Expression and biological effects of bone morphogenetic protein-15 in the hen ovary

S Elis, J Dupont, I Couty, L Persani¹, M Govoroun, E Blesbois, F Batellier and P Monget

Physiologie de la Reproduction et des Comportements, UMR 6175 INRA-CNRS-Université F Rabelais de Tours, Haras Nationaux, 37380 Nouzilly, France

¹Institute of Endocrine Sciences, University of Milan, 20122 Milan, Italy

(Correspondence should be addressed to P Monget; Email: monget@tours.inra.fr)

Abstract

The bone morphogenetic protein 15 (*Bmp15*) and growth differentiation factor 9 (*Gdf9*) genes are two members of the transforming growth factor- β superfamily. In mammals, these genes are known to be specifically expressed in oocytes and to be essential for female fertility. However, potential ovarian roles of BMPs remain unexplored in birds. The aim of the present work was to study for the first time the expression of *Bmp15* in the hen ovary, to compare its expression pattern with that of *Gdf9*, and then to investigate the effects of BMP15 on granulosa cell (GC) proliferation and steroidogenesis. We found that chicken *Bmp15* and *Gdf9* genes were preferentially expressed in the ovary. We showed using *in situ* hybridization that *Bmp15* and *Gdf9* mRNAs were specifically localized in oocytes of all ovarian follicles examined. We also

demonstrated using real-time quantitative RT-PCR that *Bmp15* and *Gdf9* expression was maintained during hierarchical follicular maturation in the germinal disc region and then progressively declined after ovulation. BMP15 was able to activate Smad1 (mothers against decapentaplegic homolog1) signaling pathway in hen GCs. Moreover, we showed a strong inhibitory effect of BMP15 on gonadotropin-induced progesterone production in hen GCs. This inhibitory effect was associated with a decrease in steroidogenic acute regulatory protein (STAR) level. Taken together, our results suggest that BMP15 may have a key role in the female fertility of birds.

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Introduction

Recent evidence indicates that bone morphogenetic proteins (BMPs) are expressed in a cell-specific manner in the mammalian ovary, and they play key roles in the regulation of folliculogenesis, ovulation, and corpus luteum functions (Knight & Glistler 2003, Su *et al.* 2004, Pierre *et al.* 2005). The *Bmp15* and growth differentiation factor 9 (*Gdf9*) genes are two members of the transforming growth factor- β (TGF- β) superfamily. They are specifically expressed in oocytes and essential for female fertility in several species, including humans, sheep, and mice (for a review, see Juengel *et al.* 2004). The importance of these factors in mice ovarian function has been confirmed by gene knockout experiments. Female mice lacking the *Gdf9* gene are infertile, with follicular growth arrested at the primary stage (Dong *et al.* 1996), whereas *Bmp15* knockout female mice are fertile, with only slight changes in folliculogenesis (Yan *et al.* 2001). Moreover, partial loss of function of *Bmp15* in ewes leads to an increase in the ovulation rate, whereas mutation leading to complete loss of function dramatically affects folliculogenesis and fertility in sheep and humans (Galloway *et al.* 2000, Juengel *et al.* 2002, Hanrahan *et al.* 2004, Di Pasquale *et al.* 2004). These factors could thus affect ovarian function in a species-specific manner (Hashimoto *et al.* 2005).

The expression pattern of these two genes differs between species, *Gdf9* being expressed in primordial follicles in bovine, ovine, and brushtail possum species (Bodensteiner *et al.* 1999, Eckery *et al.* 2002) but not in mice. Usually, the *Bmp15* gene is not expressed in primordial follicles in mice, sheep, and cattle, whereas it is found in primordial follicles in the brushtail possum (Eckery *et al.* 2002). Moreover, GDF9 and BMP15 regulate a variety of murin granulosa and theca cell functions *in vitro*. The BMP15 effects on granulosa cells (GCs) are mediated through activation of several signaling pathways, including the Smad1/5/8 (mothers against decapentaplegic homolog 1/5/8) and/or mitogen-activated protein kinase (MAPK) (extracellular signal-regulated kinase 1/2, ERK) signaling pathway (Moore *et al.* 2003, Su *et al.* 2004). In contrast to BMP4 and BMP2, BMP15 has not been shown to activate the MAPK p38 pathway (Yamaguchi 1995). In rodents, both GDF9 and BMP15 inhibit follicle-stimulating hormone (FSH)-stimulated steroid production by GCs, partly through inhibition of expression of gonadotropin receptors (Otsuka *et al.* 2000). Indeed, in rat GCs, GDF9 suppresses binding of human chorionic gonadotropin (hCG; Vitt *et al.* 2000), and BMP15 decreases FSH receptor mRNA expression (Otsuka *et al.* 2001). This inhibitory effect of BMP15 on FSH-induced progesterone secretion is associated with a decrease in the protein level of steroidogenic acute

regulatory protein (StAR), an important cholesterol carrier, and two key enzymes of steroidogenesis 3β hydroxysteroid dehydrogenase (3β -HSD) and P450_{scc} (P450 side chain cleavage; Otsuka *et al.* 2001). Moreover, GDF9 and BMP15 exert a mitogenic effect in human (Di Pasquale *et al.* 2004) and rat GCs (McNatty *et al.* 2005). In birds, oocyte-conditioned medium increased chicken GC proliferation *in vitro*. This effect is inhibited by GDF9 antibody suggesting that GDF9 also exerts a mitogenic effect in avian GCs (Johnson *et al.* 2005).

Study of the reproductive physiology of the hen shows numerous specific features which include oviparity and telolecithal oocytes (Gilbert 1971). There are several differences between mammalian and hen ovarian folliculogenesis. The ovary of reproductively active hens contains small pre-hierarchical follicles and maturing preovulatory follicles showing a hierarchy according to their size (F6–F1; Etches & Petite 1990). In laying hens, ovulation may occur almost every day, the largest follicle (F1) filled with yolk, being ovulated first, then the second largest (F2) the following day, and so on until a pause interrupts the sequence of ovipositions, generating successive multiple series of ovipositions. The avian oocyte, which can reach the size of 40 mm in diameter before ovulation, consists of a large amount of yolk and a structure called the germinal disc. The germinal disc is a white plaque about 3–4 mm in diameter on the surface of the oocyte. It contains the nucleus and 99% of organelles of the oocyte even though it occupies <1% of oocyte volume (Yao & Bahr 2001). Structurally, and therefore functionally, the germinal disc is equivalent to the mammalian oocyte. Before ovulation, the germinal disc is closely associated with its overlying GCs and forms a structure called the germinal disc region (GDR). GCs cannot be separated from it (Perry *et al.* 1978, Tischkau & Bahr 1996, Malewska & Olszanska 1999). Estrogen production by hierarchical follicles declines from F6 to F1, while progesterone production increases (Huang & Nalbandov 1979). The GCs from the largest follicles produce large amounts of progesterone. In contrast to mammalian GCs, chicken GCs produce no estrogen, which is synthesized only by theca cells (Huang & Nalbandov 1979, Marrone & Hertelendy 1983).

Few studies have been undertaken on BMP expression and roles in hen ovaries. Onagbesan *et al.* (2003) have shown that BMP4 and BMP7 increase basal and insulin-like growth factor-I (IGF-I)- and gonadotropin-induced progesterone production in hen GCs (Onagbesan *et al.* 2003). Moreover, they have demonstrated that BMP4 inhibits FSH-stimulated GC proliferation (Onagbesan *et al.* 2003). Another recent study indicated that GDF9 is expressed in both hen oocytes and GCs, where it exerts a mitogenic effect (Johnson *et al.* 2005). However, most of the presumptive ovarian roles of BMPs remain unexplored in birds.

The aim of the present study was first to determine the temporal and spatial dynamics of the *Bmp15* gene expression and to confirm those of *Gdf9* in the hen ovary and early embryogenesis, and then to investigate *in vitro* the effects of BMP15 on GC proliferation and steroidogenesis.

Materials and Methods

Animals

Laying breed hens, aged 60–70 weeks (ISA Brown, egg layer type, Institut de Selection Animale, Saint Briec, France), were housed individually in laying batteries with free access to feed and water and were exposed to a 15 h light:9 h darkness photoperiod, with lights-on at 2000 h. Individual laying patterns were monitored daily. For *in situ* hybridization, these hens and younger ones aged 10 weeks were used to provide mature and immature ovaries, in order to study follicles of each stage. Hens used to provide fertilized eggs were inseminated once a week.

Hormones and reagents

Purified ovine FSH-20 (oFSH; lot no. AFP-7028D, 4453 IU/mg, FSH activity = 175 times activity of oFSH-S1) and purified ovine luteinizing hormone (LH; lot 26; a gift from NIDDK, National Hormone Pituitary Program, Bethesda, MD, USA) and human recombinant IGF-I (Sigma) were used for culture treatment. Dulbecco's modified Eagle's medium (DMEM), penicillin, and streptomycin were purchased from Invitrogen, and thymidine methyl- H^3 from Perkin–Elmer Life and Technological Sciences (Boston, MA, USA). Human recombinant BMP15 was prepared as previously described (Di Pasquale *et al.* 2004). Human recombinant and chicken proteins showed 44% identity, but the two proteins are 72.8% identical and 79.8% homologous in the C-terminal mature peptide region (Fig. 1). Human recombinant BMP4 was purchased from R&D (R&D Systems, Oxon, UK); human and chicken BMP4 are 84% identical.

Antibodies

Rabbit polyclonal antibodies to phospho-Smad1 and Smad1 were purchased from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Rabbit polyclonal antibodies against P450_{scc} and StAR were generously provided by Dr Dale Buchanan Hales (University of Illinois, Chicago, IL, USA) and antibodies against 3β -HSD by Dr Van Luu-The (CHUL Research Center and Laval University, Canada). The monoclonal antibody to Vinculin was obtained from Sigma. All antibodies were used at 1:1000 dilution in western blot analysis.

RNA isolation and RT-PCR

Hens aged 60 weeks were used. Total RNA was extracted from different tissues (ovary, spleen, intestine, gizzard, liver, heart, skin, brain, pectoralis muscle, and lung) and GDRs (Malewska & Olszanska 1999) from different preovulatory follicles (F1–F6) as previously described, just ovulated oocytes and early embryos at 6.5, 12, 24, 36, and 48 h post-ovulation. For the last two stages, eggs were incubated at 37.8 °C for 12 and 24 h respectively. During follicular maturation, the GDR used for these studies consisted of not only the germinal disc but also the overlying layer

Figure 1 Aligned amino acid sequence. Data for chicken BMP15 compared with several species in the biologically C-terminal region. The light-grey shading indicates areas of identity and the dark-grey one indicates areas of similarity between the six species shown.

of GCs, because it is impossible to separate the germinal disc from its overlying GCs (Tischkau & Bahr 1996). However, the number of GCs could not be measured. Total RNA was extracted using TRI reagent according to manufacturer's procedure (Euromedex, Mundolsheim, France). RNA was quantified by measuring the absorbance at 260 nm. Samples were stored at -80°C until use. RT-PCR was performed to test the expression of *Bmp15* and *Gdf9* genes in different tissues and at different stages of follicular maturation and embryo development. Specific sets of primer pairs designed to amplify parts of *Bmp15* and *Gdf9* genes are shown in Table 1. PCR amplification without cDNA was performed in parallel as negative control. RT-PCR disposables were purchased from Sigma, except for Moloney murine

leukemia virus reverse transcriptase and RNase inhibitor (RNasin) which were purchased from Promega. Real-time PCR disposables were purchased from Eurogentec (qPCR Mastermix Plus for Sybr Green I, Eurogentec, Angers, France). *Efl- α* was used as reporter gene, its expression being similar in GCs and in GDR (data not shown). GCs without germinal disc and GCs localized in the vicinity of the germinal disc were studied as control for each follicle stage used.

In situ hybridization

Female chickens were killed at different stages of sexual development. Two types of tissues were used: mature ovaries

Table 1 Oligonucleotide primer sequences

	Sequence	Accession number	Product size (bp)
Primer			
<i>bmp15</i> forward 1	GGG GGA TTT GGG TTT TAG CC	AY725199	142
<i>bmp15</i> reverse 1	GGT AAA GCA GAA AGA GGT CCC G		
<i>gdf9</i> forward 1	TGA TGT GAC TTC TTT TCT GCG GC	AY672110	105
<i>gdf9</i> reverse 1	TTA GTG TTA TGT TGC GGG TTA CCC		
<i>bmp15</i> forward 2	TGG GGG ATT TGG GTT TTA GC	AY725199	727
<i>bmp15</i> reverse 2	AAA TAG GGG GTG ATG TCC GC		
<i>gdf9</i> forward 2	GGT ACG TGG AGG ATC TGT GTT TGT T	AY672110	757
<i>gdf9</i> reverse 2	CCT GTT TAG TGT TAT GTT GCG GG		
<i>efl-α</i> forward	AGC AGA CTT TGT TGT GAC CTT GCC	NM 204157	83
<i>efl-α</i> reverse	TGA CAT GAG ACA GAC GGT TGC		

containing follicles of different sizes (50 μm –7 mm) from 60-week-old hens, most follicles being larger than 300 μm , and immature ovaries containing a majority of small follicles (25–500 μm) from 10-week-old hens, most follicles being smaller than 100 μm . Mature and immature ovaries were then collected and embedded in Tissue-Tek (Sakura Finetek Europe BV, Zoeterwoude, The Netherlands). Frozen ovaries were serially sectioned with a cryostat (thickness of 10 μm) to perform *in situ* hybridization experiments using ^{35}S -labeled chicken *Bmp15* and *Gdf9* cRNA. The *bmp15* and *gdf9* antisense and sense constructs used for *in situ* hybridization were generated by inserting fragments of chicken *Bmp15* or *Gdf9* cDNA (727 and 757 bp respectively) into the pGEM-T vector (Promega), and selecting a clone with the appropriate antisense or sense orientation. The *Bmp15* and *Gdf9* cDNA fragments were generated by RT-PCR from chicken ovary mRNA using forward and reverse primers (Table 1). The *in situ* hybridization was performed as previously described (Pierre *et al.* 2005). Hybridization specificity was assessed by comparing signals obtained with the cRNA antisense probe and the corresponding cRNA sense probe.

Isolation and culture of GCs

Hens were killed 12 h before the next oviposition, and the follicles were immediately removed and placed in ice-cold sterile 1% NaCl saline solution for immediate use. GCs from F1, F2, and mixed F3 and F4 (F3/4) follicles were dispersed in 0.3% collagenase type A (Roche Diagnostic) in F12 medium containing 5% FBS. Cells were recovered by centrifugation, washed with fresh medium, and counted in a hemocytometer. The culture medium was DMEM supplemented with 100 U/ml penicillin, 100 mg/l streptomycin, 3 mmol/l L-glutamine, and 5% FBS. The cells were initially cultured for 24 h in serum-supplemented medium (5%) with no treatment and incubated in fresh culture serum-free medium with or without test reagents for the appropriate time. All cultures were performed under a water-saturated atmosphere of 95% air/5% CO_2 at 37 °C. Seven independent replicate cultures were carried out.

Western blotting

GCs were solubilized and centrifuged as previously described (Tosca *et al.* 2005). Cell extracts were then submitted to electrophoresis on 10% (w/v) SDS-polyacrylamide gel under reducing conditions and electrotransferred as previously described (Tosca *et al.* 2005). The membranes were then incubated overnight at 4 °C with appropriate antibodies (final dilution 1:1000) in Tris-buffered saline (TBS, 2 mM Tris-HCl (pH 8), 15 mM NaCl (pH 7.6)) containing 0.1% Tween 20 and 5% non-fat dry milk powder (NFDMP). After washing in TBS–Tween 20 (0.1%), nitrocellulose membranes were incubated for 2 h at room temperature with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (final dilution 1:10 000; Diagnostic Pasteur, Marnes-la-Coquette,

France) in TBS–Tween 20 (0.1%) NFDMP (5%). After washing in TBS–Tween 20 (0.1%), the signal was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech). The films were analyzed and signals quantified with the Scion Image Beta software 4.0.2 (Fuji PhotoFilm, Edison, NJ, USA).

Progesterone RIA

The concentration of progesterone in the GC culture medium was measured after 32 h of culture by an RIA protocol as previously described (Tosca *et al.* 2006), adapted to measure steroids in cell culture media (Tosca *et al.* 2006). The limit of detection of progesterone was 12 pg/tube (60 pg/well) and the intra- and inter-assay coefficients of variation were <10 and 11% respectively. Results were expressed as the amount of steroids secreted per 300 000 cells over 32 h (number of cells/well).

Thymidine incorporation into GCs

GCs were seeded in 24-well dishes (2×10^5 viable cells/500 μl). After 24 h, cells were serum starved overnight, and [^3H]thymidine (1.5 $\mu\text{Ci/ml}$) was added in the absence or presence of BMP15 (50 or 200 ng/ml), BMP4 (5 or 50 ng/ml), IGF-I (10^{-8} M), LH (10^{-8} M), or FSH (10^{-8} M). After 24 h, excess [^3H]thymidine was removed by washing twice with PBS; cells were then fixed with ice-cold trichloroacetic acid (50%) and lysed by NaOH (0.5 N). The radioactivity in the cells was determined after resuspension by scintillation counting in a β -photomultiplier. Seven independent replicate experiments were carried out.

Statistical analysis

All experimental data are presented as means $\pm$ S.E.M. One-way ANOVA was used to test differences. If ANOVA revealed significant effects, the means were compared by Fisher's test, with $P < 0.05$ considered significant. Different letters indicate significant differences.

Results

Expression of *Bmp15* and *Gdf9* mRNA in hen ovary and other tissues

RT-PCR analysis showed that *Bmp15* and *Gdf9* were preferentially expressed in the hen ovary. However, *Bmp15* mRNAs were also present in the brain, but at a low level (Fig. 2). This latter expression was confirmed by real-time PCR (data not shown). *In situ* hybridization on ovarian sections (Fig. 3) revealed that *Bmp15* mRNA was found in small oocytes from follicles of 50–100 μm from both immature (Fig. 3A) and mature ovaries, and in larger oocytes from follicles of 500 μm –6 mm from mature ovaries (Fig. 3C). *Gdf9* mRNA was also

Figure 2 RT-PCR analysis of *Bmp15* and *Gdf9* genes in hen tissues. Total RNA was isolated from whole ovary (O), spleen (Sp), intestine (I), gizzard (G), liver (Li), heart (H), skin (Sk), brain (B), muscle (M), and lung (Lu) tissues, and RT-PCR was performed as described in Materials and Methods. Ethidium bromide staining of the gel is shown for *Bmp15*, *Gdf9*, and *Efl- α* . *Efl- α* was chosen as an ubiquitous gene. (C) corresponds to the negative control (water).

observed in small oocytes from follicles of 50–100 μm from immature ovaries (Fig. 3B), and a signal was found in oocytes from all follicles of 50 μm –6 mm from mature ovaries (Fig. 3D). No significant expression was detected in somatic cells.

For larger follicles that could not be examined by *in situ* hybridization, we performed real-time PCR on GDR mRNA extracted from F6 to F1 follicles, from ovulated oocytes, and from early embryos at 6.5, 12, 24, 36, and 48 h after ovulation (fertilization occurs ~ 30 min after ovulation). The expression pattern was fairly similar for *Bmp15* and *Gdf9* (Fig. 4). During follicular maturation, *Bmp15* and *Gdf9* genes were found to be expressed at a higher level in GDR than in GCs, 7- to 30-fold and 2- to 16-fold for *Bmp15* and *Gdf9* respectively. Their expression levels did not vary from stage F5 to F1 for *Bmp15* and from stage F4 to F1 for *Gdf9*. The *Bmp15* expression level was 1.9-fold higher at stage F6 than at the other stages, and *Gdf9* expression level was five- and tenfold higher at stages F5 and F6 respectively (Fig. 4A and B). Expression of both mRNA progressively decreased after ovulation, becoming very low at 24 h after ovulation (Fig. 4C and D).

Effects of BMP15 on Smad signaling pathway in hen GCs

We investigated whether BMP15 was able to activate a signaling pathway in GCs from F3/4 and F1 follicles. BMP4 was chosen as a positive control, since it has been shown to

Figure 3 Localization of *Bmp15* and *gdf9* mRNA in the hen ovary. Localization of *Bmp15* (A and C) and *Gdf9* (B and D) by *in situ* hybridization in immature ovaries (A and B) and in mature ovaries (C and D). Bright fields are on the left and dark fields are on the right. The control sections were hybridized with the *Bmp15* or *Gdf9* sense probes. Oocytes (Oo), granulosa cells (GCs), and somatic cells (SCs) are indicated. Scale bar=100 μm .

A. *Bmp15* B. *Gdf9* C. *Bmp15* D. *Gdf9*

Figure 4 Expression of *Bmp15* and *Gdf9* genes during follicular maturation and early embryo development. Real-time RT-PCR analysis was performed on GCs and germinal disc regions of the six largest follicles of the hen ovary. Follicles are designated as F6–F1, F1 being the largest follicle taken 24 h before ovulation. A and B show the expression in GCs and germinal disc regions of *Bmp15* and *Gdf9* respectively. C and D show the expression during early embryo development, at the moment of ovulation and 6.5, 12, 24, 36, and 48 h after ovulation of *Bmp15* and *Gdf9* respectively. Expression of *Bmp15* and *Gdf9* was quantified in relation to the ubiquitous gene *Ef1-α*. Results represent means ± S.E.M. The same letters indicate that difference was not significant. Different letters indicate that difference was significant ($P < 0.05$).

have a biological function in GCs in both the hen (Onagbesan *et al.* 2003) and in different mammals (Pierre *et al.* 2004, Fabre *et al.* 2006). Western blot analysis revealed that both peptides significantly increased phosphorylation of Smad1 in GCs from F1 and F3/4 (Fig. 5). However, there was no significant effect of BMP15 on the state of MAPK p38 phosphorylation, a member of the MAPK signaling pathway (data not shown).

Effects of BMP15 on GC steroidogenesis

We next studied the effects of BMP15 on GC steroidogenesis at different stages of follicular maturation (F3/4, F2, and F1). Avian GCs produce no estradiol, so we investigated only the effect of BMP15 on the progesterone level. Surprisingly, IGF-I had no stimulatory effect on progesterone secretion for each stage considered and even a 1.7-fold inhibitory effect on F1 GCs (Fig. 6C). As expected, LH treatment caused an increase in progesterone production for each stage tested (F3/4, F2, and F1 GCs; Fig. 6). FSH treatment increased progesterone production in GCs from F3/4 and F2 follicles, but no significant effect was observed in F1 GCs. LH treatment induced 3.5- ($P < 0.0001$), 3.9- ($P < 0.0001$), and 1.5-fold ($P < 0.0001$) increases in progesterone secretion on F3/4, F2, and F1 GCs respectively (Fig. 6A–C). FSH treatment induced 1.6- ($P < 0.05$) and 1.9-fold ($P < 0.0001$)

increases in progesterone secretion on F3/4 and F2 GCs respectively (Fig. 6A and B).

BMP15 (200 ng/ml) induced a 1.3-fold ($P < 0.05$) decrease in basal progesterone production on F1 GCs (Fig. 6C). No effect on basal progesterone secretion was observed at the other stages, whatever the BMP15 concentration. On the other hand, BMP15 showed a strong inhibitory effect on LH- and FSH-induced progesterone secretion for the different stages considered (Fig. 6). In F3/4 GCs, LH-induced progesterone production decreased 4.6-fold ($P < 0.0001$), and FSH-induced progesterone production decreased 2.9-fold ($P < 0.001$) under BMP15 treatment. In F2 GCs, LH- and FSH-induced progesterone production decreased 2.8- ($P < 0.0001$) and 2.4-fold ($P < 0.0001$) respectively. In F1 GCs, only the inhibitory effect on LH-induced progesterone secretion was significant, decreasing twofold ($P < 0.0001$). Similar overall effects were observed with BMP4 (Fig. 6).

Effects of BMP15 on protein levels of StAR, 3β-HSD, and P450_{sc}

BMP15 did not have any effect on the expression of the 3β-HSD and P450_{sc} steroidogenesis enzymes as assessed by immunoblot analysis, whatever the treatment or stages

Figure 5 Effects of BMP15 on levels of Smad1 phosphorylation. GCs were cultured in the presence of BMP15 (50 ng/ml) or BMP4 (5 ng/ml) for 0, 5, 30, 60, and 120 min. Western blot analysis was then performed, and membranes were probed with an antibody against phospho-Smad and then reprobed with an antibody against Smad1 antibody. Data are represented as the phospho-Smad1/Smad1 ratio in F3/4 GCs (A) and in F1 GCs (B). Results represent means $\pm$ S.E.M. The same letters indicate that difference was not significant. Different letters indicate that difference was significant ($P < 0.05$).

studied (data not shown). Expression of StAR increased when GCs from F3/4 to F1 were treated by LH, and when GCs from F3/4 and F2 were treated by FSH (Fig. 7). Combined treatment with LH or FSH and BMP15 (50 ng/ml) had a significant inhibitory effect on the expression of StAR ($P < 0.0001$ and $P < 0.01$ respectively) in F3/4 and F2 GCs (Fig. 7A and B). In F1 GCs, only combined treatment with LH and BMP15 resulted in a significant decrease in the protein level of StAR ($P < 0.05$; Fig. 7C). Similar results were found after BMP4 treatment, but the effect was slighter.

Effects of BMP15 on GC proliferation

IGF-I had the greatest stimulatory effect on GCs, the thymidine incorporation level being increased 2.8-, 2.7-, and 4.1-fold in F3/4, F2, and F1 GCs respectively (Fig. 8). The stimulatory effect of LH on proliferation was only significant in F1 GCs (1.9-fold). FSH also had a significant stimulatory effect in F3/4 and F1 GCs (1.7- and 1.6-fold respectively). No significant effect of BMP15 alone was observed. In F3/4 GCs, combined treatment with FSH and BMP15 (50 ng/ml) resulted in a significant decrease in thymidine incorporation ($P < 0.001$; Fig. 8A). No other significant effect was observed with combined BMP15 and IGF-I or gonadotropin treatment. Only significant stimulatory effect of BMP4 on proliferation induced by IGF-I in F2 GCs was observed. No effect of BMP15 (200 ng/ml) was observed on GC viability when evaluated with Trypan blue staining (data not shown).

Discussion

The regulation of the expression and function of chicken genes involved in the endocrine-dependent maturation of the oocyte has some consequences on the development of the early embryo. Among these factors, the genes of the TGF- β family, including *Gdf9* and *Bmp15*, may be particularly important. The *Gdf9* gene has already been shown to be an ovary-specific gene and to exert positive effects on GC proliferation in many species (mice, rat, human, and hen; Juengel *et al.* 2004, Johnson *et al.* 2005). In the present study, we demonstrated that *Bmp15* was expressed in the germinal disc and in the early embryo in the hen. Moreover, we showed a strong inhibitory effect of human BMP15 on gonadotropin-induced progesterone production of hen GCs. This inhibitory effect was associated with a decrease in StAR protein level.

As in mammals (Fitzpatrick *et al.* 1998, Aaltonen *et al.* 1999, Bodensteiner *et al.* 1999, Jaatinen *et al.* 1999, Eckery *et al.* 2002), we confirmed in this study that *Gdf9* has specific ovarian expression in the hen (Johnson *et al.* 2005). The *Bmp15* gene has also been reported to be oocyte specific in most species (Juengel *et al.* 2004), and for the first time to our knowledge, we found that it was mainly expressed in the hen ovary, with weak expression also occurring in the brain. Our real-time RT-PCR data showed a slight expression in the pituitary gland (data not shown). This latter result is concordant with studies on rats which showed that the *Bmp15* gene was slightly expressed in the pituitary gland (Otsuka & Shimasaki 2002).

Figure 6 Effects of BMP15 on progesterone secretion by cultured hen GCs. GCs (300 000 viable cells) were cultured in serum-free medium as described in Materials and Methods in the presence or absence of IGF-1 (10^{-8} M), LH (10^{-8} M), or FSH (10^{-8} M) with the combination of BMP15 (50 or 200 ng/ml) or BMP4 (5 or 50 ng/ml). Progesterone secretion was measured in GCs at different stages of follicular maturation (F3/4, F2, and F1: A, B, and C respectively). After 32 h of culture, the progesterone levels in the medium were measured by RIA. Each combination of treatments was tested in quadruplicate in seven independent experiments. Results represent ng/ml of progesterone secretion by 300 000 GCs after 32 h of culture *in vitro*. Data are shown as means $\pm$ S.E.M. Different letters indicate that difference was significant ($P < 0.05$). Seven independent experiments were performed in triplicate.

We succeeded in using *in situ* hybridization on hen ovaries and specifically localized *Bmp15* and *Gdf9* mRNA in oocytes. This localization was close to that previously found in mammals (Aaltonen *et al.* 1999, Bodensteiner *et al.* 2000). The *Gdf9* and *Bmp15* genes were expressed in all hen follicles studied (50 μ m–6 mm). It was surprising to observe almost wide-spread localization of *Gdf9* mRNA throughout the oocyte, while previous immunocytochemistry studies (Johnson *et al.* 2005) localized GDF9 protein under the vitelline membrane adjacent to the granulosa layer. However, these techniques are different and do not localize the same molecules, mRNA or protein. Moreover, in our *in situ* hybridization experiments, any signal was observed with sense probes, strongly suggesting that the hybridizations are specific. However, in small oocytes (<1 mm), it is already known that the germinal disc is localized in the center of the oocyte (Sauveur 1988). Then, the germinal disc moved to the surface of the oocyte, where it stayed until the end of the follicle growth. This could explain why we found a central localization of mRNA in the oocyte.

In situ hybridization did not detect any significant signals of *Bmp15* and *Gdf9* in hen GCs. However, we also observed a slight expression in GCs by real-time RT-PCR, in accordance with previous studies in primate (Duffy 2003) and in the hen (Johnson *et al.* 2005). However, it is possible that there is a contamination of GCs dissected from perivitelline membrane by the so-called ‘extra-embryonic’ RNA, i.e., by mRNA containing oocyte cytoplasm, as previously described by Malewska & Olszanska (1999). Moreover, the different sensitivity of these two techniques could explain our results. As shown in mammals, both could be involved in the differentiation of growing follicles, rather than in early embryo development (Juengel *et al.* 2004, Li *et al.* 2007, Pennetier *et al.* 2004, Su *et al.* 2004). Indeed, as shown in the bovine model (Pennetier *et al.* 2004), the expression of both of these genes decreases after ovulation, even after embryonic genome activation. Our data suggest that the levels of *Gdf9* and *Bmp15* mRNA in germinal disc start to decrease after ovulation but they are still present till oviposition, 24 h post-ovulation, and disappear in the case of *Bmp15* after embryonic

Figure 7 Effects of BMP15 on StAR protein level. GCs (300 000 viable cells) were cultured in serum-free medium as described in Materials and Methods in the presence or absence of IGF-I (10^{-8} M), LH (10^{-8} M), or FSH (10^{-8} M) with the combination of BMP15 (50 or 200 ng/ml) or BMP4 (5 or 50 ng/ml) for 32 h. Total cellular proteins were then extracted and subjected to western blotting as described in Materials and Methods. All extracts were probed with anti-StAR antibody and then re-probed with anti-Vinculin antibody. The StAR protein level was measured in GCs from F3/4, F2, and F1 preovulatory follicles (A, B, and C respectively). Data presented are representative of four independent experiments. Data are shown as means $\pm$ S.E.M.

genome activation which occurs soon after oviposition (Zagris *et al.* 1998). The chicken embryonic genome reactivation occurs when the embryo contains 30 000–50 000 cells. The relevance of this study is reinforced by the fact that proteins and mRNA accumulated during chicken oocyte maturation are essential not only for fertilization and first cleavage but also for supporting such a high number of embryonic cell divisions. For comparison, in the cattle model, the embryonic genome is activated at the eight-cell stage (Meirelles *et al.* 2004), and in the murine model, it occurs at the two-cell stage (Eric *et al.* 1998).

Since it is difficult to realize GC culture from follicles smaller than F4, and even if BMP15 expression is higher in small than in large follicles, we studied BMP15 effects on GCs from F3/4 to F1 stage. The increase in Smad1 phosphorylation after BMP15 treatment showed for the first time that, as

in mammals (Moore *et al.* 2003), BMP15 may activate the Smad 1/5/8 pathway in chicken GCs. BMP4 exhibited the same increase in level of Smad1 phosphorylation, confirming results obtained in other vertebrate models (Baemans & Van Hul 2002). Other studies have shown that the MAP kinase pathway (ERK, p38) is involved in the intracellular transduction of BMP signals (Moore *et al.* 2003, Su *et al.* 2004). However, no significant effect of BMP15 on p38 phosphorylation was observed in the present study (data not shown). It would be interesting to see whether BMP15 activates the p38 signaling pathway in mammals.

As previously observed with perfused rabbit ovaries (Yoshimura *et al.* 1996), in our study, IGF-I alone showed no effect on progesterone secretion by GCs, whatever the follicular stage. Onagbesan *et al.* (2003) discussed the lack of effect of BMP4 and IGF-I alone on hen GCs, and hypothesized that

Figure 8 Effects of BMP15 on proliferation rate of cultured hen GCs. Thymidine incorporation was determined in GCs from F3/4, F2, and F1 preovulatory follicles cultured for 24 h in the presence or absence of IGF-I (10^{-8} M), LH (10^{-8} M), or FSH (10^{-8} M) with the combination of BMP15 (50 or 200 ng/ml) or BMP4 (5 or 50 ng/ml) as described in Materials and Methods. Results are representative of at least three independent experiments. The results are expressed as means $\pm$ S.E.M. in relation to values in the absence of treatment (basal state). Different letters indicate that difference was significant ($P < 0.05$). Seven independent experiments were performed in triplicate.

these factors act as modulators of gonadotropin action on hen GCs, as previously observed in the rat (Huang *et al.* 2001) but not in the sheep (Campbell *et al.* 1995). In addition, a study on rat ovarian theca cells showed that IGF-I alone did not stimulate androstenedione production and had only an effect when used in combination with other factors (Huang *et al.* 2001). In the present study, a strong inhibitory effect of BMP15 on LH-induced progesterone secretion was observed at all maturation stages. Such effects of BMPs have previously been reported on basal and LH-induced androgen production in bovine theca interna cells (Glister *et al.* 2005). Moreover, the fact that we studied follicles a few days before ovulation (60, 36, and 12 h before ovulation) might explain such a high sensitivity of GCs to LH action. An inhibitory effect was also observed in FSH-stimulated conditions for F3/4 and F2 stages. These BMP15 inhibitory effects were associated with a marked decrease in StAR expression in LH- and FSH-stimulated conditions. These effects associated with the increase of Smad phosphorylation showed that recombinant human BMP15 is biologically active on hen GCs. The BMP15 inhibitory effect on

FSH action in GCs has been widely documented in mammals (Otsuka *et al.* 2000, Su *et al.* 2004, McNatty *et al.* 2005). The absence of effect of BMP15 on FSH-induced progesterone secretion could be explained by a relatively low level of FSH receptor (FSHr) expression in the F1 follicles. In particular, in rat GCs, BMP15 was shown to inhibit FSH action by suppressing FSHr expression (Otsuka *et al.* 2001). It is of note that FSHr mRNA concentration is lower in F1 GCs than in smaller follicles (Yamamura *et al.* 2001). It would be interesting to investigate whether the effect of BMP15 on GC progesterone secretion is associated with a modulation of LH or FSH receptor expression, and/or with cAMP production level.

The steroidogenic enzymes 3β -HSD, P450_{scc}, and StAR were studied to investigate whether the inhibitory effect of BMP15 was due to a decrease in their expression (Otsuka *et al.* 2001). The only significant difference was obtained with StAR. Combined treatment with BMP15 (or BMP4) and LH (or FSH) caused a decrease in progesterone secretion as well as a decrease in StAR protein level. This combined treatment did not affect 3β -HSD or P450_{scc} expression, but could

possibly affect their activity. The activities of these enzymes should be investigated in order to elucidate the mechanism of BMP15 action on hen GCs.

One interesting finding in this study was, in contrast to mammals, the absence of effect of BMP15 on GC proliferation. As a positive control, we verified that our GCs were able to proliferate in our *in vitro* conditions, IGF-I exerting in particular a strong mitogenic effect on these cells. It is possible that, as suggested by Juengel *et al.* (2006), which compared the effects of other BMPs from different mammalian species, human recombinant BMP15 that we used is not fully efficient on chicken cells, due to the relatively low percentage of identity (72%) between BMP15 sequence of both species. It is also possible, as suggested by McNatty *et al.* (2005), that the presence of GDF9 is necessary for BMP15 to exert biological effect on proliferation of hen GCs. Of note, there was a significant inhibitory effect of BMP15 on proliferation of FSH-stimulated F3/4 GCs (1.7-fold less). It is therefore possible that, unlike mammalian species, there is only a slight or no effect of BMP15 on chicken GC proliferation.

In the present study, BMP4 and BMP15 presented similar effects on progesterone production and proliferation. In mammals, the effects of BMP15 and BMP4 have already been identified on progesterone secretion and GC proliferation. Both proteins have an inhibitory effect on progesterone secretion. The stimulatory effect of BMP4 on GC proliferation depends on the model studied (rat versus sheep; Juengel *et al.* 2006). Such a similar effect could reveal redundant molecules, produced in order to achieve essential functions.

Our BMP4 results differ from those of Onagbesan *et al.* (2003) reporting an intense stimulatory effect of BMP4 on LH-, FSH-, and/or IGF-I-induced progesterone secretion and on FSH- and/or IGF-I-induced proliferation. It is of note that BMP4 is known to be a potent inhibitor of progesterone secretion by GCs in every mammal studied (Pierre *et al.* 2004). In our study, BMP4 had almost the same effect as BMP15 on progesterone secretion and proliferation. One explanation for the differences between the two studies could be that the culture conditions were different. In the study of Onagbesan *et al.* (2003), M199 medium was used and the total culture period was longer (72 h) than in the present work (32 h; Onagbesan *et al.* 2003).

Previous studies have already shown that GDR destruction suppresses follicular maturation and ovulation and leads to atresia (Yoshimura *et al.* 1994, Yao *et al.* 1998). In addition, GDR produces proliferation-stimulating and steroidogenesis-inhibiting factors that influence GCs (Tischkau & Bahr 1996). In the present study, we showed that *Bmp15* was expressed by the oocyte, and recombinant BMP15 had a strong inhibitory effect on gonadotropin-stimulated progesterone secretion. The high similarity between human recombinant and chicken BMP15 could partially explain the similar effect of BMP15 on progesterone in rat and hen GCs (Otsuka *et al.* 2000). Overall, these findings suggest that BMP15 might be used by the oocyte together with other factors as EGF and GDF9 to modulate GC differentiation and progesterone

production. EGF, also produced in the germinal disc, stimulates proliferation and decrease basal progesterone production (Volentine *et al.* 1998), and GDF9 was reported to stimulate proliferation (Johnson *et al.* 2005). The fact that in chicken, BMP15 has a similar expression pattern and in part similar effects on GCs as in mammals, provides strong evidence of the conserved essential role of BMP15 in the ovarian function in mammals and birds. Moreover, GDF9 has a similar expression pattern in the chicken and in mammals, and it enhances GC proliferation (Johnson *et al.* 2005). We hypothesize that BMP15 and GDF9 play an essential role in the regulation of the ovulation rate, normal follicular development and female fertility in the hen as in mammals (Dong *et al.* 1996, Yan *et al.* 2001, Hanrahan *et al.* 2004, Fabre *et al.* 2006). Future studies should investigate whether *Bmp15* and *Gdf9* expression is associated with ovulation rate or multiple ovulations, using hen lines with different fertility parameters.

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