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Attenuation of Bone Morphogenetic Proteins (BMPs) Signaling Induces Granulosa Cell Sensitivity to Gonadotropins in Female Mouse

Sarmed Al-Samerria[1*], Suresh K. A. Palanisamy[2], Ibtisam Al-Ali[2], Cyril D. Mamotte[3], James R. McFarlane[2] and Ghanim Almahbobi[3]

[1]Laboratory of Human Growth and Reproductive Development, Department of Pediatrics, Robert Wood Johnson Medical School, Rutgers, The State University of New Jersey, New Brunswick 08901, NJ, USA; sa1595@rwjms.rutgers.edu
[2]School of Science and Technology, University of New England, Armidale 2351, NSW, Australia
[3]Curtin Medical School, Curtin Health Innovation Research Institute (CHIRI), Curtin University, Bentley 6012, WA, Australia

Abstract

Several lines of evidence support the notion that Bone Morphogenetic Proteins (BMPs) and gonadotropic hormones are major regulators of ovarian follicle development by inducing gonadotropin-mediated steroid hormone production and regulating ovarian follicle cell proliferation. Research from our laboratory and elsewhere have demonstrated that BMPs play important roles during the early stages of folliculogenesis. Despite the extensive research to reveal the mechanism of interaction between BMPs and gonadotropic hormones in the process of folliculogenesis, the exact mechanism of such an interaction is not adequately understood. Previously, we developed a unique mouse model characterized by a short-term attenuation of the BMP signaling system using passive immunization against bone morphogenetic protein 4 (BMP-4) and bone morphogenetic protein receptor 1B (BMPR1B). This model unequivocally demonstrated that the attenuation of BMP action was an effective method of enhancing fertility reserve and promoting follicle and ovulation rates in female mice. In addition, this study pointed to the possibility of bi-directional mutual regulation between BMPs, Follicle-Stimulating Hormone (FSH), and Luteinizing Hormone (LH). To gain further insight into this mechanism we used this mouse model to examine the protein expression and mRNA level of BMPR1B, Follicle-Stimulating Hormone Receptor (FSHR) and Luteinizing Hormone/Choriogonadotropin Receptor (LHCGR) in multiple stages of follicle development in female mice. Immunofluorescent analysis of female mice treated with anti-BMPR1B antibodies showed a significant upregulation of BMPR1B, FSHR, and LHCGR in the ovarian granulosa cells during the main stages of follicle development, whereas treatment with anti-BMP4 antibodies showed no effect. In addition, attenuation of BMPR1B resulted in upregulation of the FSHR (exclusively expressed in the granulosa cell) and LHCGR mRNA levels in the ovary. The present study implies that BMPs engage indirectly in regulating the later stages of folliculogenesis, in addition to their direct role in the regulation of the early stages of follicle development, by enhancing granulosa cell sensitivity to gonadotropins through upregulating the receptor expression. We propose that our mouse model is siutable to elucidate the mechanism of interaction between BMPs and gonadotropins in folliculogenesis.

Keywords: BMP-4, BMPR1B, FSHR, Gene Expression, Immunization, Immunofluorescence, LHCGR, Protein Expression,

**Author for correspondence*

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1. Introduction

Folliculogenesis is the main process associated with female fertility leading to the development of mature oocytes. The folliculogenesis process comprises a series of chronological and well-regulated steps beginning in follicle cells formed during the early stages of embryonic development and ending in ovulation and corpus luteum formation[1]. During fetal development, the Primordial Germ Cells (PGCs) are formed and migrate to the gonads to form a germ cells nest, which represents the fertility reserve for the entire female reproductive life[2,3]. The primordial follicle, the basic functional unit of the ovary, comprises an oocyte, arrested in the first prophase of meiosis, enclosed by an incomplete flattened pre-granulosa cell[2]. A limited number of primordial follicles reach the stage of ovulation, while a vast majority of the follicles die of atresia[4]. As such, the number and quality of these follicles influence fertility and reproduction in females. Previous studies using various experimental models demonstrated that gonadotropins, namely FSH and LH, interact with other intraovarian factors, including bone morphogenetic proteins (BMPs) signaling to regulate the normal ovarian functions[5–8].

In more detail, this kind of interaction is either stimulatory, such as the role of BMP-6 in increasing the expression of FSHR in human granulosa cells *in vitro*[9], or inhibitory, such as the role of BMP-2, BMP-4, and BMP-15 on FSHR modulation of granulosa cells in hens[10], ewes[11], and rats[12]. Several reports from our laboratory and others have demonstrated that the BMPs signaling system is involved in most events of folliculogenesis such as primordial follicle formation[13–16], primordial follicles recruitment[17,18], gonadotropin-mediated steroidogenesis[8,19,20], cyclic recruitment of mature follicles[1,21], ovulation[7,22], and follicular atresia[19,23,24]. Unfortunately, these studies have been conducted separately in many different experimental setups such as using different animal species, *in vivo* and *in vitro*, in one or more specific stages of folliculogenesis, and using different methodological approaches. Such diverse approaches make it difficult to arrive at a comprehensive understanding of the outcomes. Therefore, a comprehensive study demonstrating the mechanism(s) of such reciprocal regulatory interactions between BMPs signaling and gonadotropin actions in the regulation of follicle development *in vivo* is required.

We believe that there are two main reasons for this lack of understanding: 1) Most of the reported studies were conducted *in vitro*, partly because of the complex nature and physiology of ovarian folliculogenesis. 2) To overcome this difficulty and facilitate *in vivo* studies, several purpose-made transgenic knockout animal models became useful. However, the majority of these animal models with the potential to decipher the mechanism of interactions between BMPs and gonadotropins are associated with major disruption in the BMPs and/or gonadotropins' natural system, adding further limitations to the ability to elucidate the actual mechanism(s) of interaction.

For example, Abel *et al.*[25] have generated a transgenic mouse model characterized by a total body knockout of the Follicle-Stimulating Hormone Receptor (FSHRKO). The FSHRKO female mice were infertile, and the ovaries were significantly small than in their littermate's control. In addition, the inactivation of FSHR resulted in the arrest of folliculogenesis at the preantral stage, lack of response to exogenous Pregnant Mare's Serum Gonadotropin (PMSG), and significantly higher serum FSH in the mutant mice[25].

BMP receptor-1B (BMPR1B) is the main receptor utilized by many members of BMPs including BMP-2, BMP-4, BMP-7, GDF9, and BMP-15. Among them, BMP-4 was reported to play a role in the formation of PGCs[19,26], recruitment of primordial to primary follicles in *Booroola* sheep, an animal strain characterized by a natural point mutation in the BMPR-1B[27], and rats[14]. BMP-4 is particularly involved in the regulation of estrogen production, inhibition of progesterone biosynthesis, and ovulation[13,14,19]. Although it is well known that the above-mentioned processes are gonadotropins-dependent, the exact mechanism by which BMP-4 directly or indirectly exerts its regulatory action in these processes is not well elucidated. Recently, we have developed a mouse model characterized by a short-term attenuation (not knockout) of the BMPs signaling system using a passive immunization technique against the BMPR-1B and BMP-4[7,28,29]. This model has been found suitable to study the role of BMPs in the formation and maintenance of the primordial follicle reserve, controlling the unnecessary transition of the primordial follicles to developing primary follicles, and promoting the cyclic follicle development and ovulation[7]. In addition, this model demonstrated that the early stage of follicle development is mainly driven by the action of the BMPs signaling system[7]. In the present study, we aimed to investigate the reciprocal interaction between the BMPs signaling system and the action of gonadotropic hormones in the

three key limiting stages of folliculogenesis using the *in-vivo* mouse model and, more specifically, to decipher the effects of attenuation of BMPR1B signaling on the dynamic process of gonadotropin receptors (FSHR and LHCGR) expression in three stages of folliculogenesis using our mouse model[7,28,29].

2. Materials and Methods

2.1 Animal Care and Passive Immunization

The *in vivo* experiments were carried out in the Animal House Facility of the University of New England, NSW, Australia, in accordance with the Australian code of practice for the care and use of animals for scientific purposes, and approved by the University of New England Animal Ethics Committee. To study the effect of the BMPs signaling system on the process of follicle development, pre-pubertal (three weeks old) female Swiss mice were devided into six groups (n=10-12 for each group). Daily subcutaneous injections (100 µL) of the following treatments were administrated for 7 days as follows: the first group received a dose of 50 mg in 100 µL of anti-BMP4; the second group received 50 mg in 100 µL anti-BMPRIB; the third group received 1 IU in 100 µL equine chorionic gonadotropin (eCG; Bioniche Animal Health, Armidale, NSW, Australia); the fourth group received 1 IU of eCG with anti-BMP4 in 100 µL; the fifth group received 1 IU eCGC with anti-BMPRIB; and the sixth group received 50 mg purified non-immune chicken IgY in 100 µL, as the control group. Mice were euthanized by CO_2 inhalation and decapitated after being anesthetized 24 hours after the last injection. From each mouse, one ovary was collected and snap-frozen in liquid nitrogen and stored at -80°C for 2 weeks before being cryosectioned for immunofluorescent staining. The other ovary was stored in RNAlater® solution (Ambion, TX, USA) overnight at 4°C and processed for RNA extraction.

2.2 Antibody Preparation

The antibodies of BMP4 (JMCK#54) and BMPRIB (JMCK#59) were raised and characterized in our laboratory as described in detail in our previous publication. In brief, the antibodies were raised in chicken against synthetic peptides (Invitrogen Australia) equivalent to amino acids 88–102 (ISMLYLDEYDKVVLK) of mouse BMP4 and amino acids 103–117 of mouse BMPRIB (NKDLHPTLPPLKDRD)[7,28,29]. The synthetic peptides were conjugated to diphtheria toxoid (CSL) and emulsified in Freund's complete adjuvant (Sigma–Aldrich Pty Ltd) for the primary vaccination and in Freund's incomplete adjuvant for booster injections. The primary vaccination contained 50 mg of peptide, while all boosters contained 25 mg of the peptide. The antibodies were purified from egg yolk using a combination of ammonium sulfate and octanoic acid as described[30]. The cross-reactivity of BMP2 and BMP7 was <0.5% for the anti-BMP4 antibody and the specificity of the BMPRIB antibody was determined by the competitive binding ELISA test against random peptides[7,29]. The DELTA-BLAST analysis showed that the sequence was specific to mouse BMPRIB with no potential binding to other proteins. These antibodies were used to passively vaccinate mice using 50 mg of purified antibody in 100 mL of saline injected subcutaneously. The dose of antibody was similar to that we have used previously[28] and was determined by pilot studies.

2.3 Immunofluorescent (IF) Localization of BMPR1B, FSHR, and LH-CGR Protein Receptors

This procedure has been described in full detail in our previously published articles[7,31]. In brief, the entire ovaries were placed in a PCR tube and snape-frozen for 20 seconds in liquid nitrogen (N_2), then stored at -80°C for two weeks before sectioning for IF staining. After that, the whole frozen ovary was partially embedded in an optimal cutting temperature compound (OCT) and cut into 10 µm sections using a cryostat (Carl Zeiss, Sydney, NSW, Australia). Sections were mounted on Super-frost slides (HD scientific supplies Pty Ltd., Australia) and processed for immunofluorescent labeling as previously described[31]. Briefly, tissue sections were fixed in 4% paraformaldehyde at 4°C for 7 min. For the negative control, tissue sections were incubated with rabbit non-immune serum (Life Technologies VIC, Australia) instead of primary antibodies. Polyclonal goat anti-BMPR1B (sc-5679), anti-FSHR (sc-7798), and anti-LH-CGR (sc-293165) (Santa Cruz Biotechnology) were applied at 4 µg/mL overnight at 4°C in a humidified chamber. The antibodies' specificity was validated in our previous study[32]. A polyclonal rabbit anti-goat IgG second antibody conjugated to Alexa 488 fluorochrome (A-11078, Life Technology) was applied to all slides at 4 µg/mL for 45 min at room temperature. The slides were washed and mounted using Prolonged Diamond anti-fade mounting media with DAPI (P-36962,

Life Technologies, VIT, Australia). Sections were analyzed using an inverted confocal microscope (AR1+/A1+; Nikon Corporation, Tokyo, Japan). Images were captured using A1 software (Nikon Corporation, Tokyo, Japan).

2.4 mRNA Extraction and RT-PCR

The RNA was extracted using Trisol reagent according to manufacturer's instruction and as previously reported[32]. RNA quality and integrity were verified using 1% RNA agarose gel (CSBC 2011) and measured and quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE). Reverse transcription was performed by annealing 2 µg of total RNA with 20 oligos (dT)15Primer (Fisher BioTec, Subiaco WA) at 70 °C for 15 min and immediately transferred to ice.

Gene-specific primers (Table 1) were designed using NCBI Primer-Blast tools as described[33]. Quantitative RT-PCR reactions were conducted in triplicate using 16 ng complementary DNA (cDNA), in Fast Evagreen® qPCRMaster Mix (Biotium, Hayward, CA) using a CAS 1200 automated PCR setup robot (Corbett Robotics, Eight Mile Plains, QLD) and qPCR carried out using a Rotor-Gene R6 6000 Real-Time Analyzer (Corbett Life Science, Concord, NSW). Relative differences in gene expression were calculated using the formula: fold change = 2^ (control ΔCt – sample ΔCt).

2.5 Quantification of Receptor Expressions in 3D Immunofluorescent Images

The principle of this methodology lies in measuring and quantifying the background-corrected immunofluorescent signaling corresponding to the cell receptor protein at the Region Of Interest (ROI) at random locations, and normalizing that measurement against the same receptor protein that does not change under the chosen experimental condition (wild type or control animal). Previously, we used this approach in combination with computer-based 3D image analysis of the immunofluorescent signal to study the dynamic interaction between BMPs and the gonadotropins in a different stage of follicle development in sheep[32]. Briefly, the exposure time was set by omitting the primary antibody and replaced by rabbit non-immune serum to avoid autofluorescent and non-specific antibody binding. The fluorescent signals observed after using the first antibody were taken as the base to further subtract the signals generated by autofluorescence and non-specific binding. A surplus of the fluorescent signal observed after subtraction was considered a positive signal and used in the quantification analyses. In Graafian follicles, the quantification of the protein expression of the three receptors was performed on granulosa cells by placing three random ($9x10^3$) μm^3 sections in three different locations of the follicle, and the average of the quantified intensities was used in the calculation (Figure 1A). The same principle was applied to the secondary follicles with ($1x10^3$) μm^3 sections (Figure 1B) and primary follicles with 250 μm^3 in two locations of the follicles (Figure 1C). Three-D image analysis was performed using Velocity 3D Image Analysis Software, version 6.2 (Perkin Elmer; Waltham, MA, USA). All the analyses were performed on the signals generated from granulosa cells only. The selection of the follicle stages was done according to the classification scheme already published[34].

2.6 Data Analysis

Receptor protein expression intensities were assessed in multiple stages of folliculogenesis (primary, secondary and Graafian follicles), between the treated and untreated animals and were analyzed using two-way ANOVA followed by a Bonferroni post hoc test for multiple comparisons. The data are presented using Prism version

Table 1. List of gene primers used for RT-PCR

Gene	NCBI references #	Forward primer	Reverse primer	Amplicon length (bp)
β-actin	NM_007393.3	CGTCGACAACGGCTCCGGCATG	TGGGCCTCGTCACCCACATAG	150
Bmpr1b	NM_007560.3	TCAATGTCGTGACACTCCCATTCCT	TGCTGTACCGAGGTCGGGCT	245
Bmp4	NM_007554.2	*GACTACTGGACACCAGACTAGTCC*	*CTTCCCGGTCTCAGGTATCA*	*180*
Fshr	*NM_01353*	*GGAGCCTCTGGGCCAGTCGT*	*TGGAGGCGGCAAACCTCTGAA*	*165*
Lhcgr	*L36329*	*GGACGACGCTAATCTCGCTGGAG*	*GGTTGCCACCTGACCTACCC*	*82*

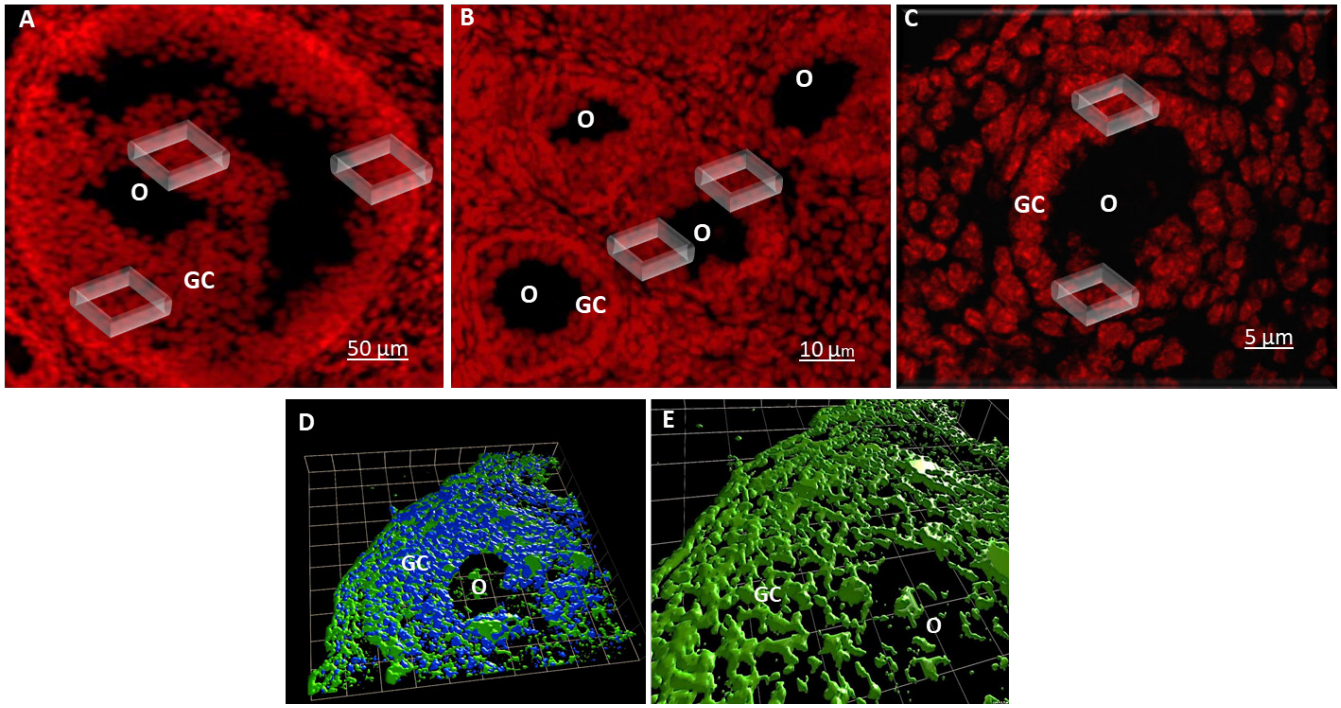

Figure 1. Schematic representation of three stages of follicles. **A.** Graffian follicle's granulosa cells stained with DAPI fluorescent nucleus photoconverted to red; **B.** secondary follicle; **C.** primary follicle; **D.** a 3D image of Graafian follicles generated by compiling 10 µm Z-stack images demonstrating the granulosa cell nucleus staining with DAPI (blue) and BMPR1B (green).

6 (Graph Pad Software, La Jolla, CA, USA) as means ± standard deviations/ standard errors. Quantification of the relative changes in the gene expression in the samples was done using the $2^{\wedge-\Delta CT}$ method adapted from Livak *et al.*[35]. Relative PCR amplification of the target genes was calculated after normalization with the housekeeping reference gene (β-actin) as described[36]. Differences in the expression level between the treated and untreated animals, in whole ovaries, were analyzed using one-way analysis of variance (ANOVA) followed by a student–Newman–Keuls multiple range tests, using the Prism version 6 (Graph Pad Software, La Jolla, CA, USA). For statistical analyses, the differences were counted as significant at $p<0.05$.

3. Results

3.1 Immunofluorescent Localization of BMPR1B, FSHR, and LH-CGR in multiple Stages of Ovarian Follicle Development

The Immunofluorescent labeling of BMPR1Bin the control (untreated) animals revealed positive staining on the granulosa cells of three different stages of the ovarian follicle: primary follicle (Figure 2A,B), secondary follicle (Figure 2C,D), and *Graafian* follicle (Figure 2E,F). Staining of FSHR showed positive staining limited to granulosa cells of primary follicle (Figure 2G,H), secondary follicle (Figure 2I,J), and Graafian follicle (Figure 2K,L). Luteinizing hormone/choriogonadotropin receptor(LHCGR) staining was absent in granulosa cells of primary follicle (Figure 2M,N), and positive staining was observed on the granulosa cells of secondary (Figure 2O,P) and *Graafian* follicles (Figure 2Q,R). In animals treated with anti-BMP-4 antibodies, there were no differences observed in the immunofluorescent staining of BMPR1B, FSHR, and LH-CGR in all stages of ovarian follicle development (data not shown) compared to the untreated control. In animals treated with anti-BMPR-1B antibody, stronger signals of BMPR1B were observed in the granulosa cells of primary (Figure 3K, L), secondary (Figure 3A,B), and *Graafian* (Figure 3E,F) follicles when compared to the staining of granulosa cells from control untreated animals (Figure 3C,D and Figure 3G,H), respectively. Similarly, immunofluorescent staining of the FSHR in granulosa cells of treated animals showed

stronger signals in secondary and *Graafian* follicles but not in granulosa cells of primary follicle (data not shown). On the other hand, no labeling signal was detected for LH-CGR in granulosa cells of primary follicles regardless of treatment (data not shown).

3.2 Quantification of BMPR1B, FSHR, and LH-CGR in Mutiple Stages of Folliculogenesis

To determine the dynamic interaction between BMPs and gonadotropins, a quantitative analysis of the intensity of the fluorescent signals was performed in multiple stages of follicle development. In the primary follicles, the expression of BMPR1B in mice treated with BMPR1B antibody (with or without eCG) was significantly higher than in the control group (Figure 4 A). On the other hand, no changes were observed in the FSHR expression in primary follicles from mice treated with anti-BMPR-1B (with or without any treatment) (Figure 4B). Consistent with the microscopic observation mentioned above, the digital analysis revealed that LGCR labeling was not detected in the granulosa cells of primary follicle (Figure 4C). In secondary follicle, animals treated with anti-

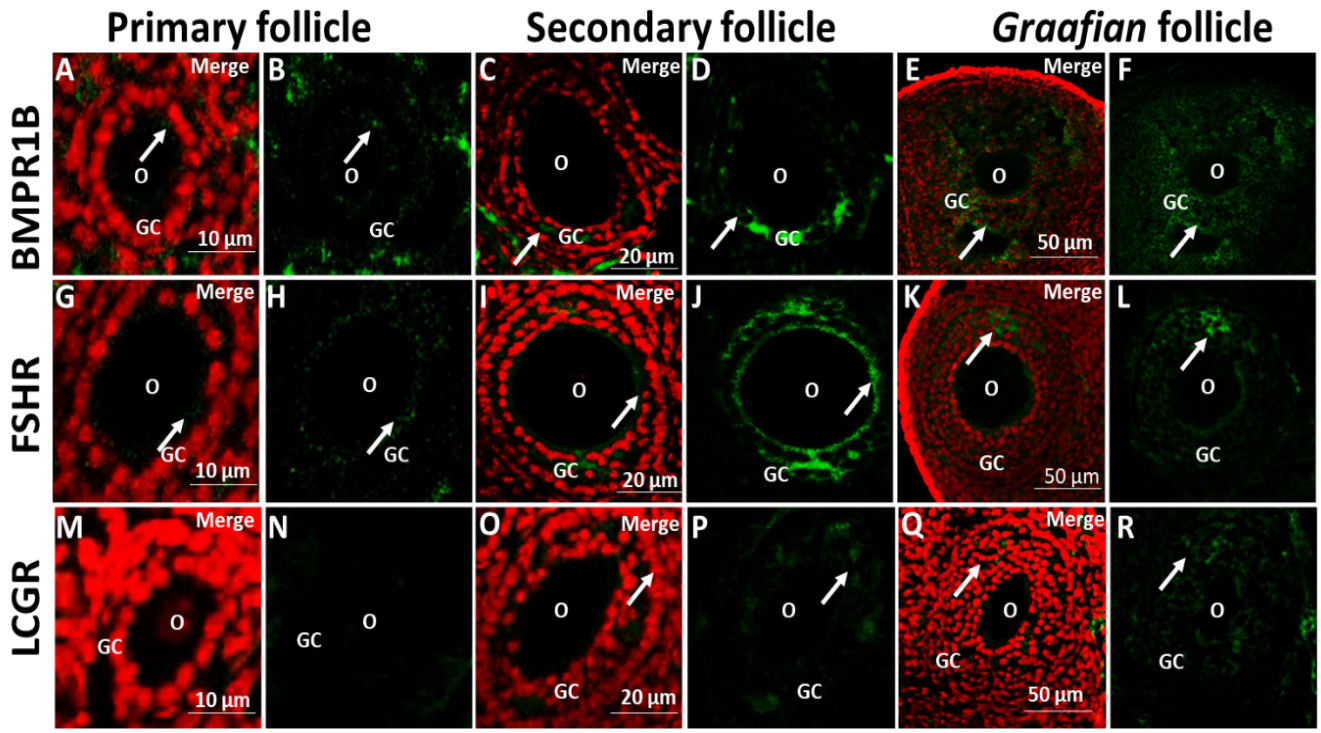

Figure 2. Immunofluorescent localization of BMPR1B, FSHR, and LH-CGR in three stages of follicles in the control group (wild type). **A G**, and **M.** Merged images (two filters) of ovary sections from the control group, showing primary follicles with positive green staining (arrows) of BMPR1B (A) and FSHR (G) but not of LGCR (M), and DAPI staining of the nucleus (red). **B** and **H.** Single filter images of BMPR-1B staining (green) of the same follicles in A and G, highlighting the cell membrane expression pattern on the granulosa cells. **N.** A single filter image of LGCR staining in the same follicle in M, showing negative labeling. **C, I,** and **O.** Merged images of the ovary sections from control group, showing secondary follicle with positive staining of BMPR1B (C), FSHR (I), and LGCR (O) (green) and DAPI staining (red) of granulosa cells. **D, J,** and **P.** Single filter images of BMPR1B, FSHR, and LH-CGR in the same follicles are shown in C, I, and O, respectively, highlighting the cell membrane expression pattern on granulosa cells (green, arrows). **E, K,** and **Q.** Two filters merged images of the ovary sections from control group, showing Graafian follicles with positive staining of BMPR1B (F), FSHR (K), and LH-CGR (Q) (green) and DAPI staining (red) of the granulosa cells. **F, L,** and **R.** Single filter images of BMPR1B (F), FSHR (L), and LH-CGR (R) in the same follicles are shown in E, K, and Q, respectively, highlighting the cell membrane expression pattern on the granulosa cells (green, arrows). GC, granulosa cells.

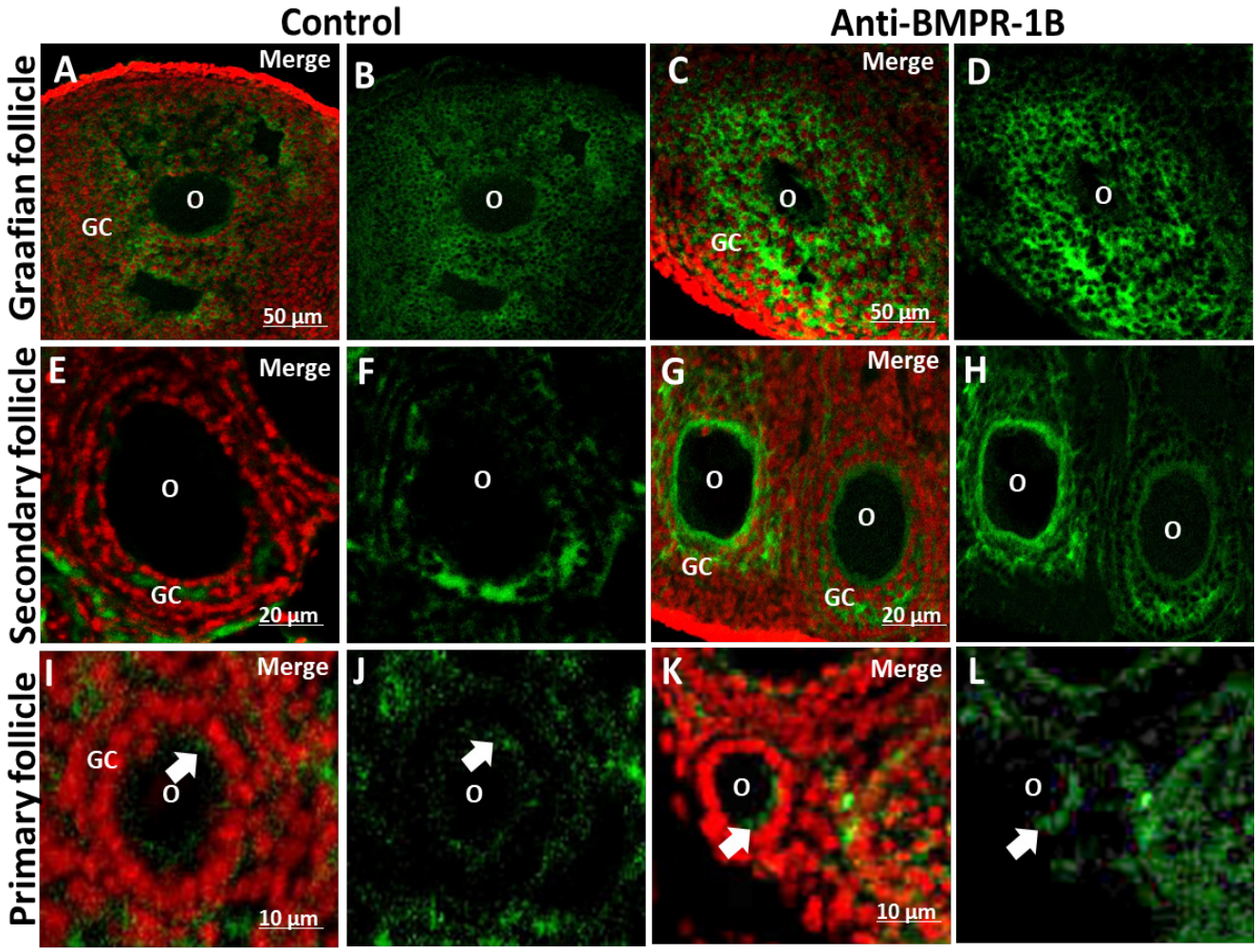

Figure 3. Comparison of the immunofluorescent staining intensity of BMPR1B in three stages of follicles in treated (immunized) and untreated (control) animal groups, using anti-BMPR1B antibody. **A** and **E.** Merged images of ovary sections from animals treated with anti-BMPR1B, the secondary follicle (A), and Graafian follicle (E) showing positive staining of BMPR1B antibody (green) and DAPI (red) of granulosa cells. **B** and **F.** Single filter images of BMPR1B staining (green) of the same follicles in A and E, highlighting the cell membrane expression pattern on the granulosa cells. **C** and **G.** Two filters merged images of the ovary sections from the control group showing secondary follicle (C) and Graafian follicle (G). **D** and **H** Single filter images of the same follicles in B and F from treated animals, showing stronger intensity of staining when compared to D and H from the untreated animal group. GC, granulosa cells; O, oocyte.

BMPR-1B (with or without eCG) showed a significant increase in BMPR1B, FSHR, and LH-CGR expression compared to control group (Figure 4D, E, F), respectively. Interestingly, treatment with eCG alone did not induce significant changes in the BMPR1B and LH-CGR protein expression in granulosa cells of secondary follicles (Figure 4D, F) whereas, FSH-R was significantly upregulated (Figure 4E). In Graafian follicle, animals treated with anti-BMPR-1B (with or without eCG) showed a significant increase in the BMPR1B, FSHR, and LH-CGR expression compared to control group (Figure 4G, H, I), respectively. Administration of eCG alone resulted in upregulation of FSHR and LH-CGR in the granulosa cells of Graafian follicles (Figure 4H,I) but not in BMPR1B expression (Figure 4G). Interestingly, no changes in receptor expression of BMPR1B, FSHR, and

LH-CGR were observed in mice treated with anti-BMP-4 (with or without eCG; data not shown) at any stage of follicle development (Figure 4A-I).

3.3 Expression of *Bmpr1b*, *Fshr,* and *Lhcgr*

To study the dynamic interaction between BMPs and gonadotropins at the molecular level, we measured the levels of mRNA gene expression of the *Fshr*, *Lhcgr*, and *Bmpr1b* in the ovaries. The expression of *Fshr* and Lhcgr genes was significantly higher in mice treated with BMPR1B antibody (with or without eCG) than in the control group (Figure 5A, B). Different from the protein expression, eCG treatment also resulted in a significant increase in *Fshr* and *Lhcgr* gene mRNA expression, respectively (Figure 5A, B). Similarly, animals treated with anti-BMP-4 antibodies showed no changes in the mRNA level of *Fshr* and *Lhcgr* genes (Figure 5A, B). The *Bmpr1b* was noted to be elevated in the ovaries of mice treated with BMPR1B antibody (with or without eCG) (Figure 3C).

4. Discussion

The present study demonstrates some aspects of the interaction between the intraovarian BMPs and gonadotropins, FSH and LH, in the regulation of folliculogenesis *in vivo* using a unique mouse model developed in our laboratory. In this model, both BMPs and gonadotropin signaling systems appeared to be normally functional. While gonadotropins in this study were responsive to exogenous eCG, the BMPs signaling system was partially attenuated, due to passive immunization against BMPR-1B and BMP-4. In addition, the mechanism of interaction was investigated for the first time the three principal stages of follicular development *in vivo*, where ovarian function is fully active. Moreover,

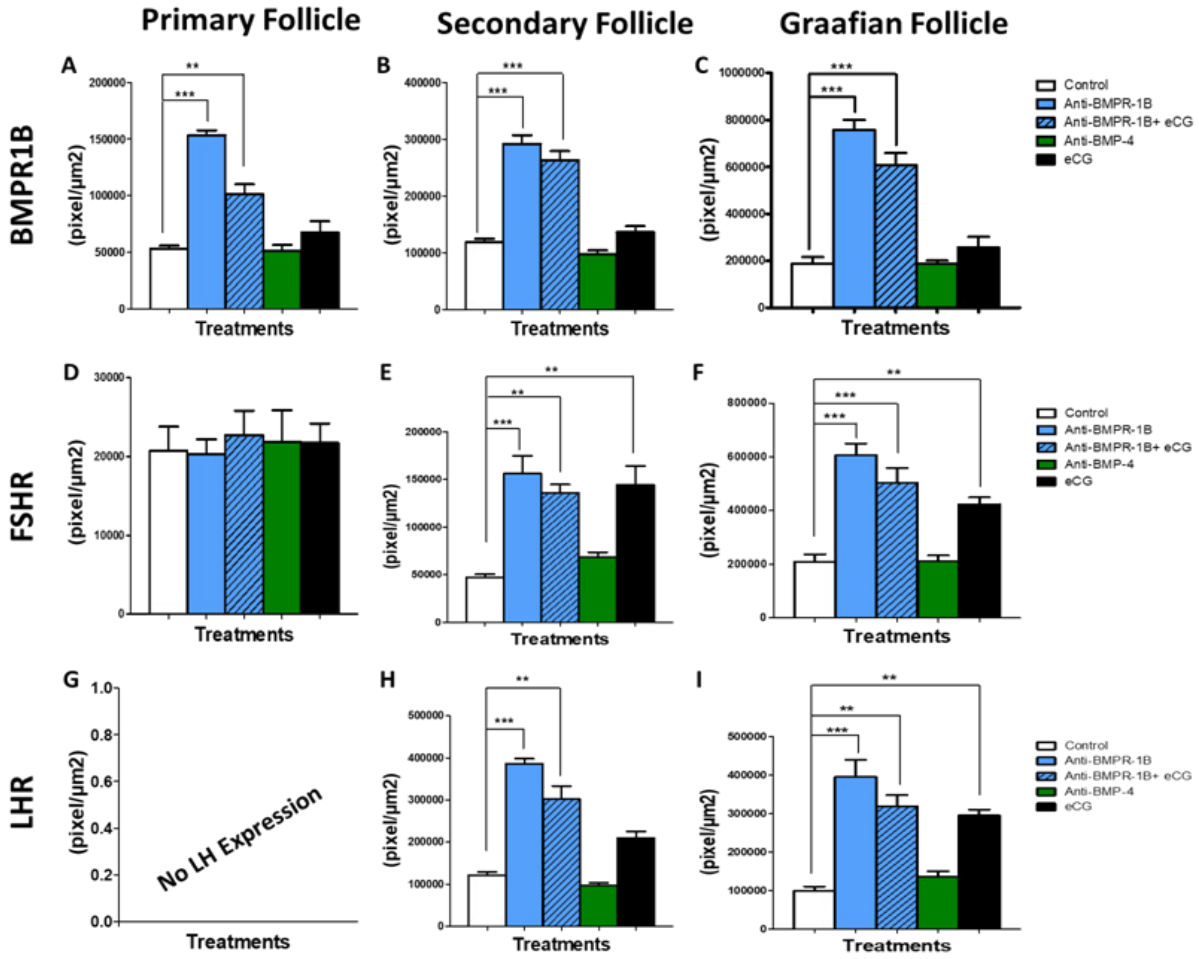

Figure 4. *In situ* quantification of the BMPR1B, FSHR, and LH-CGR receptors expression (pixel/µm^2) in three stages of follicle development after different treatments. 10-12 mice per group and 20follicles per ovary were subjected to quantification.

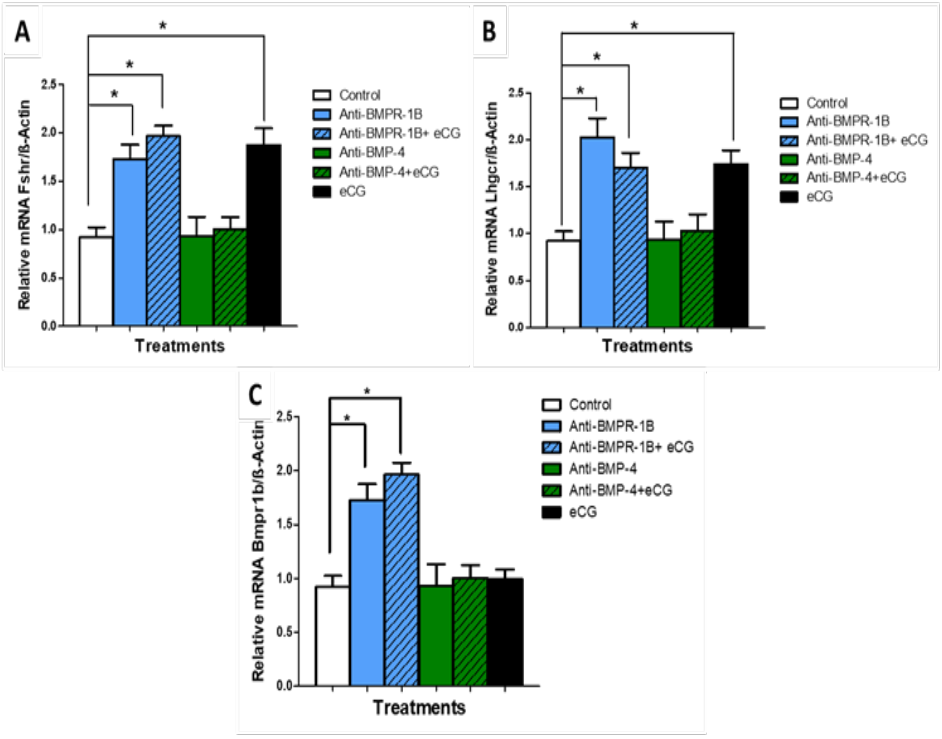

Figure 5. Relative expression (mRNA) levels of *Fsh* and *Lhcg* receptor genes in the whole ovary after different treatments. The difference was considered significant at ($p< 0.05$). **A.** *Fshr* relative mRNA expression level; **B.** *Lhcgr* relativemRNA expression levels; **C.** *Bmpr1b* relative expression level in the whole ovary (n= 10-12).

the effects of immunization on the nature of interaction, expressed by the levels of gonadotropin gene expression in the ovary, have not been previously reported.

The mouse model used in this study has previously been used as suitable to investigate many aspects of ovarian functions as reported[7,28,29]. The localization and quantification of BMPR1B, FSHR, and LH-CGR were carried out by *in situ* immunofluorescent localization combined with computer-based quantitative analysis of 3D images. This method proves to be an efficient tool to quantify the expression of a small amount of protein in a complex tissue such as ovary[31].

In this study, we report that attenuation of BMPR-1B signaling leads to up-regulation of BMPR-1B in all stages of follicular development, probably due to an auto-compensatory effect to overcome the inhibitory effect on BMPR-1B signaling function. A similar result of up-regulation was previously reported in *Booroola* sheep, which is characterized by a natural point mutation in the BMPR-1B[37].

In the primary follicles, the attenuation of BMPs system showed no effect on the FSH-R protein expression with and without eCG administration, providing further evidence that the early stage of follicle development is gonadotropin-independent but is driven by BMPs rather than FSH or LH as previously reported[7,38,39]. On the other hand, in secondary and Graafian follicles, attenuation of BMPR-1B signaling system resulted in up-regulation of FSHR and LH-CGR in granulosa cells, which explains the enhanced sensitivity of granulosa cells towards gonadotropins, as previously reported[40]. Interestingly, the administration of eCG resulted in up-regulation of FSHR expression in the granulosa cells of secondary follicles only, whereas expression of both FSHR and LH-CGR were increased in Graafian follicles. The elevation of FSHR after eCG administration can be attributed to increased FSH production[40], which in turn leads to up-regulation of FSH-R expression[41]. Using a different experimental approach we have previously reported a similar pattern of up-regulation of expression of FSHR and LH-CGR in *Booroola* sheep with a natural point mutation in BMPR-1B[37]. As a result, the increase in sensitivity of follicular cells to gonadotropins has been linked with enhanced cellular proliferation and differentiation[41], reduced apoptotic

activity[42], and luteinization in *Booroola* sheep[27] and mice[7], and increased steroidogenic activity[14,20,43,44], and improved oocyte-granulosa cells communication[45,46]. In humans, the increase in follicular cell sensitivity towards gonadotropins underlines the cyclic recruitment of mature follicles for further development towards ovulation at midcycle thereby improving fertility[47]. BMP-4 is the main BMP ligand, which signals via the BMPR-1B system, and has been implicated in the regulation of several aspects of folliculogenesis such as initial formation of ovarian follicle reserve[19,43] and recruitment of primordial follicles into the cohort of developing follicles in Booroola sheep[27] and rat[14], in addition to estrogen production, inhibition of progesterone and ovulation[13,19]. In this study, immunization against BMP-4 producedno effect on protein or RNA expression of FSHR, LHC-GR, or BMPR-1B at any stage of follicle development. Based on previous reports[7,27] and data from this study, we propose for the first time that, unlike attenuation of its signaling pathway (via BMPR-1B), dysregulation of BMP-4 ligand function by immunization has no impact on the regulatory interaction between the BMPs and gonadotropins signaling systems, such as protein and/or RNA expression of their receptors. In conclusion, the outcomes of this study imply that follicle development is tightly controlled by reciprocal interaction between intraovarian BMPs and gonadotropin signaling systems. In addition to the direct role of BMPs in follicle development, the present tudy demonstrates that BMPs also exert indirect actions in the regulation of follicle development by enhancing the granulosa cell's sensitivity to gonadotropins via up-regulation of the expression of FSHR and LH-CGR. We propose that downregulation of the intraovarian BMP signaling system appears to be a pre-requisite for the major action of gonadotropins at the later stages of folliculogenesis and can be used as a suitable clinical tool to improve human fertility by increasing primordial follicle reserves as we previously reported[7].

5. Declaration of Interest

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

6. Funding

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8. Author Contribution Statement

SA carried out the experiments. SA and GA wrote the manuscript with support from JM, CM, and SP. IA participatedin conducting the *in vivo* studies, and sample collection and preparation. GA and JM conceived the original idea with support from SA. CM, JM, and GA supervised the research. All authors discussed the results and contributed to the final manuscript.

9. Data Availability

The data that support this study are included in the article.

10. References

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