect of AMH and BMP-15 on estradiol and progesterone production from primary-cultured human luteinizing granulosa cells, to delineate what is the effect of FSH on their actions and which are the possible mechanisms involved.

*Methods* Luteinizing granulosa cells (GCs), obtained from follicular fluid of 30 women undergoing in vitro fertilization, were cultured, after a short 24-h preincubation period, in serum-free medium for 24 or/and 48 h in the presence/absence of various concentrations of AMH, BMP-15 and FSH alone or in combinations. Estradiol and progesterone production, SMAD5 phosphorylation and StAR expression were studied in parallel. Steroids were measured in culture-supernatant using enzyme-immunoassays, while Smad5-signaling pathway activation and StAR protein expression were assessed immunocytochemically.

*Result(s)* We found that the treatment of AMH in GCs for 24/48 h attenuated FSH-induced estradiol production ($p<0.001$), had no effect on basal estradiol levels, decreased basal progesterone production ($p<0.001$) and FSH-induced StAR expression ($p<0.001$). On the other hand, BMP-15 decreased basal estradiol levels ($p<0.001$) and attenuated FSH-induced estradiol production ($p<0.001$). Furthermore, BMP-15 reduced progesterone basal secretion ($p<0.001$), an effect that was partially reversed by FSH ($p<0.01$), probably via increasing StAR expression ($p<0.001$). FSH-induced StAR expression was also attenuated by BMP-15 ($p<0.001$). FSH, AMH and BMP-15 activated Smad-signaling pathway, as confirmed by the increase of phospo-Smad5 protein levels ($p<0.001$ compared to control).

*Conclusion(s)* AMH and BMP-15 by interacting with FSH affect the production of estradiol and progesterone from cultured luteinizing-granulosa cells possibly via Smad5-protein phosphorylation.

*Capsule* AMH and BMP-15 interact with FSH and affect the production of estradiol and progesterone from cultured luteinized granulosa cells possibly via Smad5-protein phosphorylation.

✉ Ioannis E Messinis
messinis@med.uth.gr

1   Department of Obstetrics and Gynaecology, School of Health Sciences, Faculty of Medicine, University of Thessaly, 41110, Viopolis Larissa, Greece

2   Department of Pharmacology, School of Health Sciences, Faculty of Medicine, University of Thessaly, Larissa, Greece

3   Department of Nuclear Medicine, School of Health Sciences, Faculty of Medicine, University of Thessaly, Larissa, Greece

## Introduction

Ovarian follicles constitute the functional unit of the human ovary; endocrine signaling via the hypothalamus-pituitary - gonadal axis is crucial for its survival, development, maturation and steroidogenic activity [1–3]. Nevertheless, local growth factors seem to have a pivotal and synergistic role in these procedures [4, 5]. Among them, Anti-Mullerian Hormone (AMH) and Bone Morphogenetic Protein 15 (BMP-15), both members of the TGF-β superfamily, regulate follicle growth and oocyte maturation, acting in an autocrine/paracrine manner [2].

AMH is a dimeric glycoprotein exclusively expressed by granulosa cells of pre-antral and small antral follicles in the

ovary [6–10]. As a TGF-β superfamily member, AMH exert its effect by activating type I (ALK2/3/6) and type II (AMHRII) serine/threonine kinase receptors, leading subsequently to Smads protein phosphorylation [11, 12]. Studies in mice have identified the necessity of AMH in the preservation of the primordial follicle pool by inhibiting the follicular activation and growth as well as the follicle sensitivity to FSH [13, 14]. Furthermore, data in the human reveal that serum AMH levels can be used as an ovarian follicular reserve marker [15, 16], as well as a predictor of the ovarian responsiveness in normo-ovulatory women undergoing ovarian stimulation for IVF [17–20]. In addition, the importance of this hormone in the follicular recruitment arises from a number of studies, which correlate serum and follicular fluid AMH levels with the high number of 2-5 mm follicles in PCOS women [21–25], thus giving another clue to the clarification of the polycystic ovary syndrome pathology.

On the other hand, BMP-15 is an exclusively oocyte-secreted growth factor [26, 27]. Similarly to AMH, BMP-15 participates in normal ovarian function of most primates and human [4, 26–29] through binding to serine/threonine receptors (ALK6/BMPRII) and subsequent activation of the Smad signaling pathway [12, 30]. A number of sheep BMP-15 mutations have been linked to increased ovulation rate and infertility [31–33]. Likewise, mutations of the BMP-15 gene in humans have been associated with the disruption of the ovulation process in women with polycystic ovary syndrome and with premature ovarian failure [26, 34, 35]. Furthermore, follicular fluid BMP-15 levels are considered to be an indicator of oocyte quality and embryo development [36]. However, in lower mammals BMP-15 has no crucial role in folliculogenesis; in mice, apart from some mild alterations in ovulation and fertilization dynamics, loss of BMP-15 activity does not affect folliculogenesis [33].

Apart from regulating follicular development, both AMH and BMP-15 are also involved in ovarian steroidogenesis. Recent studies in animals and human suggest that AMH attenuates the FSH-induced aromatase expression and estradiol production in the ovary [37–41], while AMH and AMHRII gene alterations correlate with follicular phase estradiol levels [42]. Moreover, results from animal models and immortalized human granulosa-lutein cells indicate that BMP-15 decreases the FSH-induced StAR protein levels and progesterone production [30, 43, 44].

Nevertheless, up to date, the possible regulatory role of AMH and BMP-15 in estradiol and progesterone production from human granulosa cells (hGCs) has not yet been investigated. In the present study we examined the effects of these two factors on the steroidogenic activity of primary granulosa lutein cells alone or in combination and in the presence/absence of gonadotropins as well as the potential signaling pathways which intermediate their actions.

## Materials and methods

### Subjects and tissue isolation

Human luteinizing GCs were obtained from follicle aspirates of 30 women, aged 23–45 years old, undergoing IVF because of tubal pathology or male factor infertility. All patients were recruited after giving written informed consent and the protocol was approved by local ethics. Superovulation induction protocol involved pituitary suppression by a GnRH agonist, followed by follicle stimulation with recombinant FSH. Final follicle and oocyte maturation was induced by human chorionic gonadotropin (hCG) and oocyte retrieval was performed 36 h later. After the removal of the oocyte, GCs were retrieved from the Follicular Fluid (FF) by sedimentation as described previously [45]. In brief, GCs in follicular fluid aspirates were washed several times with phosphate-buffered saline (PBS; Biochrom AG, Berlin, Germany) containing 0.1 % bovine serum albumine (BSA; Sigma, Bornem, Belgium) until cleared from blood cells contamination and then centrifuged at 500×g for 10 min. The clear GCs pellet was resuspended in serum supplemented medium 199 Earle's salts and HEPES (GIBCO, Life Technologies, BRL, Glasgow, Scotland) containing 500 IU/mL sodium G penicillin, 500 mg/mL streptomycin (Biochrom AG), 0.1 % BSA, and 5 % L-glutamine (Biochrom AG). Cell viability and concentration was determined by trypan blue exclusion.

### Cell cultures

Human luteinizing GCs were seeded ($5 \times 10^4$ cells per well in 24-well plates) and cultured at $37^o$ C in a humidified atmosphere containing 5 % $CO_2$ and 95 % air at $37^o$ C in serum added culture media 199 Earle's salts and HEPES (GIBCO, Life Technologies). A short 24-h preincubation period processed in order to allow cells to attach and form a monolayer culture as well as to regain their sensitivity to FSH [46, 47]. Subsequently, culture medium was replaced by serum free medium containing $10^{-6}$ M androstendione (A4) (NIDDK's National Hormone and Peptide Program, Harbor-UCLA Medical Centre, Los Angeles, CA) as a substrate for the aromatase, in combination with the stimulants recombinant human FSH (R&D Systems Inc. Mineapolis, USA) (10 and 100 ng/ml), recombinant human BMP-15 (R&D Systems Inc. Mineapolis, USA) (10 and 100 ng/ml) AMH (R&D Systems Inc. Mineapolis, USA) (2, 20 and 100 ng/ml). Plates were then incubated for 72 h and the medium was collected and replaced every 24 h. All collected media were stored at −20 C until assayed. In pilot experiments, the medium was also collected and replaced every 24 h (data not shown). Nevertheless, because the greatest response of the cultured GCs to the stimulants in terms of steroids production was seen at 48 h and the viability of the cells seems to attenuate after 48 h in serum free medium conditions, the point of 72-h sampling was abandoned.

## Immunohistochemistry

SMAD signaling pathway and StAR protein expression were studied immunohistochemically after 48 h of culture, because at the 48 h versus 24 h an effect on FSH-induced estradiol production is revealed (Fig. 1), using specific antibodies. Rabbit monoclonal Anti-SMAD5 1/100 (phospho S463+S465 [MMC-1-104-3] - BSA and Azide free ab168252, Abcam Inc. Cambridge, USA) primary antibody and Mouse monoclonal Anti-StAR 1/100 (ab58013, Abcam Inc. Cambridge, USA) primary antibody were obtained. Cells were seeded in 2 % (w/v) gelatin coated cover slides (12 mm diameter) during cultivation with the protocol mentioned above. After blocking in 02 M Phosphate Buffered Saline (PB) pH 7.4 slides were fixed with 2 % Paraformaldehyde (PFA) pH 7.4 for 10 min. Each slide was incubated with primary antibody in 0.1 M Tris-buffered saline (TBS) containing 0.5 % normal donkey serum (NDS) and 0.3 % Triton X-100 overnight at room temperature. Subsequently, the slides were washed in TBS and incubated in CF™488A labeling highly cross-adsorbed donkey anti-rabbit IgG (H+L) (Biotium, USA) and CF™594 highly cross-adsorbed donkey anti-mouse IgG (H+L) (Biotium, USA) secondary antibody for 1 h in room temperature. Finally, cell cultures were mounted with DAPI containing mounting medium (UltraCruz™ Mounting buffer; sc-24941; Santa Cruz Biotechnology Inc. Europe) and cover slipped. For avoiding non-specific binding and auto-fluorescence, negative control studies with primary antibodies were omitted and cells were processed immunohistochemically as mentioned above.

## Microscopy and image analysis

Cells were examined by light microscopy (Zeiss Axioskop with Plan-Neofluar 10×/0.25 and 40×/0.75
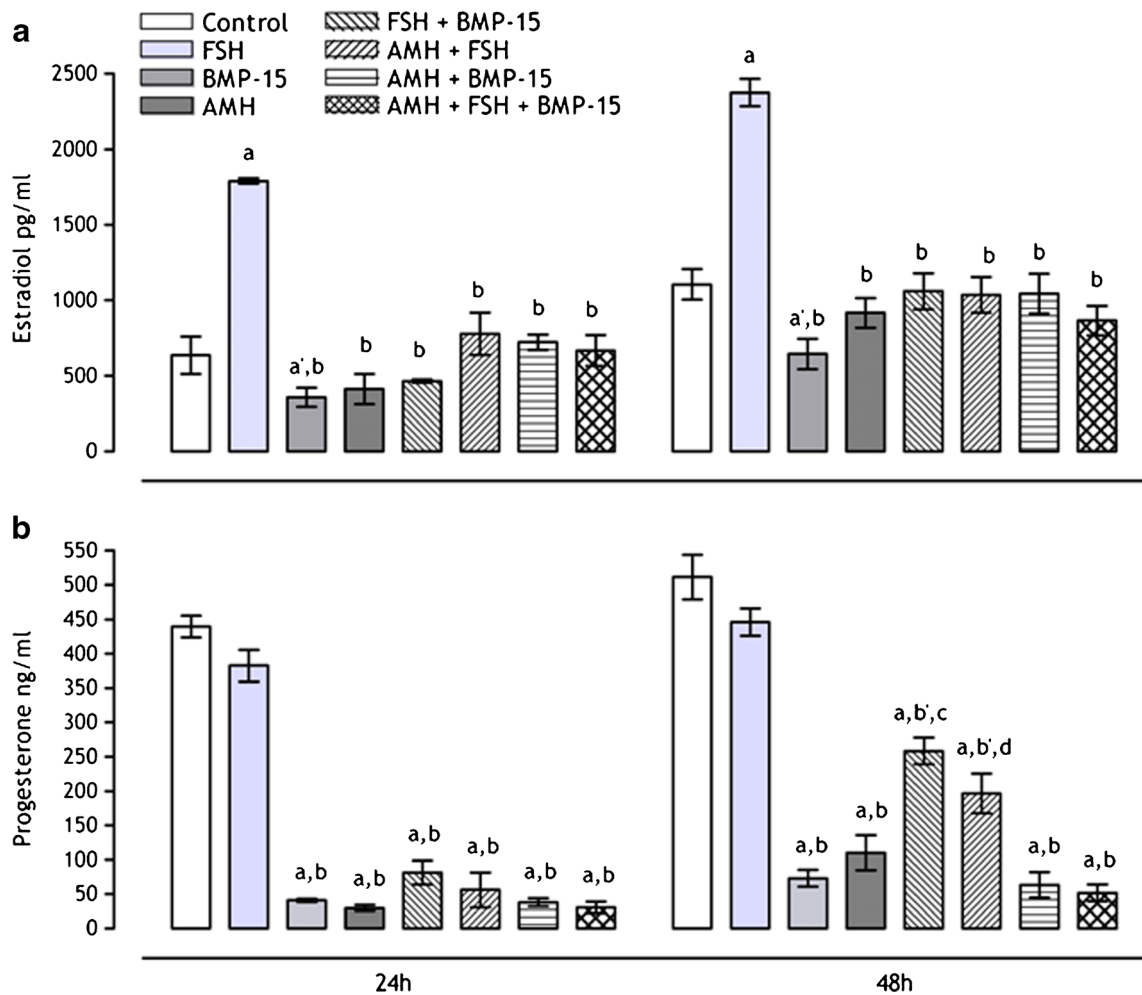


**Fig. 1** Effects of FSH (10 ng/ml), AMH (2 ng/ml) and BMP-15 (10 ng/ml) and their combinations on estradiol and progesterone production by human luteinizing granulosa cells after 24 and 48 h of culture. Data are in mean (±SEM), $n=30$ patients. Statistical comparison using one-way ANOVA followed by Bonferroni post-test analysis: (a) $p<0.001$ and (a ) $p<0.05$ compared to control; (b) $p<0.001$ and (b ) $p<0.01$ compared to FSH; (c) $p<0.05$ compared to BMP-15; (d) $p<0.05$ compared to AMH

objectives, Oberkochen, Germany). Image analysis was conducted with the use of Image J programme for Light Microscopy (1.47r, Wayne Rasband National Institutes of Health, USA). All images (RBG pictures) were similarly analysed. The mean signal intensity of each culture was recorded by employing the "Analyze/Histogram" command. Results were expressed as intensity values (0–255 integers scale from minimum to maximum luminosity intensity).

### Steroid assay

Steroids, 17b-estradiol and progesterone were measured in culture media by commercially available enzyme immunoassays (Assay Designs, Inc., Ann Arbor, MI). The lower limits of detection for 17b-estradiol and progesterone were 37 pmol/L and 0.6 nmol/L, respectively. The intra- and interassay coefficients of variation for 17b-estradiol were 6.6 and 6.2 % and for progesterone, 5.4 and 8.3 %, respectively.

### Statistical analysis

Data were normally distributed (one sample Kolmogorov-Smirnov test). Statistical comparison was performed using two-way ANOVA for randomized-block data in order to locate the source of variation (time and/or treatment) in estradiol and progesterone release from human GCs; while, one-way ANOVA followed by the Bonferroni post-test was used to study the significance of estradiol and progesterone changes between treatments. For the statistical analysis of the data, GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA) was used and p-values <0.05 were considered statistically significant. Data are expressed as means±SEM.

## Results

### Effect of FSH, AMH and BMP-15 on basal estradiol and progesterone production from human luteinizing granulosa cells

As shown in Table 1, FSH and BMP-15 affect estradiol release from human GCs while AMH has no effect on the production of this steroid hormone. In particular, FSH at 10 ng/ml increased estradiol release after 48 h in culture ($p<0.001$ compared to control) while at 100 ng/ml it has a statistical significant effect as early as 24 h ($p<0.01$). BMP-15 decreased estradiol release in both time points and concentrations used (10 and 100 ng/ml; 24 h: $p<0.01$; 48 h: $p<0.001$).

On the other hand, progesterone release was affected by AMH and BMP-15 while FSH had no effect on its production (Table 1). Specifically, AMH and BMP-15 in both time points and all concentration used (AMH: 2, 20 and 100 ng/ml; BMP-15: 10 and 100 ng/ml) decreased progesterone release in a statistical significant manner ($p<0.001$ in all cases).

Based on these results, in order to further study the effect of AMH and BMP-15 on human GCs steroidogenesis in the presence/absence of FSH we used the minimal possible concentration of each factor that had a statistically significantly effect on steroid production; that is to say, AMH: 2 ng/ml; BMP-15: 10 ng/ml and FSH: 10 ng/ml (Table 1).

### Effect of AMH and BMP-15 on FSH-induced steroidogenesis in human luteinizing granulosa cells

As mentioned above (Table 1), AMH had no effect on basal estradiol release after 24 and 48 h of culture, on the other hand it significantly reduced FSH-induced estradiol production at both time points (24 h: $p<0.001$, 48 h: $p<0.001$; Fig. 1A). On the other hand, BMP-15 significantly reduced the basal

**Table 1** Effects of different concentrations of AMH, BMP-15 and FSH on basal estradiol and progesterone production from human luteinizing granulosa cells

| Treatment | | Estradiol | | Progesterone | |
|---|---|---|---|---|---|
| | | 24 h | 48 h | 24 h | 48 h |
| Control | | 643.6±49.8 | 1089.0±16.6 | 439.6±15.8 | 511.5±32.0 |
| FSH | *10 ng/ml* | 1157.1±156.9[c] | 2627.8±100.3[c] | 382.5±43.1 | 446.0±32.1 |
| | *100 ng/ml* | 1067.6±16.9[b] | 2896.4±90.3[c] | 342.6±35.6 | 408.7±30.1 |
| AMH | *2 ng/ml* | 590.0±25.8 | 978.7±31.4 | 29.7±6.8[c] | 110.0±23.6[c] |
| | *20 ng/ml* | 588.3±74.5 | 896.0±50.7 | 22.5±15.7[c] | 104.7±34.7[c] |
| | *100 ng/ml* | 473.3±84.1 | 885.4±87.5 | 21.7±22.9[c] | 100.0±32.6[c] |
| BMP-15 | *10 ng/ml* | 356.2±63.1[a] | 644.6±84.6[c] | 40.7±2.5[c] | 73.0±26.4[c] |
| | *100 ng/ml* | 320.5±75.9[a] | 602.4±75.3[c] | 35.5±4.3[c] | 65.9±22.4[c] |

Data are in mean (± SEM) from $n=5$ patients. a: $p<0.05$; b: $p<0.01$; c: $p<0.001$ statistical significant difference from control using one-way ANOVA followed by Bonferroni post-test analysis

estradiol secretion at both time points (Fig. 1A; Table 1) and similarly to AMH, attenuated the FSH-induced estradiol production (24 h: $p<0.001$, 48 h: $p<0.01$). Combined administration of AMH and BMP-15, had a negative impact on the FSH-induced estradiol production (24 h: $p<0.001$; 48 h: $p<0.001$; Fig. 1A) while, no effect of AMH plus BMP-15 on basal estradiol synthesis was revealed (Fig. 1A).

As far as progesterone release is concerned, AMH had a negative effect on basal progesterone production in both time points studied ($p<0.001$; Fig. 1B, Table 1) and this effect was partially reversed with the addition of FSH after 48 h of culture ($p<0.05$; Fig. 2). Similarly BMP-15 reduced basal secretion at 24 and 48 h of culture ($p<0.001$; Fig. 1B, Table 1) and the addition of FSH partially reversed this effect in 48 h of culture ($p<0.05$; Fig. 1B). In contrast, the combined administration of BMP-15 and AMH, significantly suppressed basal progesterone production ($p<0.001$; Fig. 1B) but this effect

could not be reversed with the addition of FSH in both time points (Fig. 1B).

## Pathways involved in AMH and BMP-15 effect on human GCs steroidogenesis

To investigate further the actions of AMH and BMP-15 on steroidogenesis we studied immunohistochemically the Smad5 protein activation and StAR protein expression. The results here indicate that AMH and BMP-15 increased Phospho $S^{463+465}$Smad5- immunoreactivity (IR) ($p<0.001$; Fig. 2) and the combined administration of these two factors had a similar effect ($p<0.001$; Fig. 2).

In regards to StAR protein expression, administration of AMH and BMP-15 separately, significantly attenuated the FSH-induced StAR-IR and interestingly the combined

Fig. 2 **a** Specific Phospho $S^{463+465}$ Smad5 protein expression by immunofluorescence in human luteinizing granulosa cells incubated for 48 h in serum free medium (Control) or in medium containing FSH (10 ng/ml), BMP-15 (10 ng/ml) and AMH (2 ng/ml). **b** Effects of FSH (10 ng/ml), AMH (2 ng/ml), BMP-15 (10 ng/ml) and their combinations on Smad5 phosphorylation in human luteinizing granulosa cells after 48 h of culture. Data are in mean (±SEM), $n=30$ patients. Statistical comparison using one-way ANOVA followed by Bonferroni post-test analysis: (**a**) $p<.001$ compared to control
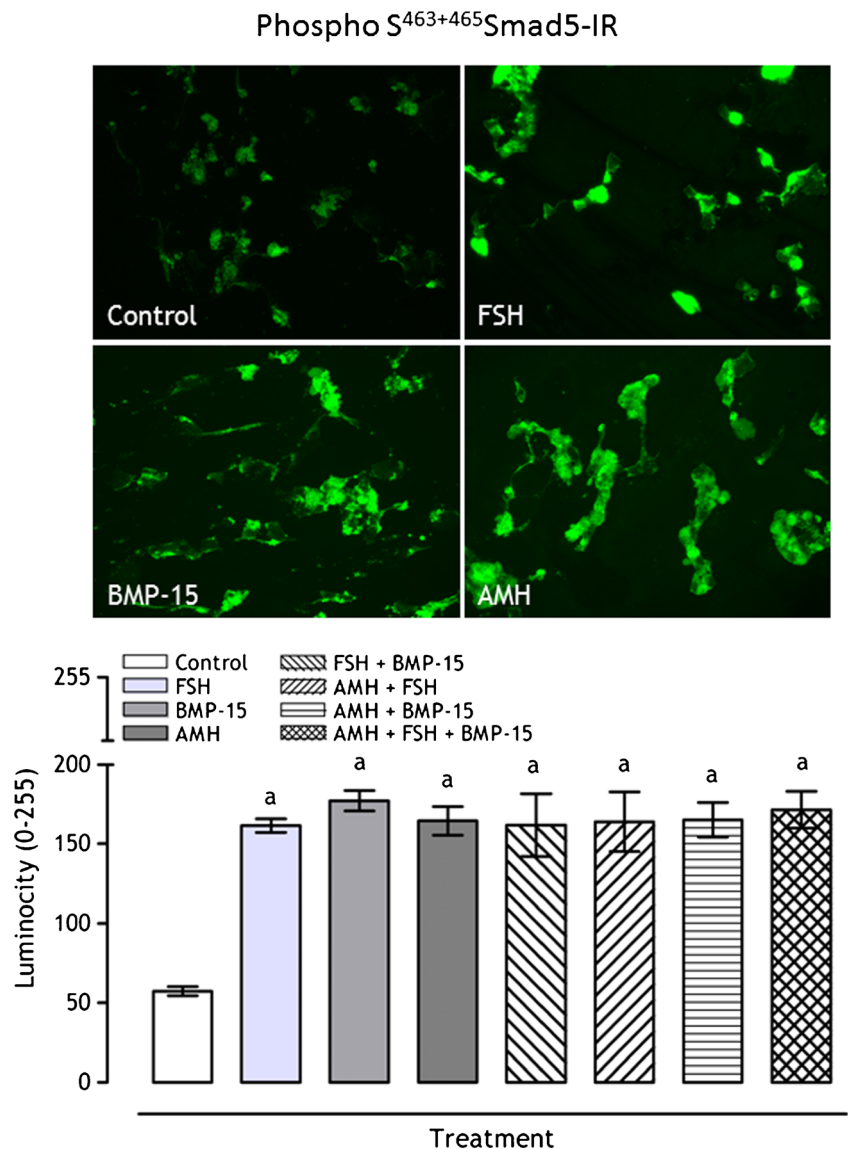
administration did not revealed any further attenuation on StAR protein expression as response to FSH ($p<.001$; Fig. 3).

## Discussion

There is compelling evidence suggesting that communication between the oocyte and the surrounding somatic cells of the follicle is crucial for a number of functions, which are important for oocyte developmental competence [1]. One of these functions, the steroidogenic activity of granulosa cells has recently been the focus of several studies.

In the present study we investigated the effect of AMH (a granulosa derived-factor), BMP-15 (an oocyte secreted-factor) and their interactions on basal and FSH-induced steroid hormones production from primary-cultured human luteinizing granulosa cells. The possible intracellular mechanisms involved in their actions were also investigated. We applied a rather short pre-incubation period of 24 h. According to previous data, this might not be sufficient to allow luteinizing granulosa cells to regain their basal functionality [48]. Nevertheless, the same methodology was applied in this study in the test groups and the controls, in agreement with a previous study from our laboratory [45, 49].
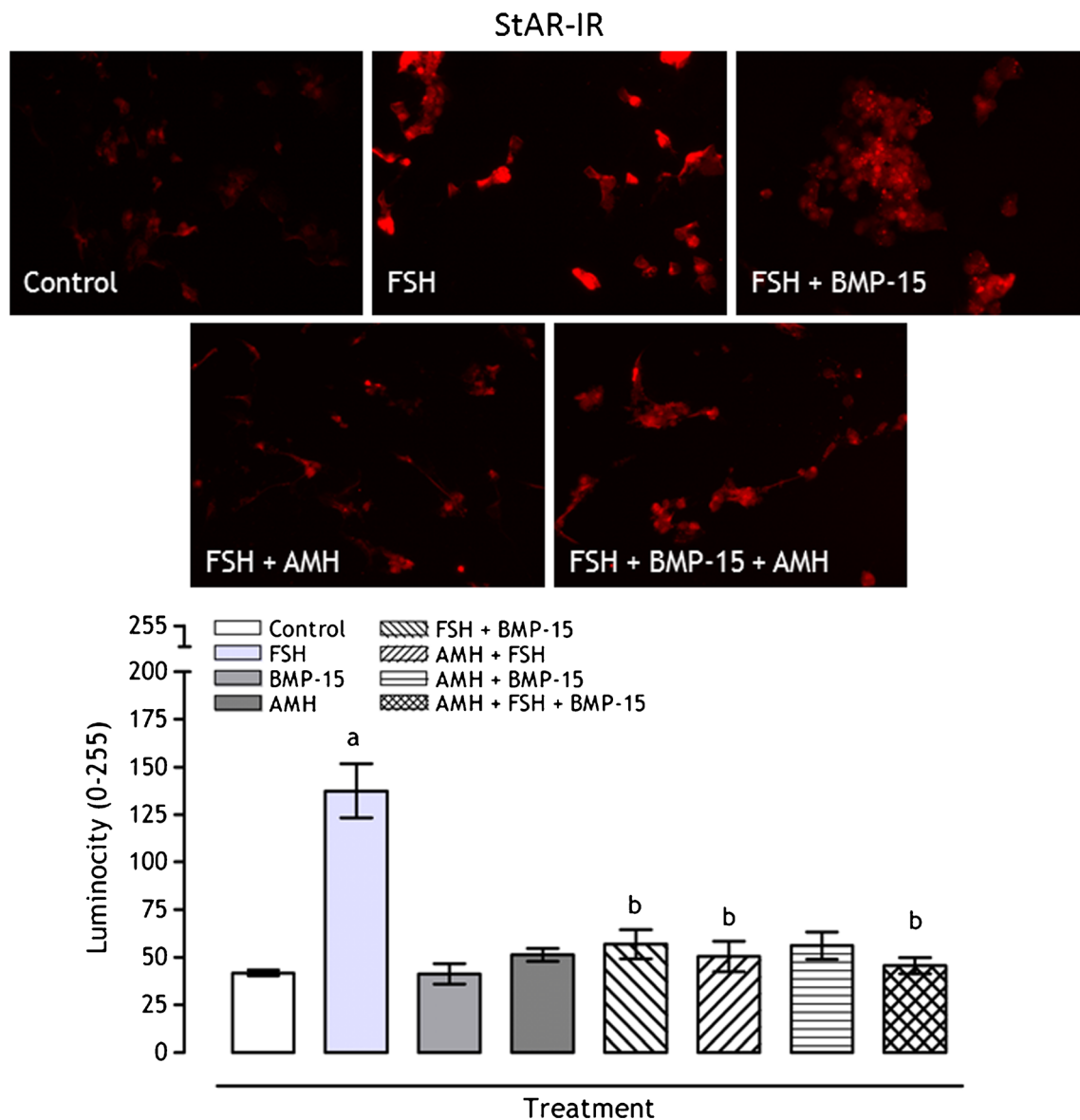


Fig. 3 **a** Specific StAR protein expression by immunofluorescence in human luteinizing granulosa cells incubated for 48 h in serum free medium (Control) or in medium containing FSH (10 ng/ml) alone or in combination with BMP-15 (10 ng/ml) and AMH (2 ng/ml.) **b** Effects of FSH, AMH, BMP-15 and their combinations on StAR protein levels in human luteinizing granulosa cells from 30 patients after 48 h of culture. Data are in mean (±SEM). (**a**) $P<.001$ compared to control, (**b**) $P<.001$ compared to FSH. Statistical comparison using one-way ANOVA followed by Bonferroni post-test analysis

According to our results, BMP-15 reduced by half basal estradiol release from hGCs while AMH had no effect on the basal production of this steroid hormone. On the other hand, both BMP-15 and AMH alone or in combination decreased FSH-induced estradiol release while their combined administration had no additive effect. To our knowledge, this is the first report on BMP-15 effect on basal and FSH-induced estradiol secretion from hGCs indicating a crucial role of this oocyte derived factor on steroidogenesis in humans. Regarding FSH-induced estradiol production, our results are in agreement with previous studies in rats, which showed that BMP-15 decreases the FSH-stimulated estradiol secretion possibly via reduction of the stimulatory effect of FSH on FSH-R synthesis [43, 50]. The fact that BMP-15 lowers estradiol production even in the absence of FSH in hGCs, indicates a different from FSH-R synthesis interaction, affecting possibly the synthesis of another factor in the steroidogenic process. Further studies in this direction are necessary in order to delineate the intracellular mechanisms involved between BMP-15 and estradiol secretion. Our observations on the autocrine role of AMH on basal and FSH-induced estradiol release are in agreement with previous reports where AMH decreased human granulosa cells sensitivity to FSH via reduction of the P450 aromatase activity [37, 38, 51] and FSH receptor mRNA expression [39].

As far as progesterone is concerned, basal secretion was reduced by AMH and BMP-15 and this effect was partially alleviated with the addition of FSH in culture. To our knowledge, this is the first report on AMH effect on progesterone release in the presence/absence of FSH from hGCs indicating that AMH is essential not only for estradiol but also for progesterone secretion regulation and strengthens further its importance in steroidogenesis. In terms of BMP-15, the observed attenuation of progesterone secretion in the presence/absence of FSH from primary human granulosa cells is in agreement with data from a tumor-derived human granulosa cell line KGN [30] and rat granulosa cells [43, 50] providing further evidence on its importance on progesterone secretion. Furthermore, when AMH and BMP-15 were administrated together, FSH had no effect and progesterone secretion was almost totally blocked. This finding indicates a possible interaction between these two factors in diminishing FSH action on progesterone production.

Though, the present data show a clear interaction between BMP-15 and AMH with FSH on progesterone secretion yet in our study at 24 and 48 h of culture an FSH-induced progesterone release was not observed. In an attempt to delineate this further, we studied the effect of FSH on StAR-IR in the presence/absence of BMP-15 and/or AMH at 48 h of culture where we noticed the highest interaction between FSH and these factors. Previous studies in rats and human have demonstrated that progesterone production in response to FSH activity is related to an increase in StAR mRNA [52] and

protein levels [53]. According to our results, BMP-15 and AMH had no effect on basal StAR-IR, FSH significantly increased StAR protein expression while BMP-15 and AMH alone or in combination, strongly attenuated this effect, which confirms an AMH and BMP-15 reduction of FSH-induced progesterone production. The discrepancy between the effect of FSH on progesterone release for 48 h and StAR-IR observed after 48 h of culture might be due to the origin of the cells used; that is, from patients that had been previously in vivo stimulated by hCG during IVF treatment. It is well known that hCG is an luteinizing hormone (LH) analog which shifts the metabolic profile of granulosa cells to high progesterone production level. Though based on data from our laboratory [45, 49] prolonged incubation periods lead to lowering of basal progesterone levels and a clear FSH-induced progesterone secretion, we have chosen to limit our study up to 48 h of culture due to the better viability of granulosa cells as well as to a clearly revealed attenuating effect of AMH and BMP-15 on progesterone secretion as early as 24 h of culture.

As mentioned above, both AMH and BMP-15 decreased the FSH-induced StAR-IR. To our knowledge this is the first report on AMH effect on StAR protein levels, while previous studies in rat and hen granulosa cells have also shown an inhibitory effect of BMP-15 on FSH-induced StAR mRNA [43] and protein expression [44].

Based on our observations, administration of AMH and BMP-15 seems to alternate the steroidogenic profile of human luteinizing granulosa cells in vitro, as a reduction in estradiol and progesterone production was observed. In order to add evidence that the actions mentioned above were due to the effect of AMH and BMP-15, by binding to their cellular receptor and activating downstream their signaling pathway, we also studied the intracellular pathway mechanisms involved. Both AMH and BMP-15 are members of the TGF-β superfamily exerting their actions through Smads protein activation [2]. TGF-β pathway activation was confirmed through Smad5-IR studies, a common target for both factors. Indeed, both AMH and BMP-15 demonstrated a 3-fold augmentation of, indicating a downstream activation of the serine/threonine receptor pathway. This evidence comes in agreement with previously published data from rodents [7, 11, 12] and the tumor-derived human granulosa cell line KGN [54], where it has been shown that AMH activates Smad5 phosphorylation by binding to the AMHRII/-ALK2/3/6 complex, as well as, with studies in COV434 ovarian granulosa tumor cells were it has been demonstrated that BMP-15 facilitates its own effect through binding to the BMPRIB/ALK3 receptor complex and subsequently activating the Smad1/5/8 phosphorylation [55]. The fact that Smad5 is a common target for both AMH and BMP-15 in our system can explain why combined administration of these factors did not lead to an additive effect on steroids production.

Finally, we found that FSH augmented PhosphoS$^{463+}$$^{465}$Smad5-IR in our system. This is in line with a previous study in human ovarian granulosa-like tumor cell line KGN [56] where it was found that FSH administration increased Smad5 mRNA. Further studies are needed in order to delineate the importance of Smad5 protein augmentation and activation in response to FSH of granulosa cells in steroidogenesis.

## Conclusions

The results of the present study provide evidence that two members of the TGF-$\beta$ superfamily; AMH (a granulosa-derived factor) and BMP-15 (an oocyte-secreted factor) play both an important role in granulosa cell steroidogenesis by affecting the actions of FSH through Smad5 protein activation and StAR protein expression attenuation. This is the first report concerning AMH effects on basal and FSH-induced progesterone production from granulosa cells, as well as the synergistic action of AMH and BMP-15 on FSH-induced progesterone production. It is important to note that our data were obtained in vitro from short-term luteinizing granulosa cell cultures, processed only for a 24 h preincubation period due to better viability of the cells, and therefore may not necessarily apply to the physiology of folliculogenesis in vivo. Further studies on the communication between FSH and TGF-$\beta$ signaling are essential for the elucidation of the steroidogenic activity of granulosa cells.

**Ethical statement** • The manuscript has not been submitted to more than one journal for simultaneous consideration.

• The manuscript has not been published previously (partly or in full), unless the new work concerns an expansion of previous work (please provide transparency on the re-use of material to avoid the hint of text-recycling ("self-plagiarism")).

• A single study is not split up into several parts to increase the quantity of submissions and submitted to various journals or to one journal over time (e.g., "salami-publishing").

• No data have been fabricated or manipulated (including images) to support your conclusions.

• No data, text, or theories by others are presented as if they were the author's own ("plagiarism"). Proper acknowledgements to other works must be given (this includes material that is closely copied (near verbatim), summarized and/or paraphrased), quotation marks are used for verbatim copying of material, and permissions are secured for material that is copyrighted.

**Important note**: the journal may use software to screen for plagiarism.

• Consent to submit has been received explicitly from all co-authors, as well as from the responsible authorities - tacitly or explicitly - at the institute/organization where the work has been carried out, **before** the work is submitted.

• Authors whose names appear on the submission have contributed sufficiently to the scientific work and therefore share collective responsibility and accountability for the results.

## References

1. Knight PG, Glister C. Local roles of TGF-beta superfamily members in the control of ovarian follicle development. Anim Reprod Sci. 2003;78(3–4):165–83. **Review**.
2. Knight PG, Glister C. TGF-beta superfamily members and ovarian follicle development. Reproduction. 2006;132(2):191–206. **Review**.
3. Messinis IE, Messini CI, Dafopoulos K. The role of gonadotropins in the follicular phase. Ann N Y Acad Sci. 2010;1205:5–11. **Review**.
4. Juengel JL, McNatty KP. The role of proteins of the transforming growth factor-beta superfamily in the intraovarian regulation of follicular development. Hum Reprod Update. 2005;11(2):143–60.
5. Lutz M, Knaus P. Integration of the TGF-beta pathway into the cellular signalling network. Cell Signal. 2002;14(12):977–88. **Review**.
6. La Marca A, Broekmans FJ, Volpe A, Fauser BC, Macklon NS. ESHRE special interest group for reproductive endocrinology–AMH round table. Anti-mullerian hormone (AMH): what do we still need to know? Hum Reprod. 2009;24(9):2264–75.
7. Visser JA, Themmen AP. Anti-Müllerian hormone and folliculogenesis. Mol Cell Endocrinol. 2005;234(1–2):81–6. **Review**.
8. Durlinger AL, Gruijters MJ, Kramer P, Karels B, Ingraham HA, Nachtigal MW, et al. Anti-Müllerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. Endocrinology. 2002;143(3):1076–84.
9. Themmen AP. Anti-Müllerian hormone: its role in follicular growth initiation and survival and as an ovarian reserve marker. J Natl Cancer Inst Monogr. 2005;34:18–21. **Review**.
10. Salmon NA, Handyside AH, Joyce IM. Oocyte regulation of anti-Müllerian hormone expression in granulosa cells during ovarian follicle development in mice. Dev Biol. 2004;266(1):201–8.
11. Visser JA. AMH signaling: from receptor to target gene. Mol Cell Endocrinol. 2003;211(1–2):65–73. **Review**.
12. Mazerbourg S, Hsueh AJ. Genomic analyses facilitate identification of receptors and signalling pathways for growth differentiation factor 9 and related orphan bone morphogenetic protein/growth differentiation factor ligands. Hum Reprod Update. 2006;12(4):373–83.
13. Durlinger AL, Gruijters MJ, Kramer P, Karels B, Kumar TR, Matzuk MM, et al. Anti-Müllerian hormone attenuates the effects of FSH on follicle development in the mouse ovary. Endocrinology. 2001;142(11):4891–9.
14. McGee EA, Hsueh AJ. Initial and cyclic recruitment of ovarian follicles. Endocr Rev. 2000;21(2):200–14. **Review**.
15. Kallio S, Aittomäki K, Piltonen T, Veijola R, Liakka A, Vaskivuo TE, et al. Anti-Mullerian hormone as a predictor of follicular reserve in ovarian insufficiency: special emphasis on FSH-resistant ovaries. Hum Reprod. 2012;27(3):854–60.
16. de Vet A, Laven JS, de Jong FH, Themmen AP, Fauser BC. Antimüllerian hormone serum levels: a putative marker for ovarian aging. Fertil Steril. 2002;77(2):357–62.
17. Visser JA, de Jong FH, Laven JS, Themmen AP. Anti-Müllerian hormone: a new marker for ovarian function. Reproduction. 2006;131(1):1–9. **Review**.
18. Arabzadeh S, Hossein G, Rashidi BH, Hosseini MA, Zeraati H. Comparing serum basal and follicular fluid levels of anti-Müllerian hormone as a predictor of in vitro fertilization outcomes in patients with and without polycystic ovary syndrome. Ann Saudi Med. 2010;30(6):442–7.
19. Dumesic DA, Lesnick TG, Stassart JP, Ball GD, Wong A, Abbott DH. Intrafollicular antimüllerian hormone levels predict follicle responsiveness to follicle-stimulating hormone (FSH) in normoandrogenic ovulatory women undergoing gonadotropin

releasing-hormone analog/recombinant human FSH therapy for in vitro fertilization and embryo transfer. Fertil Steril. 2009;92(1): 217–21.

20. Silberstein T, MacLaughlin DT, Shai I, Trimarchi JR, Lambert-Messerlian G, Seifer DB, et al. Mullerian inhibiting substance levels at the time of HCG administration in IVF cycles predict both ovarian reserve and embryo morphology. Hum Reprod. 2006;21(1):159–63.

21. Cook CL, Siow Y, Brenner AG, Fallat ME. Relationship between serum müllerian-inhibiting substance and other reproductive hormones in untreated women with polycystic ovary syndrome and normal women. Fertil Steril. 2002;77(1):141–6.

22. Laven JS, Mulders AG, Visser JA, Themmen AP, De Jong FH, Fauser BC. Anti-Müllerian hormone serum concentrations in normoovulatory and anovulatory women of reproductive age. J Clin Endocrinol Metab. 2004;89(1):318–23.

23. Pellatt L, Hanna L, Brincat M, Galea R, Brain H, Whitehead S, et al. Granulosa cell production of anti-Müllerian hormone is increased in polycystic ovaries. J Clin Endocrinol Metab. 2007;92(1):240–5.

24. Pigny P, Merlen E, Robert Y, Cortet-Rudelli C, Decanter C, Jonard S, et al. Elevated serum level of anti-mullerian hormone in patients with polycystic ovary syndrome: relationship to the ovarian follicle excess and to the follicular arrest. J Clin Endocrinol Metab. 2003;88(12):5957–62.

25. Wachs DS, Coffler MS, Malcom PJ, Chang RJ. Serum anti-mullerian hormone concentrations are not altered by acute administration of follicle stimulating hormone in polycystic ovary syndrome and normal women. J Clin Endocrinol Metab. 2007;92(5): 1871–4.

26. Di Pasquale E, Beck-Peccoz P, Persani L. Hypergonadotropic ovarian failure associated with an inherited mutation of human bone morphogenetic protein-15 (BMP15) gene. Am J Hum Genet. 2004;75(1):106–11.

27. Elvin JA, Yan C, Matzuk MM. Oocyte-expressed TGF-beta superfamily members in female fertility. Mol Cell Endocrinol. 2000;159(1–2):1–5. **Review**.

28. Dube JL, Wang P, Elvin J, Lyons KM, Celeste AJ, Matzuk MM. The bone morphogenetic protein 15 gene is X-linked and expressed in oocytes. Mol Endocrinol. 1998;12(12):1809–17.

29. Aaltonen J, Laitinen MP, Vuojolainen K, Jaatinen R, Horelli-Kuitunen N, Seppä L, et al. Human growth differentiation factor 9 (GDF-9) and its novel homolog GDF-9B are expressed in oocytes during early folliculogenesis. J Clin Endocrinol Metab. 1999;84(8): 2744–50.

30. Chang HM, Cheng JC, Klausen C, Leung PC. BMP15 suppresses progesterone production by down-regulating StAR via ALK3 in human granulosa cells. Mol Endocrinol. 2013;27(12):2093–104.

31. Galloway SM, Gregan SM, Wilson T, McNatty KP, Juengel JL, Ritvos O, et al. Bmp15 mutations and ovarian function. Mol Cell Endocrinol. 2002;191(1):15–8. **Review**.

32. Hanrahan JP, Gregan SM, Mulsant P, Mullen M, Davis GH, Powell R, et al. Mutations in the genes for oocyte-derived growth factors GDF9 and BMP15 are associated with both increased ovulation rate and sterility in Cambridge and Belclare sheep (Ovis aries). Biol Reprod. 2004;70(4):900–9.

33. Otsuka F, McTavish KJ, Shimasaki S. Integral role of GDF-9 and BMP-15 in ovarian function. Mol Reprod Dev. 2011;78(1):9–21.

34. Liu J, Wang B, Wei Z, Zhou P, Zu Y, Zhou S, et al. Mutational analysis of human bone morphogenetic protein 15 in Chinese women with polycystic ovary syndrome. Metabolism. 2011;60(11): 1511–4.

35. Tiotiu D, Alvaro Mercadal B, Imbert R, Verbist J, Demeestere I, De Leener A, et al. Variants of the BMP15 gene in a cohort of patients with premature ovarian failure. Hum Reprod. 2010;25(6):1581–7.

36. Wu YT, Tang L, Cai J, Lu XE, Xu J, Zhu XM, et al. High bone morphogenetic protein-15 level in follicular fluid is associated with high quality oocyte and subsequent embryonic development. Hum Reprod. 2007;22(6):1526–31.

37. Li L, Mo YQ, Chen XL, Li Y, Chen YX, Zhong JM, et al. Effect of anti-Müllerian hormone on P450 aromatase mRNA expression in cultured human luteinized granulose cells. Zhonghua Fu Chan Ke Za Zhi. 2009;44(3):191–5.

38. Grossman MP, Nakajima ST, Fallat ME, Siow Y. Müllerian-inhibiting substance inhibits cytochrome P450 aromatase activity in human granulosa lutein cell culture. Fertil Steril. 2008;89(5 Suppl):1364–70.

39. Pellatt L, Rice S, Dilaver N, Heshri A, Galea R, Brincat M, et al. Anti-Müllerian hormone reduces follicle sensitivity to follicle-stimulating hormone in human granulosa cells. Fertil Steril. 2011;96(5):1246–51.e1.

40. Lyet L, Louis F, Forest MG, Josso N, Behringer RR, Vigier B. Ontogeny of reproductive abnormalities induced by deregulation of anti-müllerian hormone expression in transgenic mice. Biol Reprod. 1995;52(2):444–54.

41. di Clemente N, Ghaffari S, Pepinsky RB, Pieau C, Josso N, Cate RL, et al. A quantitative and interspecific test for biological activity of anti-müllerian hormone: the fetal ovary aromatase assay. Development. 1992;114(3):721–7.

42. Kevenaar ME, Themmen AP, Laven JS, Sonntag B, Fong SL, Uiterlinden AG, et al. Anti-Müllerian hormone and anti-Müllerian hormone type II receptor polymorphisms are associated with follicular phase estradiol levels in normo-ovulatory women. Hum Reprod. 2007;22(6):1547–54.

43. Otsuka F, Yamamoto S, Erickson GF, Shimasaki S. Bone morphogenetic protein-15 inhibits follicle-stimulating hormone (FSH) action by suppressing FSH receptor expression. J Biol Chem. 2001;276(14):11387–92.

44. Elis S, Dupont J, Couty I, Persani L, Govoroun M, Blesbois E, et al. Expression and biological effects of bone morphogenetic protein-15 in the hen ovary. J Endocrinol. 2007;194(3):485–97.

45. Karamouti M, Kollia P, Kallitsaris A, Vamvakopoulos N, Kollios G, Messinis IE. Growth hormone, insulin-like growth factor I, and leptin interaction in human cultured lutein granulosa cells steroidogenesis. Fertil Steril. 2008;90(4 Suppl):1444–50.

46. Földesi I, Breckwoldt M, Neulen J. Oestradiol production by luteinized human granulosa cells: evidence of the stimulatory action of recombinant human follicle stimulating hormone. Hum Reprod. 1998;13(6):1455–60.

47. Lambert A, Harris SD, Knaggs P, Robertson WR. Improved FSH sensitisation and aromatase assay in human granulosa-lutein cells. Mol Hum Reprod. 2000;6(8):677–80.

48. Breckwoldt M, Selvaraj N, Aharoni D, Barash A, Segal I, Insler V, et al. Expression of Ad4-BP/cytochrome P450 side chain cleavage enzyme and induction of cell death in long-term cultures of human granulosa cells. Mol Hum Reprod. 1996;2(6):391–400.

49. Karamouti M, Kollia P, Kallitsaris A, Vamvakopoulos N, Kollios G, Messinis IE. Modulating effect of leptin on basal and follicle stimulating hormone stimulated steroidogenesis in cultured human lutein granulosa cells. J Endocrinol Investig. 2009;32(5):415–9.

50. Otsuka F, Yao Z, Lee T, Yamamoto S, Erickson GF, Shimasaki S. Bone morphogenetic protein-15. Identification of target cells and biological functions. J Biol Chem. 2000;275(50):39523–8.

51. Chang HM, Klausen C, Leung PC. Antimüllerian hormone inhibits follicle-stimulating hormone-induced adenylyl cyclase activation, aromatase expression, and estradiol production in human granulosa-lutein cells. Fertil Steril. 2013;100(2):585–92.e1.

52. Minegishi T, Tsuchiya M, Hirakawa T, Abe K, Inoue K, Mizutani T, et al. Expression of steroidogenic acute regulatory protein (StAR) in rat granulosa cells. Life Sci. 2000;67(9):1015–24.

53. Tajima K, Hosokawa K, Yoshida Y, Dantes A, Sasson R, Kotsuji F, et al. Establishment of FSH-responsive cell lines by transfection of

pre-ovulatory human granulosa cells with mutated p53 (p53val135) and Ha-ras genes. Mol Hum Reprod. 2002;8(1):48–57.

54. Anttonen M, Färkkilä A, Tauriala H, Kauppinen M, Maclaughlin DT, Unkila-Kallio L, et al. Anti-Müllerian hormone inhibits growth of AMH type II receptor-positive human ovarian granulosa cell tumor cells by activating apoptosis. Lab Investig. 2011;91(11):1605–14.

55. Pulkki MM, Mottershead DG, Pasternack AH, Muggalla P, Ludlow H, van Dinther M, et al. A covalently dimerized recombinant human bone morphogenetic protein-15 variant identifies bone morphogenetic protein receptor type 1B as a key cell surface receptor on ovarian granulosa cells. Endocrinology. 2012;153(3):1509–18.

56. Miyoshi T, Otsuka F, Suzuki J, Takeda M, Inagaki K, Kano Y, et al. Mutual regulation of follicle-stimulating hormone signaling and bone morphogenetic protein system in human granulosa cells. Biol Reprod. 2006;74(6):1073–82.