BMPs and BMPRs in chicken ovary and the effects of BMP-4 and –7 on granulosa cell proliferation and progesterone production in vitro.

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Abstract

Bone Morphogenetic Proteins (BMP) and their receptors (BMPR) are now known to have important roles in mammalian ovarian folliculogenesis. This study determined the expression of the mRNA encoding for BMPs and their receptors in the chicken ovary and explored possible roles for them. The expression of the mRNA for BMP-2, -4, -6, -7 and BMPR-IA, IB, and II was determined and quantified by a semi-quantitative RT-PCR. The mRNAs for all the BMPs and receptors determined were present in both the granulosa (G) and theca (T) cells of the F1, F2 and F3 follicles. All BMP mRNAs increased in granulosa cells with follicular development whereas only BMP-7 mRNA has this trend in the theca. BMP-2, -4 and –6 mRNAs in theca were similar between follicles. BMPR-IA mRNA was similar in F2G and F3G but lower in F1G. BMPR-IB mRNA was similar in granulosa of all follicles and BMPR-II mRNA increased with development. In the theca, each receptor subtype showed equal distribution between follicles. mRNA levels for BMPR-IB and –II were higher in granulosa than in theca suggesting that the granulosa is a major target for BMPs. BMP-4 and –7 stimulated basal, IGF-I-, and gonadotrophin-stimulated progesterone production by cultured granulosa cells with differential responses between cells from the F1 and F3/4. This suggests involvement in follicular differentiation. BMP-4 and –7 reversed the inhibitory effects of TGFα on basal and gonadotropin-stimulated granulosa cell progesterone production with greater effect in the F1 compared to the F3/4. This effect suggests an important role for BMPs interacting with TGFα in modulating the effects of gonadotrophins and IGF-I on follicular differentiation. Finally, BMP-7 stimulated granulosa cell proliferation but BMP-4 inhibited TGFα + IGF-I or + FSH- stimulated granulosa cell proliferation suggesting a role in the control of follicular growth during development. These effects of BMP-4 and –7 on
the granulosa cell function showed relationships with the expression levels of the BMPs and the BMPR-II.

Introduction

The Bone Morphogenetic Proteins (BMP) are a group of growth and differentiating factors belonging to the Transforming growth factor-β superfamily. By molecular cloning, at least 15 members of the group have been identified and are called BMP-2 through to BMP-15. The BMPs signal via complexes of serine/threonine kinase types-I and –II receptors that have been designated as BMPR-IA, BMPR-IB and BMPR-II. In vertebrates, the BMPs have been implicated in several functions during both embryonic and post-embryonic life. These functions include formation of bones, cartilage (25), neurons (1), tooth (7), eyes, kidney (8) and feathers (2). They are also involved in the delivery of positional information including organogenesis, tissue patterning and remodelling (32, 14). However, a virtually unexplored role in the reproductive tissues is emerging. Recently, it was shown that the BMPs and their receptors are involved in the autocrine/paracrine regulation of ovarian folliculogenesis in some mammals. BMP-2, -4, -6, -7 and –15, and their receptors have been specifically implicated in rat, sheep and cattle ovarian function. In rat, the mRNA encoding BMP-4, and -7 are expressed in the theca while the BMP-6 and –15 are confined to the oocytes of the follicles. All BMP receptors are expressed in the granulosa, theca and oocyte (28, 4, 11). In this species, BMP-4, –7 and –15 regulate granulosa cell steroidogenesis while BMP-6 and –15 induce cell proliferation (28, 10, 21, 22). In sheep, a crucial role for the BMPs in the regulation of ovarian folliculogenesis has been established since the discovery that the presence of heterozygous mutation in BMP-15 gene results in improved prolificacy in Inverdale
(FecX) ewes (5). On the other hand, carrying a Q249R mutations in the BMPR-IB also confer improved prolificacy on Booroola (FecB) ewes (30, 15). Except for BMP-15, there is a dearth of information on the types of BMPs that are expressed in the sheep ovary and their functional significance. Souza et al (31) reported the localization of BMPR-IA, IB and II in the granulosa and to a lesser extent in the theca of the sheep ovary. These authors and Wilson et al (37) have demonstrated that BMP-2 and -4 regulated granulosa cell steroidogenesis in sheep.

In the avian species, the gene expression or a role for the BMPs in the ovary is still unexplored. Previous studies have shown that other growth factors like IGF-I, EGF, TGF-α, TNF-α and FGF are expressed in the chicken ovary and perform important roles alone or in their interaction with LH, FSH or GH in the regulation of follicle growth and differentiation (18, 19, 17, 24, 39). The aim of the current study was to determine the expression of mRNA encoding BMP-2, -4, -6, and -7, and their receptors, BMPR-IA, -IB and –II in the chicken ovary. Having found the transcripts of the BMPs and BMPRs, we further determined the effects of two of the highly expressed BMPs (BMP-4 and -7), on granulosa cell progesterone production and proliferation in culture. The effects of their interaction with LH, FSH and other growth factors (IGF-I, IGF-II and TGFα) on these functions were also determined.

Materials and Methods

a) Gene Expression of BMPs and BMPRs

Animals and tissue collection for gene expression

Laying ‘Label’ broiler breeder hens (Hubbard-ISA, Quintin, France) of 35-40 weeks of age were kept in cages under a photoperiod of 16hr light: 8hr darkness. Chickens
were allowed food and water ad libitum and laying was monitored daily. Hens were killed by cervical dislocation 4 - 8 hrs before the next oviposition. The largest (F1), second largest (F2) and the third largest (F3) follicles were excised from the ovary into warmed (37°C) phenol-free Hanks balanced salt solution (pfHBSS)(Sigma-Aldrich, Belgium). The granulosa cell layer of the follicles was dissected out as previously described (6). Tissues were immediately snap-frozen in liquid nitrogen and stored at –80°C until used for total RNA isolation.

**RNA Isolation**

Total RNA was extracted from chicken granulosa and theca tissues using the Trizol® reagent (Invitrogen, Belgium). The RNA concentration was estimated by reading the absorbance at 260 nm and was checked for purity at 280 nm in a spectrophotometer (GeneQuant, Pharmacia, Biotech Ltd, Cambridge UK). The samples were stored at –80°C.

**Primer sets**

The primer sets used for this study were designed using a computer program (DNAMan for Windows, Lynnon Biosoft, USA). Primers were selected from published chicken and quail sequences. The upstream and downstream primer sets, their sources, the predicted product sizes are as shown in Table 1.

**Protocol**

The expression of the mRNA for each BMP and BMPR was first determined in the granulosa (G) and theca (T) of the different follicle sizes (F1G, F2G, F3G, F1T, F2T and F3T). Then using the housekeeping gene glyceraldehyde-3-phosphate
dehydrogenase (GAPDH) as control to demonstrate equal loading of the lanes, mRNA level in the tissues of the different follicle sizes was determined.

**Reverse transcription**

Complementary DNA was synthesized by the extension of respective anti-sense primers. Nineteen microliters of RT master mix for each sample (1 µg total RNA) was prepared by using single-strength first-strand buffer (Invitrogen), 5 U RNasin (Promega, Leiden, The Netherlands), 10 U Moloney murine leukemia virus reverse transcriptase (Invitrogen), 0.5 mM PCR nucleotide mix (Promega), 5 mM DTT (Invitrogen), and 1µM of anti-sense primer separately. The RT reaction was carried out in a DNA thermal cycler (MJ-PTC-200, Biozym, Landgraaf, The Netherlands) at 37°C for 45 min followed by 5 min at 95 °C. The samples were then placed on ice immediately and used further for amplification.

**PCR Amplification and quantification of mRNA**

PCR was performed for the each BMP and BMPR by adding the following components to the reaction mixture: 10x REDTaq DNA Polymerase buffer (Sigma, Saint Louis, USA), 0.25 mM PCR nucleotide mix (Promega), 0.5 µM of each primer and 7.5 U REDTaq™ DNA polymerase (Sigma) in a total volume of 40 µl. The PCR reaction for BMPR-IA was performed as follows: 95 °C for 3 min; 30 cycles at 94 °C for 45 s, 52.5 °C for 45 s, and 72 °C for 20 s; and a final extension for 5 min at 72°C. The PCR reaction for BMP-2, -4, -6 and -7 was performed as follows: 95 °C for 3 min; 30 cycles at 94 °C for 45 s, 54 °C for 45 s, and 72 °C for 25 s; and a final extension for 5 min at 72°C. The PCR reaction programme for BMPR-IB and BMPR-II was: 95 °C for 3 min; 30 cycles at 94 °C for 45 s, 54.5 °C for 45 s, and 72 °C for 25
s; and a final extension for 5 min at 72°C. Fifteen microliters of PCR products were electrophoresed on a 2% (wt/vol) agarose gel (Invitrogen) containing 1% ethidium bromide. A 100-bp DNA ladder (Eurogentec, Herstal, Belgium) was used as molecular weight standard. Negative control RT-PCR with diethyl pyrocarbonate-treated water was included in all experiments.

After electrophoresis, the gel was scanned and the intensities of the different bands were measured with a densitometer (Image Master VDS, Pharmacia Biotech, The Netherlands) as previously described (26, 33). Data were normalized to GAPDH mRNA abundance and expressed as a percentage.

b) Effects of BMP-4 and –7 on cultured granulosa cells.

*Chicken granulosa cell preparation and culture*

Laying ‘Label’broiler breeder hens of 35-40 weeks of age were kept in cages under a photoperiod of 16hr light : 8hr darkness. Chickens were allowed food and water ad libitum and laying was monitored daily. Hens were killed by cervical dislocation 4 - 8 hrs before the next oviposition. The largest (F1), third largest (F3) and the fourth largest (F4) follicles were excised from the ovary into warmed (37°C) phenol-free Hanks balanced salt solution (pfHBSS)(Sigma-Aldrich, Belgium). The granulosa cell layer of the follicles were dissected out as previously described (6) and dispersed in Ca\(^{++}/\)Mg\(^{++}\) free Hanks (Sigma) containing 0.1mg ml\(^{-1}\) collagenase (Sigma) as described previously (23). Cells from follicles of the F1 or F3 + F4 position were pooled from 5 - 6 hens and the cell density was determined by measuring the DNA in aliquots using the method of Labarca & Paigen (9) in which 1µg DNA =10\(^5\) cells. Cell viability was assessed by the trypan blue method and was usually > 90%. Cells were resuspended in Medium 199 (M199)(Sigma-Aldrich) supplemented with 2mmol
glutamine $\text{L}^{-1}$, 40mmol sodium bicarbonate $\text{L}^{-1}$, 1% (v/v) PSA (antibiotic-antimycotic solution)(Sigma) and 1% BSA (Sigma). During preliminary studies, experiments were conducted to test the response of freshly isolated cells under short-term 3 hr incubation. 1 x $10^5$ cells were incubated in 0.5ml medium at 39°C in presence of different concentrations of ovine LH, ovine FSH (from Dr Parlow, NIDDK, USA), recombinant chicken IGF-I (IBT-Immunological and Biochemical Testsystems GmbH, Reutlingen, Germany), human recombinant BMP-4 and BMP-7 supplied with carrier proteins (R&D Systems Europe Ltd, Abingdon, UK) and human recombinant TGFα (Sigma-Aldrich NV/SA, Bornem, Belgium). Due to the lack of effect of the BMPs and IGF-I on progesterone production whereas LH and FSH dose-dependently increased progesterone production under the short-term incubation conditions, a cell culture method was adopted. For cultures, cells were resuspended in Medium 199 (M199)(Sigma-Aldrich) supplemented with 2mmol glutamine $\text{L}^{-1}$, 40mmol sodium bicarbonate $\text{L}^{-1}$, 1% (v/v) PSA (antibiotic-antimycotic solution)(Sigma), and 5% foetal bovine serum (FBS)(Invitrogen) and plated at 1.0 x $10^5$ viable cells per well (equivalent to 1 µg DNA/well) in 24-well plastic plates (Iwaki). Cells were cultured for 24hrs in a humidified atmosphere of 5% CO₂ in air at 37°C to establish cultures and to facilitate firm cell attachments especially for the F1 cells. After this initial period of culture, the medium was discarded and monolayer cells were washed with pHBSS. Some plates were withdrawn for the evaluation of number of cell attached to plates at the beginning of the final culture phase for testing hormone effects. (The DNA content of wells was between 0.7 and 0.82 µg/well for the F1 and F3/4 cultures). This was followed by a further 48hrs of culture in serum-free M199 supplemented with 2mmol glutamine $\text{L}^{-1}$, 40mmol sodium bicarbonate $\text{L}^{-1}$, 1% PSA,
6.25µg ml⁻¹ transferrin, 5ng ml⁻¹ selenium and 0.1% (w/v) BSA (Sigma). All additions of test hormones were made in the second period of culture for 48hrs.

**Effect of BMPs on progesterone production by granulosa cells**

Granulosa cells from F1 (F1G) and F3/4G follicles were cultured for 48hrs in the presence of increasing concentrations (0 – 500ng ml⁻¹) of recombinant human BMP-4 or -7, initially to determine the dose effect of the BMPs. As the effect of the BMPs was maximal at 50ng/ml (see Figure 5), subsequent tests were conducted to test the minimum (1ng/ml), and a submaximal (5ng/ml) effective dose. Cells were cultured in the presence of these doses of BMP with or without a submaximal concentration of either recombinant chicken IGF-I (5ng ml⁻¹), recombinant human TGFα (0.05ng/ml), ovine LH or FSH (5ng ml⁻¹). Various combinations of these hormones with the BMPs were also tested. The total incubation volume in all experiments was always 500µl per well. At the end of the incubation period, medium was collected and stored at –20°C until assayed by radioimmunoassay for progesterone content.

At the end of all experiments, the final DNA content of the culture wells was determined and used to correct the progesterone production data, since the values of progesterone production will depend on the number of cells in the wells.

**Progesterone assay**

Progesterone concentration in the culture medium was determined by radioimmunoassay without extraction. Assay was conducted with a commercial kit (ICN Biomedicals Inc., Costa Mesa, CA). The crossreactivities of the antiserum in the kit with other ligands are 20α-dihydroprogesterone 5.46%, desoxycorticosterone
3.8%, and < 1% for other steroids. The sensitivity of the assay was 0.05 ng/ml. The intra-assay and inter-assay coefficients of variation were 5.20% and 8.44%.

**DNA assay**

Cultures for DNA determination were washed in pfHBSS. The cells were disaggregated with 200µl trypsin-EDTA (ROCHE) in TNE buffer (10mmol Tris l⁻¹, 1mmol EDTA l⁻¹, 2M NaCl, pH 7.0) followed by sonication. The DNA in aliquots of the lysate was determined by the method of Labarca & Paigen (9) using calf thymus DNA (Sigma) as standard and H33258 (bisbenzimide; Sigma) as fluorogen. The DNA was quantified on a TKO 100 mini fluorometer (Hoeffer Scientific Instruments, San Francisco, CA). The sensitivity of the assay was 0.25 ng/ml and the standard curve ranged between 0.0 and 10 ng/ml.

**Statistical analysis**

Data for mRNA expression represent the means±SEM of experiments repeated four times, each using pools of total RNA from the F1, F2 and F3 follicles of 2 hens.

Data presented for progesterone and DNA assays are means±SEM of four experiments each with four replicate culture wells. Data were analysed by analysis of variance (ANOVA). Statistical differences were assessed by Duncan’s multiple range test (General Linear Models procedure; SAS Institute, 1985). P-values of < 0.05 were considered statistically significant.

**Results**

*Presence of mRNA encoding BMPs and BMPRs*
Figures 1A and B show the transcripts of BMP-2, -4, -6, -7 and BMPR-IA, -IB and –II in the granulosa and theca tissues of the chicken follicle. The mRNA for all BMPs and BMPRs was detected in the granulosa and theca of the F1, F2 and F3 follicles. Gel electrophoretic analysis showed that the transcripts were of the exact molecular size predicted. The products were confirmed further by restriction site digestion and sequencing.

**BMP mRNA levels in granulosa and theca cells**

The comparative levels of the mRNA in the granulosa and theca of all follicles was determined by reference to GAPDH levels. Figure 2 shows a typical assay for comparing the level of BMP-7 or BMPR-IA with GAPDH levels in the granulosa or theca of F1, F2 and F3 follicles.

The comparative levels of BMP-2, -4, -6 and –7 in the granulosa or theca of F1, F2 and F3 are shown in Figure 3. BMP-2, -4, -6 and –7 mRNAs were significantly higher in F1G and F2G than in the F3G, but there was no difference in the levels between the F1G and F2G.

In the theca cells, there were no significant differences in the expression of BMP-2 or BMP-4 in the F1, F2, or F3. However, the level of BMP–7 was higher in the F1 and F2 than in the F3. The expression levels in the F1 and F2 were similar. BMP-6 mRNA was higher in the F2 than in the F3 with the level in the F1 in between.

Between tissues, BMP-2 was not significantly different in all follicles between the granulosa and the theca. BMP-4 in the F3 was similar between the granulosa and theca tissues but was higher in the granulosa cells of the F1 and F2. BMP-6 was significantly higher in the theca of follicles compared to the levels in the granulosa
cells. BMP-7 showed similar level of transcripts in the theca and granulosa of all follicles.

**BMPR mRNA levels in granulosa and theca cells**

The comparative transcript levels of BMPR-IA, -IB and –II in the granulosa and theca cells of follicles are shown in Figure 4. In the granulosa cells, there were no differences in the expression of BMPR–IB between follicle sizes but BMPR-IA was lower in the F1 compared with the F2 and F3 follicles. However, BMPR-II expression was higher in the F1G and F2G compared to the F3G. In the theca cells, BMPR-IA, -IB and –II were expressed at similar levels between follicles. Between tissues, the theca tended to express the mRNA for BMPR-IA more than in the granulosa. Conversely, the granulosa had higher levels of BMPR-IB and BMPR-II in all follicles than the theca except in the F3 where BMPR-II levels were similar.

**Effect of BMP-4 and BMP-7 on granulosa cell progesterone production**

The results of our preliminary experiments with freshly isolated cells under short-term incubation of 3 hr showed that whereas LH and FSH dose-dependently increased progesterone production, IGF-I, BMP-4 and –7 had no effect on at all doses tested (0.01 – 500ng/ml). TGFα however, inhibited progesterone production at a maximal dose of 1ng/ml (data not shown). However, in culture, both IGF-I and BMPs-4 and -7 dose-dependently stimulated progesterone production by granulosa cells cultured in serum-free medium 199. The effect of the BMPs and IGF-I were evident after 24 hr in culture, was maximal at 48 hr and then decreased at 72 hr. The maximum effective concentration was 50 ng/ml when either BMP-4 or BMP-7 was used. Figure 5 shows
the dose effect of the BMPs on F1 or F3/4 granulosa cells. BMP-4 and –7 at 100 ng/ml or greater decreased progesterone production below that observed for 50 ng/ml. There was a differential responsiveness between the cells of the more mature F1 and the F3/4. At most doses except at the maximal dose, the response of cells to either BMP-4 or –7 was similar. At the maximum dose, the F1 cells were 3-fold more responsive to BMP-4 compared to the F3/4, whereas the response to BMP-7 was 4.5-fold.

Figures 6 and 7 show the effects of BMP-4 or –7 on progesterone production at doses of 1 ng or 5 ng/ml and their combination with LH, FSH or IGF-I. LH, FSH or IGF-I significantly increased progesterone production individually. The addition of either BMP-4 or BMP-7 along with LH or FSH or IGF-I dose-dependently enhanced the effect of the gonadotrophins or IGF-I significantly. However, under a combination of LH or FSH with IGF-I, the higher dose of BMPs (5ng/ml) had no increased effect beyond that obtained with the lower dose (1ng/ml). At all doses and combinations with LH, FSH or IGF-I, the F1 cells showed significantly higher response than the F3/4 cells. It is noteworthy that BMP-4 or –7 at a similar dose as IGF-I (5 ng/ml) was significantly more effective in enhancing LH- or FSH-induced progesterone production than IGF-I. Comparatively, progesterone production in response to the BMPs in combination with FSH was significantly lower to that produced in combination with LH.

The effect of the BMP-4 in combination with TGFα, LH, or IGF-I is shown in Figure 8. TGFα significantly suppressed basal progesterone production by the cells of the F1 and F3/4. It also significantly decreased production in the presence of LH or IGF-I alone. The effect of TGFα was greater in the F3/4 than in the F1. However, there was little effect of TGFα on BMP-4 induced progesterone production in F1 cells as the
effect was only a marginal decrease in progesterone. But in the F3/4 cell TGFα significantly decreased progesterone in the presence of BMP-4. Although TGFα significantly decreased LH + IGF-I-enhanced progesterone in both the F1 and F3/4, it had no effect on BMP-4 + LH-enhanced production in the F1 but decreased production from the F3/4. In the presence of BMP-4/LH/IGF-I, TGFα, again, had no effect on progesterone enhancement from the F1 but lower the progesterone secretion from the F3/4. Thus the effect of TGFα in the presence of BMP-4 was dissimilar between the F1 and F3/4 cells: the F1 being not responsive to TGFα in the presence of BMP-4 whereas the F3/4 was. Similar effects were seen when BMP-7 was substituted for BMP-4 in the culture (data not shown).

**Effect of BMPs on granulosa cell proliferation in culture**

Figure 9 (A – D) shows the DNA content of culture wells after cells have been cultured for 48 hrs in the presence of BMP-4 or –7 with or without LH, FSH, IGF-I, TGFα or their combinations. Cell proliferation was greater in control wells of the F3/4 cultures than in the F1 cultures as measured by the DNA content of wells. DNA content of the F1 cultures increased by 54% whereas that in the F3/4 wells increased by 107% over the 48 hr culture period. BMP-7 at concentrations of 1 and 5 ng/ml stimulated granulosa cell proliferation. Although BMP-4 marginally increased the DNA content of wells, the increase was not significant. Cell proliferation in the presence of BMP-7 was significantly greater in the culture of F3/4 cells than in the F1 cells. IGF-I alone enhanced cell proliferation in both F1 and F3/4 cells. FSH alone also enhanced cell proliferation in the F3 cells but not in the F1 cells. LH alone had no effect on cell proliferation either in the F1 or F3/4. However, the addition of either LH or FSH to IGF-I enhanced the proliferative effect of IGF-I significantly especially in
F3/4 follicles. In a similar fashion, BMP-7 significantly enhanced IGF-I effect. Furthermore, the addition of LH or FSH enhanced the proliferative effect of BMP-7 significantly. The inclusion of BMP-7 along with combination of LH or FSH + IGF-I did increased the effect of the combination of the gonadotropins with IGF-I. It is worthy of note here too that the effect of BMP-7 at a similar dose as IGF-I (5 ng/ml) in the F3/4 was greater in the different combinations with LH and FSH.

Figure 9C shows that TGFα enhanced cell proliferation in both the F1 and F3/4 cultures but the effect was significantly greater in the F3/4. BMP-4 had no effect on TGFα or IGF-I enhanced DNA synthesis. Similarly LH had no effect on TGFα enhanced cell proliferation but FSH significantly increased TGFα effect in the F3/4 cultures. The combination of IGF-I and TGFα had a synergistic effect on F3/4 proliferation but not the F1. BMP-4 significantly decreased the effect of the combination of TGFα and IGF-I in both the F1 and F3/4. It also decreased TGFα + FSH effect in the F3/4.

Figure 9D shows that TGFα, IGF-I and BMP-7 independently stimulated DNA synthesis in both F1 and F3/4 cultures. Again, TGFα in synergy with BMP-7 or IGF-I further increased cell proliferation in the F3/4 wells but not in the F1 cultures. However, the combination of TGFα, IGF-I and BMP-7 resulted in decreased cell proliferation compared to TGFα + IGF-I or TGFα + BMP-7. The addition of LH or FSH in the presence of TGFα combined with BMP-7 depressed the enhancing effect of TGFα with BMP-7 on cell proliferation in the F1.

**Discussion**

The results from these studies demonstrate that the genes encoding the mRNA for the BMPs screened for are expressed in the chicken ovary. The expression of the
receptors was also detected suggesting that the BMPs may have a role to play in the regulation of folliculogenesis in the avian ovary. The functional implication of the presence of these BMPs was demonstrated by their support for progesterone production on their own and enhanced gonadotropin-induced production. The BMP-7 also induced alone and enhanced IGF-I and/or gonadotropin-induced cell proliferation, and thus indicating a participatory role in follicle growth. This is the first report, to our knowledge, that BMPs are expressed in the avian ovary and involved in the regulation of ovarian function in this species.

BMP-2, -4, -6 and -7 were detected in both the granulosa and theca cells of the ovary. BMP expression levels were different between follicles; the two larger F1 and F2 having higher levels than in the F3. There were also differences in expression levels between tissues suggesting possible paracrine roles. The differential levels of BMP-4 and -7 between follicles may be related to their roles in the follicles as will be discussed later in this text. The mRNAs for the receptors mediating the actions of these BMPs were present in both granulosa and theca cells. Although no major differences in expression levels of BMPR-IB were found between follicles, the level of BMPR-IA was lower in the F1 granulosa cells. In both tissues, however, the level of BMPR-II was lower in the F3 compared with the F1 and F2. This may mean a reduced role of some BMPs that are ligands for this receptor type. As with the ligands, receptor distribution was different between the granulosa and theca. This may also mean differential functional levels of the BMPs between the tissues. Although, this is a first indication that BMPs are expressed in the avian ovary, there was a recent report that ActRII and ActRIIB, both of which are type-II serine/threonine kinase receptors that not only bind activin but also BMPs, are present in chicken follicles (29). The elucidation of the expression of BMP-2, -4, -6, -7, and receptor types-IA,
IB, and II in our study follow in the path of a similar study in the rat where BMP-4, -6, -7 and all the receptors have been identified (28, 34). In the sheep ovary, the immunolocalization of the receptors has also been reported (31). However, a major difference exists in compartmentalisation of the BMPs. Whereas the BMP-4 and -7 are confined to the theca alone and BMP-6 to the oocyte in mammals, the BMPs were expressed in both granulosa and theca in the chicken. Our data show that BMPR-IB and II expression are higher in the granulosa than in the theca suggesting that the granulosa may be a major target for BMP actions. Furthermore, the type–IB are expressed uniformly in granulosa of all follicle sizes. These findings are consistent with the distribution of mRNA and immunostaining in both rat and sheep ovaries. In contrast, BMP-II mRNA levels were different between follicles in the chicken granulosa. It showed increasing levels with follicle development. BMPR-II has been shown to perform an important role in mediating the actions of BMPs. BMP-4, -7 and –15 (GDF-9) bind first to the type-II receptor and subsequently phosphorylating the type-I receptors to trigger the Smad-protein signalling pathways (12, 13, 34). It is however not clear which type-I receptor is specific for which ligand. Blocking the synthesis of BMPR-II completely prevented granulosa cell thymidine incorporation by BMP-15 (GDF-9)(34). Thus the lower expression level of BMPs in the F3 granulosa coupled with the lower BMPR-II may lower the biological activity in the F3 compared with F2 and F1. This may be a mechanism for maintaining differential development in the follicular hierarchy of the chicken ovary.

The lack of effect of BMP-4 and –7, and IGF-I on progesterone production under short-term incubation as against that of the LH and FSH suggests a role as differentiation factors rather than involvement in acute processes. It also suggests that these differentiation factors act through different intra-cellular signalling pathways.
that a different from those that mediate the actions of LH, FSH and TGFα. Both BMP-4 and BMP-7 increased progesterone production by cultured granulosa cells. Effects were dose-dependent. They also enhanced LH, FSH, IGF-I and LH or FSH+IGF-I effects on progesterone production. The synergistic effect of the BMPs with LH or FSH is striking. It is more surprising that the BMPs showed greater synergism with the gonadotropins than did the IGF-I at a similar dose of 5 ng/ml. At this dose, both cIGF-I and the BMPs alone had comparable effects on progesterone production. Moreover, the ED50 of both were almost similar (2 ng/ml). Whether this means that the chicken follicles have preferential requirement for BMP-4 and –7 than IGF-I for differentiation needs to be determined. The lack of significant difference between the effects of BMP-4 and BMP-7 on progesterone production at the doses tested with LH, FSH or IGF-I suggests that both may equally function in follicular differentiation. Significantly, the response of the less mature F3 cells was lower than that of the F1 in all treatments. This differential response may be connected with the lower level of expression of the BMPR-II discussed earlier. Thus, in vivo, the lower expression/secretion of BMP-4 or –7 detected in the presence of lower BMPR-II will also result in lower progesterone production and therefore keep the F3 less differentiated. Considering progesterone as the major ovarian feedback hormone for LH release and ovulation in the chicken (an equivalent of oestradiol in mammals), the effects of BMP-4 and BMP-7 on progesterone in this study agrees with the results of a similar study in the rat (28). They showed that BMP-4 and –7 stimulated the secretion of oestradiol by rat granulosa cells. Recently, Souza et al (31) also reported that BMP-2 enhanced oestradiol production from sheep granulosa cells. In contrast, however, BMP-4 and –7 inhibited progesterone production in both rat and sheep (28, 15). There is currently no report on the role of BMPs in the theca cells. However, a recent study
using human theca-like tumour cell (HOTT) cultures showed that BMP-4 inhibits basal, or forskolin- and c’AMP-stimulated androstenedione but increased progesterone production (3). Although we did not determine BMP-4 or -7 effects in the theca in the current study, the detection of the mRNA for the peptides and receptors suggests that they might have a role in theca cell androgen or estrogen production and cell proliferation. This remains to be tested.

EGF and TGFα are paracrine growth factors that regulate follicular development in the ovary of the chicken (39, 40). The antagonistic effect of EGF/TGFα on basal or gonadotropin-stimulated progesterone production by chicken granulosa cells is well known (23, 18, 35, 36). Its effects decreased with follicular development, thus allowing follicles to increase basal and gonadotropin-stimulated progesterone production and differentiation. In the present study, TGFα inhibited basal, LH and IGF-I stimulated progesterone secretion but its effect on progesterone production was different between F1 cells and F3 cells in the presence of BMP-4 or –7. TGFα effect on progesterone production was voided in the F1 in the presence of BMPs with or without LH + IGF-I, but was still effective in the F3/4 in the presence of the BMPs with or without LH+IGF-I. This interaction between BMP and TGFα, we suspect, may be the underlying mechanism for the differential effects of TGFα as the follicle matures through the hierarchy to become the F1. Thus, the normal (in vivo) lower responsiveness of the F3 cells to LH, FSH, IGF-I or their combinations may be related to the intra-ovarian interaction between BMPs and EGF/TGFα that modulates the responsiveness of granulosa cells to endocrine factors with follicular maturation. Indeed, our data showed that BMP-4 and BMP-7 also increase with follicular development, an increase that may serve to counteract the effect of TGFα. The mechanism by which BMP suppresses TGFα effect is not yet known but may involve
the suppression of TGFα/EGF receptor expression since this receptor level also decreases with follicular growth (16).

BMP-7, but not BMP-4, exerted stimulatory effect on granulosa cell proliferation, which indicates its involvement in follicular growth. However, its effect alone was higher compared to IGF-I at a similar concentration, and had similar effect in synergism with LH or FSH in the F1. In the F3/4, the synergism with FSH was higher compared with IGF-I + LH or BMP + LH. This may be due to the greater concentration of FSH receptors in this follicle size than the F1. TGFα, like IGF-I and BMP-7, enhanced cell proliferation in both F1 and F3/4 cultures. It also had synergistic effects with IGF-I or BMP-7 in the F3/4. Although TGFα, BMP-7 and IGF-I individually enhanced F1 cell proliferation in culture, there was no synergism between TGFα and BMP-7 or IGF-I. BMP-4, although did not affect cell proliferation on its own, it seemed to show inhibitory effect in the presence of TGFα and IGF-I in both F1 and F3/4 cultures. In contrast to LH effect with BMP-7 or IGF-I, LH did not enhance TGFα effect in both F1 and F3/4 and in fact LH and FSH depressed the enhancing effect of BMP-7 + TGFα in F1 culture. These results suggest that there is an array of complex mechanisms that control the enhancement of cell proliferation during growth period and the reduction/inhibition of growth towards maturation. Thus BMP-4 and –7 and indeed the LH and FSH may be candidates for regulating TGFα and IGF-I effects on growth of follicles during development. BMPs are known to induce bone cell proliferation and differentiation (27, 38). In mammalian studies, only the BMP-6, 7 and –15 had been shown to stimulate ovarian cell proliferation (21, 22 34, 20). Otsuka and Shimasaki (20) showed that BMP-7 and –15 promoted rat granulosa cell mitotic activity and that this effect is enhanced in the presence of an oocyte. The significantly higher response by the F3/4 cells in this chicken study is
consistent with previous reports that the less mature chicken cells have greater proliferative capability than cells from more mature follicles (18, 23, 35). In the current study, the F3/4 cultures had significantly greater DNA content than the F1 either with or without stimulation. This indicates greater proliferation of cells in the F3/4 and that BMP-7, IGF-I, TGFα and their combination enhanced this capacity. It would seem therefore that the major role of BMP-7 in the F3 is to enhance growth while maintaining a lower level role in differentiation. On the other hand its major roles in the F1 and F2 may be more of differentiation rather than growth, together with BMP-4 and both binding to the same receptors. BMP-4 also seem to be involved in inhibiting too rapid growth during the growth phase and probably in inhibiting growth to allow for differentiation processes during the final stages of maturation.

In conclusion, we have shown that BMP-2, -4, -6 and –7 and the receptors that mediate their actions are present in the chicken follicles. In the granulosa, BMP-4 and –7 were shown to enhance basal, and gonadotropin-stimulated progesterone production, antagonise TGFα actions and therefore may be involved in follicular differentiation and maintenance of the follicular hierarchy. BMP-4 and -7 also showed a role in the regulation of granulosa cell proliferation and could therefore be involved in follicular growth.

ACKNOWLEDGEMENTS

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Reference


30. Souza CJK, MacDouglas C, Campbell BK, McNeilly AS, Baird DT. The Booroola (FecB) phenotype is associated with a mutation in the bone


**Figure Legends**

**Table 1**: Primer pairs used for the detection of mRNAs, their sources and the expected product sizes.

**Figure 1**: Expression of the mRNAs encoding for BMP-2, -4, -6, -7 and the receptors BMPR-IA, -IB and –II in the granulosa and theca of the chicken ovary determine by RT-PCR.

**Figure 2**: Representative semi-quantitative RT-PCR of mRNAs of the BMPs and BMPRs in the granulosa and theca of the chicken ovary. Photos shows transcripts from BMPR-IA (top panel) and BMP-7 (bottom panel) compared with the expression of GAPDH.

**Figure 3**: The levels of expression of the mRNA for BMP-2, -4, -6, and –7 in the granulosa (top panel) and theca (bottom panel) cells of the F1, F2 and F3 follicles of the chicken ovary (n=4). The superscripts abcde represent statistical comparison between levels of BMPs. Bars with different letters are significantly different (P< 0.05).

**Figure 4**: The levels of expression of the mRNA for BMPR-IA, -IB and –II in the granulosa (top panel) and theca (bottom panel) cells of the F1, F2 and F3 follicles of the chicken ovary (n=4). The superscripts abcde represent statistical comparison between levels of BMPRs among all receptor types. Bars with different letters are significantly different (P< 0.05).
**Figure 5:** Effects of different concentrations (0.01 –500 ng/ml) of BMP-4 (top panel) and BMP-7 (bottom panel) on progesterone production by granulosa cells cultured from the F and F3/4 follicles of the chicken (n=16).

**Figure 6:** Effects of BMP-4 and BMP-7 at 1 ng/ml and 5 ng/ml alone or in combination with LH (5ng/ml), IGF-I (5 ng/ml) or LH + IGF-I (5ng/ml each) on progesterone production by granulosa cells of the F1 and F3 in vitro (n=16). The superscripts abcdefgh or ABCDEF represent statistical comparison between treatments within the F1 (lower case) or the F3/4 (upper case) follicle cells respectively. Bars with different letters are significantly different (P< 0.05).

**Figure 7:** Effects of BMP-4 and BMP-7 at 1 ng/ml and 5 ng/ml alone or in combination with FSH (5ng/ml), IGF-I (5 ng/ml) or FSH + IGF-I (5ng/ml each) on progesterone production by granulosa cells of the F1 and F3/4 in vitro (n=16). The superscripts abcdef or ABCDEFG represent statistical comparison between treatments within the F1 (lower case) or the F3/4 (upper case) follicle cells respectively. Bars with different letters are significantly different (P< 0.05). For enhanced comparisons, some data points depicted here are the same data points as shown in Figure 6.

**Figure 8:** Effects of BMP-4 and TGFα individually or in combination with LH and/or IGF-I on progesterone production by granulosa cells of the F1 and F3/4 follicles (n=16). The superscripts abcdefgh or ABCDEGGH represent statistical
Comparison between treatments within the F1 (lower case) or the F3 (upper case) follicle cells respectively. Bars with different letters are significantly different (P<0.05). For enhanced comparisons, some data points depicted here are the same data points as shown in Figure 6 and 7.

**Figure 9**: Effects of BMP-4 and BMP-7 at 1 ng/ml and 5 ng/ml alone or in combination with LH or FSH (5ng/ml), IGF-I (5 ng/ml) or LH/FSH + IGF-I (5ng/ml each) on DNA synthesis by granulosa cells of the F1 and F3/4 in vitro (n=16). The DNA content of wells for the F1G and F3/4G controls was 1.08 ± 0.12 µg/well and 1.74 ± 0.08 µg/well respectively. The superscripts abcdef or ABC represent statistical comparison between treatments within the F1 (lower case) or the F3 (upper case) follicle cells respectively. Treatments with superscripts are those that are significantly greater than the controls. Bars with different letters are significantly different (P<0.05).
Table 1

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**Figure 1**

**GRANULOSA**

**THECA**
Figure 2

BMPR type 1A

GRANULOSA

THECA

GAPDH

BMP 7
Figure 3

BMPs

mRNA Expression levels (% BMP/GAPDH)

0 50 100 150 200 250

F1G  F2G  F3G

F1T  F2T  F3T

BMP-2  BMP-4  BMP-6  BMP-7

a a a a
b b b b
c d c d
d cd cd e

c

BMPs
Figure 4

![Graph showing mRNA expression levels of BMP receptors (BMPR-IA, BMPR-IB, BMPR-II) across different conditions (F1G, F2G, F3G) and treatments (F1T, F2T, F3T). The graph compares the expression levels as a percentage of BMPR/GAPDH, with annotations indicating statistical differences (a, b, c, ab, bc).]
Figure 5

BMP-4

Dose of BMP-4 (ng/ml)

Progesterone production (ng/µg DNA)

F1G

F3/4G

BMP-7

Dose of BMP-7 (ng/ml)

Progesterone production (ng/µg DNA)

F1G

F3/4G
Figure 6

**BMP-4**

- Control
- BMP-4 1 ng
- BMP-4 5 ng
- LH 5 ng
- IGF-I 5 ng
- BMP-4 1 ng/LH
- BMP-4 5 ng/LH
- IGF-I/LH
- BMP-4 1 ng/IGF-I
- BMP-4 5 ng/IGF-I
- BMP-4 1 ng/IGF-I/LH
- BMP-4 5 ng/IGF-I/LH

**Progesterone production (ng/μg DNA)**

- 0
- 10
- 20
- 30
- 40
- 50
- 60

**Treatments**

**BMP-7**

- Control
- BMP-7 1 ng
- BMP-7 5 ng
- LH 5 ng
- IGF-I 5 ng
- BMP-7 1 ng/LH
- BMP-7 5 ng/LH
- IGF-I/LH
- BMP-7 1 ng/IGF-I
- BMP-7 5 ng/IGF-I
- BMP-7 1 ng/IGF-I/LH
- BMP-7 5 ng/IGF-I/LH

**Progesterone production (ng/μg DNA)**

- 0
- 10
- 20
- 30
- 40
- 50
- 60
Figure 7

**BMP-4**

- **Treatments:**
  - Control
  - BMP4 1ng
  - BMP4 5ng
  - FSH 5ng
  - BMP4 1ng/FSH
  - BMP4 5ng/FSH
  - BMP4 1ng/IGF1
  - BMP4 5ng/IGF1
  - BMP4 1ng/IGF1/FSH
  - BMP4 5ng/IGF1/FSH

- **Progestosterone production (ng/µg DNA):**
  - 0
  - 5
  - 10
  - 15
  - 20
  - 25
  - 30
  - 35

**Results:**

- Data points with different letters indicate significant differences.

**BMP-7**

- **Treatments:**
  - Control
  - BMP7 1ng
  - BMP7 5ng
  - FSH 5ng
  - BMP7 1ng/FSH
  - BMP7 5ng/FSH
  - BMP7 1ng/IGF1
  - BMP7 5ng/IGF1
  - BMP7 1ng/IGF1/FSH
  - BMP7 5ng/IGF1/FSH

- **Progestosterone production (ng/µg DNA):**
  - 0
  - 5
  - 10
  - 15
  - 20
  - 25
  - 30
  - 35

**Results:**

- Data points with different letters indicate significant differences.
Figure 8

BMP-4

Progestosterone production (ng/µ DNA)

Treatments
Figure 9

BMP-4

BMP-7

Treatments

100% control

LH

FSH

IGFI

BMP

1 ng

5 ng

IGFI/LH

IGFI/FSH

IGFI/BMP 5 ng

LH/BMP 5 ng

FSH/BMP 5 ng

BMP/IGFI/LH

BMP5ng/IGFI/FSH

µg DNA content of well (% over control)

100 150 200 250 300 350 400

F1G

F3/4G

100%