Molecular basis of bone morphogenetic protein-4 inhibitory action on progesterone secretion by ovine granulosa cells

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Abstract

We have recently reported that bone morphogenetic protein-4 (BMP-4) can inhibit progesterone production by ovine granulosa cells (GCs). Here, we have investigated the underlying mechanisms of this effect in basal as well as in FSH-induced conditions. We have confirmed that treatment with BMP-4 decreased basal GC progesterone secretion and totally abolished FSH-stimulating action. This inhibitory action was associated with a decrease in the expression of cAMP-regulated genes, steroidogenic acute regulatory protein (StAR) and P450 side-chain cleavage (P450 scc) at mRNA and protein levels. However, BMP-4 did not alter basal cAMP production by GCs. In contrast, BMP-4 decreased by half the FSH-induced cAMP production and strongly inhibited cAMP-induced progesterone production. Thus, the inhibitory effect of BMP-4 was exerted both upstream and downstream of cAMP signalling. We next examined the downstream effect, focusing on cAMP-dependent transcription factors, steroidogenic factor-1 (SF-1) and CREB, through the BMP factor signalling intermediary, Smad1. As expected, BMP-4 induced phosphorylation and transcriptional activity of Smad1 in ovine GCs. BMP-4-activated Smad1 did not affect CREB activity but inhibited the transcriptional activity of SF-1 on the canonical SF-1 responsive element. Interestingly, this transcriptional inhibitory mechanism occurred on transfected StAR and P450 scc promoter. Based on these results, we propose that SF-1 is a key target in the inhibitory mechanism exerted by BMP-4 on progesterone synthesis by ovine GCs in culture. Because SF-1 plays an essential role in the differentiation of GCs, our findings could have new implications in understanding the role of BMP family members in the control of ovarian folliculogenesis.

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Introduction

Follicle-stimulating hormone (FSH) plays a central role in ovarian folliculogenesis, particularly in regulating granulosa cell (GC) differentiation. In particular, FSH enhances the expression of steroidogenic enzymes such as cytochrome P450 side-chain cleavage (P450 scc) and P450 aromatase (Richards & Hedin 1988, Winters et al. 1998) and these actions are importantly modulated by various growth factors, acting in a paracrine or autocrine way (Khamsi et al. 2001). From recent accumulating in vivo and in vitro evidence, it appears that members of the bone morphogenetic protein (BMP) family of cytokines and their receptors are strongly implicated in ovarian function, controlling folliculogenesis and ovulation rate (Monget et al. 2002). A study of invalidated mice for growth and differentiation factor-9 (GDF-9), an oocyte-derived growth factor of the BMP family, has shown that this factor is required during early ovarian folliculogenesis enabling primordial and primary to secondary follicle transition (Dong et al. 1996). In sheep, two loss of function mutations in the gene encoding BMP-15, another oocyte-derived factor closely related to GDF-9, have been shown to be responsible for a strong alteration of follicular growth in a dose-dependent manner. An increase
in ovulation rate was observed in the heterozygous carrier Inverdale and Hanna ewes, the homozygous being sterile (Galloway et al. 2000). Moreover, it has been shown that a substitutive mutation (Q249R) in the gene encoding BMP receptor (BMPR)-1B (also called Alk6), one of the type-1 receptors triggering the BMP intracellular signalling, is responsible for an increase in ovulation rate (threefold in heterozygous carrier ewes and more than fivefold in homozygous carriers) in the Booroola strain (Mulsant et al. 2001, Souza et al. 2001, Wilson et al. 2001).

In vitro, recombinant BMP-4, BMP-6, BMP-7, BMP-15 and GDF-9 are able to strongly inhibit FSH-induced progesterone secretion by rat GCs in culture (Shimasaki et al. 1999, Otsuka et al. 2000, 2001a,b, Vitt et al. 2000, Lee et al. 2001, Souza et al. 2001). In contrast, BMP-4 and BMP-7 enhance FSH-dependent estradiol production, suggesting that BMP factors regulate GC differentiation, particularly in delaying the luteinization process (Shimasaki et al. 1999).

Several studies in the rat and the ewe using in situ hybridization or immunohistochemistry have detected the expression of several elements of the BMP signalling pathway in the ovary. For example, BMP-4 and BMP-7 are expressed by theca cells (Shimasaki et al. 1999), whereas BMP-6, GDF-9 and BMP-15 are oocyte-derived factors (for review, Elvin et al. 2000). BMP type I receptors, BMPRIA/Alk3 and BMPR1B/Alk6 and type II receptor BMPRII are expressed in GCs and oocytes (Shimasaki et al. 1999, Wilson et al. 2001, Souza et al. 2002). Transducing molecules of the Smad family have been detected in all compartments of follicles in the rat ovary (Drummond et al. 2002, Xu et al. 2002). Altogether, these observations indicate the presence of a complete BMP signalling pathway in ovaries, enabling autocrine and paracrine regulation.

Less is known about the mode of action of BMP family members on target cells in the ovary, particularly in GCs. In the rat, most actions of the BMP molecules on GC steroidogenesis are FSH dependent. The proposed mechanism of action implicates down-regulation of FSH receptor expression or decrease in adenylyl cyclase activity (Otsuka et al. 2001a,b). In contrast, we have recently demonstrated that BMP-4, a known ligand of Alk6, inhibits progesterone secretion by ovine GCs in the absence of FSH (Mulsant et al. 2001, Fabre et al. 2003). However, the precise intracellular mechanism that underlies the inhibiting action of BMP-4 has not been determined.

In the present study, we have investigated the mechanism of action by which BMP-4 exerts an inhibitory action on basal as well as FSH-induced progesterone secretion through modulation of the cAMP signalling pathway and expression of genes involved in steroidogenesis. We propose that BMP-4 modulates progesterone by inhibiting the steroidogenic factor-1 (SF-1) transaltional activity on steroidogenic gene promoters. In FSH-induced conditions, this mechanism might be reinforced by an inhibition of adenylyl cyclase activity.

Materials and methods

Reagents and supplies

Fluorogestone acetate sponges used to synchronize oestrous cycles were obtained from Intervet (Angers, France). Porcine FSH (pFSH) from pituitary extracts (pFSH activity=1·15 times the activity of NIH pFSH-P1) used for injections to animals was obtained from Dr Y Combarnous (Nouzilly, France). Purified ovine FSH-20 (oFSH) (lot no. AFP-7028D; 4453 IU/mg; FSH activity=175 times the activity of oFSH-S1) used for culture treatment was a gift from the NIDDK (National Hormone Pituitary Program, Bethesda, MD, USA). Recombinant human FSH (rhFSH) used in adenylyl cyclase activity experiments was obtained from Serono (Boulogne, France). Recombinant human BMP-4 was obtained from R&D Systems Europe (Lille, France). DibutyrylcyclicAMP (db-cAMP) and isobutyl methylxanthine (IBMX) were purchased from Sigma (L’Isle d’Abeau Chesnes, France). Rabbit polyclonal anti-Smad1 and anti-phospho-Smad1 were obtained from Upstate Biotechnology (Euromedex, Mundolsheim, France). Rabbit polyclonal anti-StAR were kindly provided by Dr V Luu-The (Quebec, Canada) and Dr D B Hales (Chicago, IL, USA) respectively.

Plasmid designated pSG-SF1, constructed by inserting cDNA encoding the murine SF-1 into...
pSG5 vector (Stratagene, Amsterdam, The Netherlands) and pGL3 TkLHβ, a luciferase reporter gene under the control of two copies of the SF-1-responsive element of the luteinizing hormone (LH)-β promoter gene, were a gift from Dr A Martinez (Val et al. 2003). Plasmid pSG5-Smad1, constructed by inserting a flag-tagged murine Smad1 into pcdef3 vector was kindly provided by Dr M Kawabata (Ishida et al. 2000). Plasmid pGal4-tk80-luc was a luciferase reporter gene under the control of the Gal4-responsive element and plasmid pGal4-Smad1 was a human Smad1 sequence fused with the DNA-binding domain of Gal4 (Pearson et al. 1999). P450 scc luciferase reporter constructs containing fragments of the human P450 scc gene spanning from nucleotides –110 to +49, with or without the mutated SF-1-binding site, subcloned in pGL3 vector, were kindly provided by Dr B Staels (Gizard et al. 2003). StAR luciferase reporter construct containing fragments of human StAR gene spanning nucleotides –235 to +39 was a gift from Dr J Strauss (Sugawara et al. 1996). The cAMP-sensitive reporter construct of the somatostatin gene promoter (pSOM-luc) was kindly provided by Dr B Peers (Liège, Belgium).

**Animals**

Fifty adult cyclic Romanov ewes were treated with intravaginal sponges impregnated with progestagen (fluorogestone acetate, 40 mg) for 15 days to mimic a luteal phase. Ovaries were collected from animals in the luteal phase of the oestrous cycle (10 days after sponge removal), stimulated by intramuscular injections of 6 IU and 5 IU pFSH administered 24 h and 12 h prior to slaughter respectively. All procedures were approved by the Agricultural and Scientific Research Government committees in accordance with the guidelines for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (approval A37801).

**Isolation and culture of GCs**

Briefly, in each independent culture experiment, ovarian follicles from three to four Romanov ewes were quickly dissected, pooled and classified according to size. GCs were recovered from small antral follicles (1–3 mm in diameter) as previously described (Le Bellego et al. 2002). For progesterone and cAMP production, GC suspensions were seeded at 10 000 viable cells/well in 96-well plates and cultured for 144 h at 37 °C with 5% CO₂ in serum-free McCoy’s 5a medium (Sigma) according to a previously described method (Campbell et al. 1996). Cultures were performed with or without different exogenous factors (BMP-4, oFSH, dB-cAMP or IBMX), each alone or in combination. Each combination of treatments was tested in triplicate in at least four independent culture experiments. Culture media were partially replaced (180 over 250 µl) at 72 h. Media conditioned between 72 and 144 h of culture were collected at 144 h and stored at −20 °C prior to radioimmunoassays. At the end of the culture, the number of cells per well was estimated after trypsinization by counting an aliquot of each resulting cell suspension with a haemacytometer under a phase contrast microscope.

Alternatively, for phospho-Smad1 detection or transient transfection experiments, GCs were cultured in McCoy’s 5a medium supplemented with 3% fetal ovine serum during the 72 h prior to specific treatments.

**Northern blot analysis**

For RNA blot analysis, GCs were harvested 72 h after treatment with oFSH (5 ng/ml) or BMP-4 (50 ng/ml) alone or in combination, and total RNAs were isolated using Rnable solution (Eurbio, Les Ulis, France). Twenty micrograms of total RNAs were separated by denaturing formaldehyde electrophoresis, then transferred to a nylon membrane by capillary action overnight and immobilized by exposure to u.v. light. Blots were prehybridized for 2 h at 42 °C in a buffer containing 50% formamide, 5×Denhardt’s, 1% SDS, 5×SSC and 100 µg/ml denatured salmon sperm. The probes were labelled using the Rediprime labelling kit (Amersham Pharmacia Biotech, Orsay, France). cDNA probes for ovine StAR, P450 scc and 3β-HSD were generated by RT-PCR on ovine whole follicle mRNA using the following primers: StAR sense 5'-GGTGCT GAGTAAAGTGATCC-3' and StAR antisense 5'-CATCTCCTCGTAGAGTGTGG-3'; P450 scc sense 5'-TCCTTTAAGTTCGAGGGATC-3' and P450 scc antisense 5'-TCACTTTTCAGGGGATAT CTCTG-3'; 3β-HSD sense 5'-CAGTCTTGTGCTGC CTTGAGGCC-3' and 3β-HSD antisense 5'-CAG
GAAGCCAAGCAGAAAAC-3'. The radioactivity was quantified using a STORM apparatus and ImageQuant software (Amersham Pharmacia Biotech). The integrity and the quantification of different transcripts were assessed using the human RNA 18S probe as a control (Ambion, Huntingdon, Cambs, UK).

**Western blot analysis**

GC whole cell extracts were obtained by resuspension in lysis buffer (10 mM Tris, pH 7·4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA and 0·5% Igepal) containing several protease inhibitors (2 mM phenylmethylsulphonyl fluoride, 10 mg/ml leupeptin and 10 mg/ml aprotinin) and phosphatase inhibitors (100 mM sodium fluoride, 10 mM sodium pyrophosphate and 2 mM sodium orthovanadate (Sigma)). Lysates were centrifuged at 15 000 g for 20 min at 4 °C, and the protein concentration in the supernatants was determined by a colorimetric assay (BC Assay kit; Uptima Interchim, Montluçon, France). The protein samples (30–60 µg) were fractionated using SDS-PAGE in 12% polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell, Ecquevilly, France). For steroidogenic protein detection, GCs were harvested after 144 h of treatment with FSH and/or BMP-4 in serum-free condition. The same samples were run and transferred on three independent membranes to allow individual detection of P450 scc and 3β-HSD and dual detection on the same membrane of StAR and actin was used as a loading control. Proteins of the steroidogenic pathway were detected using rabbit polyclonal antibodies raised against StAR (1:500 final dilution), P450 scc (1:1000) and 3β-HSD (1:500). Actin was revealed using a mouse monoclonal anti-actin antibody (1:1000; Sigma). For Smad1 detection, GCs were cultured for 72 h in McCoy’s 5a medium supplemented with 3% fetal ovine serum. Then, the different plasmids expressing Smad1 or SF-1 (500 ng/well) and luciferase reporter genes for Smad1 or SF-1 activity (1µg/well) were transiently transfected to cells using DAC30 transfection reagent (Eurogentec, Seraing, Belgium) for 24 h with a DNA/DAC30 ratio of 1/2 (w/w) as specified by the manufacturer. After 24 h, media were changed with fresh McCoy’s 5a medium supplemented with 3% fetal ovine serum cells for an extra 24 h with or without BMP-4 (50 ng/ml) before luciferase assay (Promega, Charbonnières, France). Each combination of plasmids was tested in triplicate in each culture and in four independent experiments.

**Assessment of progesterone and cAMP production**

The amount of progesterone (ng/50 000 cells) in the culture media conditioned between 72 and 144 h from each experiment was measured by radioimmunoassay as previously described (Saumande 1991).

For cAMP measurement, GCs from small antral follicles were cultured for 144 h with or without BMP-4 (50 ng/ml). At the end of the culture, media were removed and cells were stimulated for 2 h with 0·2 mM IBMX in the presence or absence of 50 ng/ml rhFSH in fresh McCoy’s 5a medium. Extracellular amounts of cAMP (pmol/50 000 cells) in the media conditioned during these last 2 h were measured using the cAMP radioimmunoassay kit (Perkin Elmer, Courtaboeuf, France) following the manufacturer’s specifications. IBMX treatment was used to prevent the metabolic effect of phosphodiesterase on cAMP and then cAMP production was a direct measurement to adenylate cyclase activity.

**Transient transfection and luciferase assay**

GCs from small antral follicles were seeded at 200 000 cells/well in 12-well plates and cultured for 72 h in McCoy’s 5a medium supplemented with 3% fetal ovine serum. Then, the different plasmids expressing Smad1 or SF-1 (500 ng/well) and luciferase reporter genes for Smad1 or SF-1 activity (1µg/well) were transiently transfected to cells using DAC30 transfection reagent (Eurogentec, Seraing, Belgium) for 24 h with a DNA/DAC30 ratio of 1/2 (w/w) as specified by the manufacturer. After 24 h, media were changed with fresh McCoy’s 5a medium supplemented with 3% fetal ovine serum cells for an extra 24 h with or without BMP-4 (50 ng/ml) before luciferase assay (Promega, Charbonnières, France). Each combination of plasmids was tested in triplicate in each culture and in four independent experiments.

**Data analysis**

All experimental data are presented as means ± S.E.M. The effects of hormones on progesterone secretion and cAMP production were

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analyzed using two-way ANOVA in order to appreciate the ‘hormone effect’ as well as the ‘culture effect’. For luciferase assays, data are expressed as relative to control condition. The effect of different combinations of plasmids was assessed by two-way ANOVA to allow for ‘experiment effect’ and ‘plasmid effect’ or ‘BMP-4 effect’. One-way ANOVA was used to appreciate the treatment effect on Northern blot analysis. Post-hoc comparisons were performed with Scheffe’s and Newman–Keuls tests. For all analyses, differences with $P > 0.05$ were considered as not significant.

Results

Effect of BMP-4 on basal and FSH-stimulated progesterone production by GCs

As shown in Fig. 1, basal progesterone production between 72 and 144 h of culture was decreased by 57% ($P < 0.001$) by BMP-4. As expected, treatment with oFSH (0.5–50 ng/ml) dose-dependently increased progesterone production by GCs. In these FSH-stimulated conditions, the addition of BMP-4 totally abolished oFSH action ($P < 0.001$). The same results were observed with GCs from prevovulatory follicles (5–6 mm in diameter) (data not shown).

Effect of BMP-4 on steroidogenic enzyme genes

In order to understand the inhibiting role of BMP-4 on GC progesterone production, we have studied the expression of genes implicated in progesterone synthesis, StAR, P450 scc and 3β-HSD, at mRNA and protein levels (Fig. 2). Northern blot analysis (Fig. 2A) showed a consistent decrease in StAR mRNA level after 72 h of BMP-4 treatment (50 ng/ml) in both basal ($P < 0.05$) and FSH-induced condition ($P < 0.01$). Moreover, BMP-4 led to a decrease in P450 scc mRNA levels in FSH-induced condition only ($P < 0.05$). In contrast, the 3β-HSD mRNA level was not clearly affected. This pattern of regulation on StAR, P450 scc and 3β-HSD mRNAs at 72 h was well correlated with the results observed for protein levels at 144 h, as shown by Western blotting analysis (Fig. 2B).

Effect of BMP-4 on cAMP production and activity

The inhibitory effect of BMP-4 was investigated on the cAMP signalling pathway, a well-known regulator of steroidogenesis in GCs. After 144 h of culture, basal cAMP production of GCs, measured in the presence of IBMX, was not affected by BMP-4 treatment. In contrast, rhFSH-stimulated (50 ng/ml) cAMP production at the end of the culture was significantly reduced (50%) by the presence of BMP-4 during the culture ($P < 0.001$; Fig. 3 left-hand panel). Moreover, BMP-4 strongly inhibited db-cAMP or IBMX stimulation of progesterone secretion (Fig. 3 right-hand panel). Together, these results indicated that BMP-4 could act negatively on both pre- and post-cAMP signalling events.

Searching for the BMP-4 inhibitory mechanism

Since basal and cAMP-dependent StAR, P450 scc and 3β-HSD gene expression are regulated by a
**Figure 2** Effect of BMP-4 and FSH on expression of steroidogenic genes implicated in progesterone synthesis. GCs from small antral follicles were cultured for 72 h (mRNA) or 144 h (protein) in serum-free condition with BMP-4 (50 ng/ml) or oFSH (5 ng/ml), each alone or in combination. Each combination of treatments was tested in at least three independent experiments. (A) Representative Northern blots and quantification of StAR, P450 scc and 3β-HSD mRNA expression. Quantitated mRNA levels are expressed relative to ribosomal 18s RNA used as control. *P<0.05, **P<0.01, BMP-4 vs untreated in basal or FSH condition; $P<0.05$, FSH vs untreated. (B) Representative Western immunobloting analysis of GC whole cell extracts using anti-StAR, anti-P450 scc and anti-3β-HSD specific antibodies. Actin was used as gel loading control.

First, we checked the ability of BMP-4 to trigger the phosphorylation and the functional activation of Smad1 in ovine GCs in culture (Fig. 4). As shown in Fig. 4A, BMP-4 treatment for 30 min was able to phosphorylate Smad1. We investigated the transcriptional activity of overexpressed Smad1 protein fused to the Gal4-DNA-binding domain on the Gal4-responsive element driving the luciferase gene. Stimulation with BMP-4 for 24 h strongly increased Smad1-dependent luciferase activity (Fig. 4B). These results indicated that Smad1, like other cell types, could act as an effector of BMP-4 action in ovine GCs.

Next, we investigated the effect of BMP-4, through Smad1, on SF-1 transcriptional activity (Fig. 5). Transient transfection experiments on ovine GCs showed that the SF-1-responsive construct pGL3 TkLHβ (Fig. 5A) activity was enhanced (4.5-fold, P<0.001) by overexpression of SF-1. This stimulatory effect was lowered by BMP-4 treatment (P<0.01) or Smad1 expression (P<0.001) and totally abolished by Smad1 expression and BMP-4 treatment. In contrast, the transcriptional activity of CREB (another cAMP-dependent factor implicated in steroidogenic gene regulation), checked by the pSOM-luc construct (Fig. 5B) was not altered by overexpression of Smad1. Thus, it appears that Smad1 was able to specifically suppress SF-1 activity on SF-1 responsive elements.

In order to demonstrate that this Smad1 antagonistic effect on SF-1 could participate in the inhibitory mechanism of steroidogenic gene expression by BMP-4, we studied the role of Smad1 on StAR and P450 scc promoter activity (Fig. 5C and D). Overexpression of SF-1 enhanced P450 scc and StAR promoter activity and these stimulations were reduced by Smad1 co-expression (P<0.001). Interestingly, basal activity of the P450 scc promoter was significantly reduced by Smad1 overexpression (P<0.05), suggesting a possible direct negative action of Smad1 on the P450 scc promoter (Fig. 5C). The mutant P450 scc promoter in which the SF-1 binding site has been disrupted exhibited a great decrease in basal activity in comparison with the wild-type promoter and was not sensitive to SF-1.
overexpression. On this mutant promoter, the Smad1 inhibitory action was minimized but not totally abolished ($P<0.05$).

Discussion

There is accumulating evidence supporting the inhibitory action of BMP molecules on progesterone production by ovarian GCs. However, most studies using the rat model have indicated that BMPs exerted their action only in FSH-induced condition. In the present study, using ovine GCs, we have confirmed the strong inhibitory action of BMP-4 on FSH-stimulated progesterone production but, in contrast to the rat, we also observed inhibition of progesterone production on its own in the absence of FSH. Based on knowledge about the implication of BMP in regulating ovulation rate (Galloway et al. 2000, Mulsant et al. 2001, Souza et al. 2001, Wilson et al. 2001, Hanrahan et al. 2004), this result suggests some species differences in the role of the BMP system on ovarian function between the high ovulating rodent and the low ovulating ruminant.

The objective of the present study was to explore the molecular basis by which BMP-4 exerted its inhibitory action on ovine GC basal and FSH-stimulated progesterone synthesis. First, BMP-4 reduced basal and/or FSH-induced increases in mRNA and the protein levels of StAR and P450 scc, a finding consistent with the decrease in progesterone production. Secondly, BMP-4 exerted its inhibitory effect mainly downstream of cAMP signalling and to a lesser extent by decreasing FSH-dependent cAMP production. Thirdly, BMP-4 was able to activate the Smad1 signalling pathway in primary ovine GCs. Fourthly, Smad1 was able to inhibit the transcriptional activity of SF-1 on the LHβ/afii9826 promoter as well as on the StAR and P450 scc promoter. Based on these findings, we propose that part of the negative action of BMP-4 on progesterone secretion by ovine GCs would be exerted through the activation of Smad1 that would inhibit the SF-1-dependent expression of StAR and P450 scc genes, leading to a decrease in progesterone synthesis activity. In FSH-stimulated condition, this inhibitory mechanism would be reinforced by a decrease in FSH-dependent cAMP production.

In order to appreciate the regulation of progesterone production, we examined the steady-state level of mRNA for StAR, P450 scc and 3β-HSD genes implicated in the progesterone synthesis pathway. As observed in other species (Urban et al. 1991, Pescador et al. 1997, Eimerl &
Orly 2002), mRNA levels of StAR, P450 scc and, to a lesser extent, 3β-HSD increased under FSH treatment. This up-regulation at the mRNA level was followed by the same regulation at the protein level. FSH-induced expression of StAR and P450 scc mRNAs and proteins was clearly down-regulated by BMP-4. These results are in agreement with those observed on FSH-stimulated

Figure 5 Effect of Smad1 expression on SF-1 and CREB transcriptional activity. GCs were cultured for 72 h in McCoy’s 5a medium supplemented with 3% fetal ovine serum prior to transient transfection. (A) Co-tranfection of the SF-1 responsive element of the LHβ gene promoter construct and vectors expressing or not expressing SF-1 or Smad1, each alone or in combination and treated with or without BMP-4 (50 ng/ml) for 24 h. (B) Co-tranfection of the CREB responsive element construct of the somatostatin gene promoter and vectors expressing or not expressing CREB or Smad1, each alone or in combination and treated with oFSH (5 ng/ml) for 24 h to obtain transcriptional activity of CREB. (C) Co-tranfection of the wild-type (wt) P450 scc gene promoter constructs or a mutated form of the SF-1 responsive element (SF-1 m) or (D) StAR gene promoter construct and vectors expressing or not expressing SF-1 and/or Smad1. Results are expressed as luciferase activity relative to control condition (reporter construct alone). Each combination of treatments was tested in triplicate in each of four to six independent experiments. Data represent means±S.E.M. of measurements. $P<0.05$, $$P<0.01$$, $$$P<0.001$$, BMP-4 vs basal, *$P<0.05$, ***$P<0.001$, Smad1 vs empty vector.
rat GCs using BMP-15, BMP-6 or BMP-7, even if BMP-7 seems to inhibit only StAR mRNA without affecting P450 scc (Lee et al. 2001, Otsuka et al. 2001a,b).

Since StAR, P450 scc and 3α-HSD are cAMP/protein kinase A (PKA)-regulated genes (Lauber et al. 1993, Sugawara et al. 1997, Rodway et al. 1999), we have focused on the BMP-4 regulation of this signalling pathway. BMP-4 was able to inhibit the FSH-stimulated adenylate cyclase activity of GCs, as measured by cAMP production in the presence of IBMX. This result indicates a regulation of the cAMP signalling pathway by BMP-4 upstream of cAMP as previously observed for BMP-15 and BMP-6 in rat GCs (Otsuka et al. 2001a,b). BMP-15 and BMP-6 have been shown to inhibit FSH-stimulated progesterone production by two different mechanisms implying down-regulation of the FSH receptor expression and inhibition of adenylate cyclase activity respectively. Using RT-PCR, we were unable to demonstrate any down-regulation of FSH receptor expression by BMP-4 in ovine GCs (data not shown). In the absence of FSH, BMP-4 was without effect on cAMP production while it inhibited progesterone synthesis, supporting the hypothesis that BMP-4 is also acting downstream of cAMP. The strong inhibitory effect of BMP-4 observed on cAMP-stimulated progesterone secretion also argues in favour of this hypothesis.

Most of the transforming growth factor-β/BMP effects at the cellular level are triggered by Smad-related factors. Among the Smad family members, Smad1 is considered to be a BMP-responsive Smad, because most BMPs identified to date, including BMP-2, -4, -6, -7 and -15, have been shown to activate the Smad1 pathway in different cell types (Candia et al. 1997, Yamamoto et al. 1997, Macias-Silva et al. 1998, Henningfeld et al. 2000, Moore et al. 2003). Based on this fact, we investigated whether BMP-4 activates the Smad1 pathway in primary ovine GCs. Treatment of cells with BMP-4 caused a clear increase (i) in the level of phosphorylated Smad1 as determined by immunoblotting and (ii) in Smad1 transcriptional ability as shown by transient transfection experiments. Hence Smad1 can transduce the BMP-4 signal in ovine GCs. Although the Smad pathway is the main canonical pathway for BMP ligands, there is much evidence on the activation of alternative pathways, particularly the p38 kinase of the MAPK family in various cell types (for a review see Nohe et al. 2004). In agreement, we have observed a weak increase in phosphorylated p38 MAPK under BMP-4 treatment in ovine GCs (Pierre et al. 2002). However, the inhibition of p38 phosphorylation in vitro by a specific inhibitor (SB203580) had no effect on BMP-4 suppression of progesterone production (A Pierre, unpublished data), suggesting that activation of the p38 MAPK pathway by BMP-4 cannot be implicated in the mechanism of progesterone inhibition.

The ability of BMP-4 to regulate post-cAMP steps and to activate the Smad1 factor led us to hypothesize the existence of functional interactions between the transcription factors of these two pathways in the regulation of steroidogenic gene expression. To date, no Smad binding element (SBE) has been described on the regulatory region of StAR or P450 scc or 3α-HSD genes. Our present transient transfection experiments did not detect any effect of Smad1 on the basal activity of the human StAR proximal promoter. In contrast, basal activity of the P450 scc proximal promoter appeared to be sensitive to Smad1 overexpression even in the context of a disrupted SF-1-binding site, indicating a possible direct inhibitory action of Smad1 on the P450 scc promoter. Alternatively, cAMP/PKA-activated transcription factors such as CREB and SP-1, known to regulate genes of the steroidogenic pathway (Mukherjee et al. 1996, Leers-Sucheta et al. 1997, Liu & Simpson 1997, Sugawara et al. 2000) have also been shown to functionally interact with Smad factors. Indeed, Warner et al. (2003) recently demonstrated that phosphorylated CREB could be associated through the CREB-binding protein (CBP) with a Smad2 SBE-bound transcriptional complex. Moreover, SP-1 can co-operate with Smad3 to regulate the α2(I) collagen promoter gene (Poncelet & Schnaper 2001) and with Smad2, 3 and 4 to control the promoter of the p15Ink4a gene (Feng et al. 2000). Nevertheless, FSH-activated CREB did not co-operate with Smad1 to drive the CREB-dependent somatostatin promoter gene in our transient transfection experiments with ovine GCs. In contrast, we have clearly demonstrated that the transcriptional activity of SF-1, another factor implicated in basal as well as in cAMP-induced regulation of steroidogenic genes (Liu & Simpson 1997, Sugawara et al. 1997), was altered by Smad1.

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This is the first demonstration of a functional negative crosstalk between Smad factors and SF-1 in any cell type. Such a negative alteration of a transcription factor activity by Smad factors has been described only for the nuclear factor κB (NFκB) (DiChiara et al. 2000). The inhibitory mechanism implies a competitive interaction between Smad2 and NFκB mediated by the transcriptional coactivator CBP, without Smad2/ NFκB direct interaction. Of note, in our model, co-immunoprecipitation experiments with overexpressed Smad1 and SF-1 failed to detect any direct interaction between Smad1 and SF-1 (A Pierre, data not shown). Hence, one can hypothesize about the possible implication of an intermediary factor such CBP, known to interact with Smad factors (Pouponnot et al. 1998) and SF-1 (Monte et al. 1998). Further experiments are needed to validate this hypothesis on the inhibitory mechanism of Smad1 on SF-1 in the context of GCs, and to study the mechanism that underlies this inhibition.

Nevertheless, the inhibiting action of Smad1 on SF-1, checked by the SF-1 responsive element of the LHβ subunit promoter gene, occurs also on the SF-1-dependent response of human StAR and P450 scc promoter constructs. These results need to be considered together with the observed inhibition of StAR and P450 scc mRNA expression by BMP-4. Therefore, one may hypothesize that part of the BMP-4-induced inhibition of StAR and P450 scc genes expression, and ultimately progesterone secretion, would pass through a Smad1-dependent inhibition of SF-1 activity. Since SF-1 is a key regulator of GC differentiation, our present data reinforce the hypothesis that BMP factors control ovarian follicle development in delaying GC differentiation.

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