Dynamic regulation of pituitary mRNAs for bone morphogenetic protein (BMP) 4, BMP receptors, and activin/inhibin subunits in the ewe during the estrous cycle and in cultured pituitary cells

C Sallon^{1,2,3,4}, M O Faure^{1,2,3,4}, J Fontaine^{1,2,3,4} and C Taragnat^{1,2,3,4}

¹Centre INRA de Tours, UMR85 Physiologie de la Reproduction et des Comportements, F-37380 Nouzilly, France

²CNRS, UMR 6175, F-37380 Nouzilly, France

³Université de Tours, F-37041 Tours, France

⁴Haras Nationaux, F-37380 Nouzilly, France

(Correspondence should be addressed to C Taragnat at Centre INRA de Tours; Email: catherine.taragnat@tours.inra.fr)

Abstract

Recently, bone morphogenetic protein (BMP) 4 has been shown to inhibit FSH secretion in ewe. The detection of BMP4 mRNA and BMP receptors in the pituitary suggests that BMP4 can exert paracrine actions on FSH production. This work aimed at determining whether BMP4 and/or BMP receptor mRNA as well as activin/inhibin subunit mRNA fluctuates during the estrous cycle when FSH β mRNA and FSH release changed. The estrous cycles of ewes were synchronized with progestagen sponges. Ewes were killed in late follicular stage ($n=5$), before the secondary FSH surge ($n=4$), and in luteal phase ($n=4$). Using quantitative reverse transcription-PCR, we showed that the levels of mRNA for BMP4, BMP receptor, the inhibitor of differentiation 2 (*Id2*), a target gene of BMP4, and noggin did not change significantly across the estrous cycle.

In contrast, the level of activin β B mRNA and the percentage of immunoreactive cells for activin β B-subunit were higher before the secondary surge of FSH compared to other groups. In ewe pituitary cell cultures, activin, GnRH, or estradiol-17 β (E_2) did not significantly affect the levels of BMP4, BMP receptor, and *Id2* mRNA. E_2 , but not GnRH, increased the level of activin β B mRNA. Moreover, the *in vitro* FSH release was not modified by noggin, a BMP antagonist. In contrast, SB431542, an inhibitor of activin pathway, inhibited FSH release. Collectively, our data showed that pituitary BMP4 would not play a crucial role in the regulation of FSH production during the estrous cycle, whereas local activin B would be a major stimulus of FSH synthesis necessary for the secondary FSH surge.

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Introduction

In mammals, the gonadotropins FSH and LH are essential components for reproduction (Morel *et al.* 1981). Synthesis and release of LH and FSH by gonadotrope cells are regulated by a complex interplay of hypothalamic, gonadal, and pituitary factors. The hypothalamic GnRH is the major regulator of LH and FSH secretion. While the LH secretion is mainly dependent of GnRH and gonadal steroids, additional factors are involved in regulation of FSH production. Particularly, activins and inhibins, members of the transforming growth factor- β (TGF- β) superfamily, are able to stimulate (Ling *et al.* 1986) and inhibit (Carroll *et al.* 1989) FSH synthesis and release respectively. Activin is a dimer of two highly related β -subunits (β A and/or β B), resulting in three possible molecular species: activin A (β A β A), activin B (β B β B), and activin AB (β A β B). Inhibin is a heterodimer composed of one α -subunit and one β -subunit (β A or β B). The activin and inhibin subunits are produced in both ovary and pituitary gland in different species, including rat

(Kogawa *et al.* 1991, Wilson & Handa 1998), human (Uccella *et al.* 2000), and sheep (Baratta *et al.* 2001). Activin is thought to play an autocrine/paracrine action on FSH secretion (Padmanabhan & McNeilly 2001). Indeed, monoclonal antibodies to activin B inhibit FSH release from plated pituitary cells as well as from hypophysectomized, pituitary-grafted rats (Corrigan *et al.* 1991, DePaolo *et al.* 1992). Inhibin is produced by gonads and exerts an endocrine action on FSH secretion (de Kretser & Robertson 1989), mainly through antagonism of activin action (Lewis *et al.* 2000, Bilezikjian *et al.* 2004).

Recently, other members of the TGF- β superfamily, the bone morphogenetic proteins (BMPs), were shown to regulate FSH production *in vitro*. In rat pituitary cells and murine L β T2 gonadotrope cell lines, BMP6 and BMP7 at high concentrations or BMP2 and BMP15 at lower concentrations stimulate basal FSH secretion and FSH β promoter activity (Huang *et al.* 2001, Otsuka & Shimasaki 2002, Lee *et al.* 2007). Moreover, BMP4 increased the release of FSH in response to activin and activin+GnRH

(Nicol *et al.* 2008). In contrast, in ovine pituitary cells, BMP4 inhibits the FSH and is able to antagonize the effects of activin (Faure *et al.* 2005, Young *et al.* 2008). To initiate their intracellular signaling cascade, BMPs bind to two types of serine–threonine kinase receptors, BMP receptor type I (BMPRI1A or BMPRI2B also known as ALK3 and ALK6 respectively) and BMP receptor type II (BMPRI2; Kawabata *et al.* 1998, Miyazono *et al.* 2001, Allendorph *et al.* 2006). Some ligands such as BMP2 first bind BMPRI1A (ALK3). Upon ligand binding, the constitutively phosphorylated type II receptor transphosphorylates the type I receptor. Consequently, activated type I receptors phosphorylate intracellular mediators Smad1, Smad5, or Smad8, which then interact with Smad4. This complex translocates to the nucleus, where it regulates gene expression (Zwijnsen *et al.* 2003).

In the ewe pituitary, the mRNA encoding BMP4 is present, as well as BMPRI1A (ALK3), BMPRI1B (ALK6), and BMPRI2 (Faure *et al.* 2005). Furthermore, both BMPRI1A (ALK3) and BMPRI2 are immunodetected on the gonadotrope cells (Faure *et al.* 2005), suggesting that BMP4 may exert paracrine/autocrine actions on FSH production and participate with activin in FSH regulation. However, the physiological relevance of pituitary BMP4 in the control of the FSH synthesis and release has not yet been established.

If BMP4 is a regulator of FSH, the expression of the ligand, receptors or antagonists may exhibit changes at mRNA or protein level leading to corresponding changes in FSH secretion. In the ewe, it is true for activin receptors since the mRNA for ActR1A (ALK2), ActR1B (ALK4), and ActR2B fluctuates across the estrous cycle (Fafioffe *et al.* 2004). Whether activin ligands are changing in the pituitary is not known.

In order to gain an insight into potential intrapituitary role of BMP4 in the regulation of FSH synthesis and release, the present study was designed to determine whether changes in the patterns of *FSHβ* gene expression and FSH secretion observed *in vitro* and *in vivo* during the ewe estrous cycle were associated with variations in the expression of the BMP4 system, i.e. ligand, BMP receptors, noggin antagonist, and *Id2*, a target gene for BMP4. In the absence of available antibodies and protein immunoassays for ovine BMPs, we focused on mRNA levels. To compare the BMP system to those of activin, the pattern of activin subunit expression in the pituitary during the estrous cycle and *in vitro* was investigated.

Materials and Methods

Reagents

Cell culture reagents used were DMEM and F12 (nutrient mixture F-12 Ham) from Sigma. FCS and BSA were purchased from Sigma. Collagenase A and DNase I were obtained from Roche Diagnostics Ltd. Human recombinant activin-A and BMP4 were obtained from R&D Systems

(Lille, France). Estradiol-17β (E₂) and SB431542 were obtained from Sigma. GnRH (Relefact LH-RH) was obtained from Aventis (Frankfurt, Germany). Human noggin was kindly provided by Regeneron (Tarrytown, NY, USA).

Pituitary collection during the estrous cycle

The estrous cycles of 15 Ile de France ewes were synchronized by insertion of vaginal sponges impregnated with synthetic progestagen during the breeding season. Sponges remained in place for 12 days. Ewes were killed at different stages of the estrous cycle: 36 h (group A), 48 h (group B), 66 h (group C), or 240 h (group D) after sponge removal. Prior to killing, blood samples were collected from the jugular vein at 2 h intervals between 32 and 36 h for group A, 32 and 48 h for group B, and 32 and 66 h for group C. For group D, a single blood sample was collected just prior to killing. Plasma was recovered from these samples and assayed for LH, FSH, and E₂. Anterior pituitaries were collected immediately after slaughtering and hemisected midsagittally. One half of pituitary was stored at −80 °C until RNA extraction, and the other half was fixed for 72 h in Bouin-Hollande fixative containing HgCl₂ for immunohistochemistry as previously described (Taragnat *et al.* 1998). All procedures were approved by the Agricultural and Scientific Research agencies, and conducted in accordance with the guidelines for Care and Use of Agricultural Animals in Agricultural Research and Teaching.

Pituitary collection for cell culture

Pituitaries from Ile de France ewes were collected during the non-breeding season and dissociated for cell cultures as previously described (Faure *et al.* 2005). Pituitaries were finely sliced and placed in F12 supplemented with collagenase A (0.4 mg/ml) and DNase I (0.025 mg/ml), and then incubated for 1 h 30 min in a shaking water bath at 37 °C followed by manual dispersion through different sizes of syringe. Cells were then centrifuged at 100 *g* for 5 min, and the pellet was resuspended in culture medium (DMEM containing 5% FCS). Cells were plated in 6-well plates at 3 × 10⁶ cells/well in 2 ml of culture medium (DMEM with 5% FCS), and allowed to attach for 2 days in a humidified atmosphere with 5% CO₂ at 37 °C. Media were replaced with serum-free DMEM containing 0.1% BSA. One hour later, media were changed and replaced by DMEM–0.1% BSA alone (control) or supplemented with test substances at different concentrations (see Results). The choice of concentrations was based on our previous studies determining 10^{−8}, 10^{−9}, and 10^{−9} M as optimal doses for GnRH, activin, and E₂ respectively to regulate FSH secretion *in vitro*. Media were collected for measurement of secreted FSH by ELISA. Total RNA was extracted from cells for measurement of studied mRNA levels by quantitative reverse transcription (RT)–PCR.

Gonadotropin measurements by ELISA

The concentrations of ovine gonadotropins in blood plasma or in culture cell media were determined using double-antibody ELISA for all experiments, as previously described by Faure *et al.* (2005) with a sensitivity of 0.1 ng/ml for ovine LH (oLH) and 0.4 ng/ml for ovine FSH (oFSH). For LH, the intra- and inter-assay coefficients of variation (CV) of the control averaged 4 and 10% respectively. The cross reaction with oFSH was 0.01%. For FSH, the intra- and inter-assay CV of the control were 2.6 and 2.1% respectively. The cross reaction with oLH was 0.07%.

E₂ measurements by RIA

For steroid assays, 600 µl plasma was extracted with ethyl acetate–cyclohexane as described (Monniaux *et al.* 2008), and the dried extracts containing steroids were measured by RIA. E₂ was assayed with the E₂ RIA kit following the manufacturer's specifications (Diasorin SA, Antony, France). The limit of detection of the assay was 0.08 pg per tube. The intra- and inter-assay CV of the control were 3.8 and 8.7% respectively.

Quantitative RT-PCR from tissue and from pituitary cell cultures

Levels of mRNA for BMP4, BMP receptors, Id2, inhibin α -subunit, activin β A- and activin β B-subunits were measured using SYBR Green RT-PCR. Total RNA from

6-well plates (plated at 3×10^6 cells/well) and from tissue was extracted using TRI Reagent (Sigma) and kit NucleoSpin RNA L (Macherey-Nagel, Hoerd, France) respectively according to the manufacturer's instructions. Remaining DNA was removed by RNase-free DNase treatment. cDNA was synthesized from 1 µg RNA in a volume of 20 µl containing 150 ng oligodT (Promega), 1 mM dNTPs, 20 U of RNasin, 1× RT PCR buffer, and 12 U M-MLV reverse transcriptase (Promega). For the negative control, the reverse transcriptase was omitted. The RNA denaturation was performed at 70 °C for 10 min, and the RT was performed at 37 °C for 1 h.

For PCR, each reaction consists of a final reaction volume of 20 µl containing iQ SYBR Green Supermix (Bio-Rad) (1×), 3 µM of each gene-specific primer, and cDNA. Primers were designed using Beacon Designer Software (Premier Biosoft International, Palo Alto, CA, USA), and sequences are described in Table 1. The equivalent of 5–50 ng of starting RNA was used in each reaction. Each sample was assayed in duplicate. Quantitative real-time PCR was run on an iCycler from Bio-Rad. The amplification program consisted of 95 °C for 30 s followed by up to 40 cycles of 95 °C for 30 s, 58–61 °C for 30 s, and 72 °C for 20 s. Annealing temperatures are given in Table 1.

Prior to analysis, amplification efficiency was determined for each gene by generating a standard curve using serial dilutions of the cDNA, obtained after reverse transcriptase of the RNA, in abscissa and the corresponding cycle

Table 1 Oligonucleotide primer sequences used for real-time PCR

	GenBank	Sequence	Annealing temperature (°C)	Product size (bp)
Primer				
L19	AY158223	5'-AATCGCCAATGCCAACTC 3'-CCCTTTCGCTTACCTATACC	60	156
FSH β	NM_001009798	5'-GTAGCCTCAATGCCTAACACTC 3'-ATAGCCTCTAACATCCTCTCTCAG	58	123
BMP4	DQ192013	5'-GCCGACCATCAACTCAAC 3'-AGAACCACCTTGTCTACTCATCC	59	146
BMPR1A	NM_001076800	5'-TGTCGGACCAACTATGTAACCAG 3'-TGAGCAAAGCCAGCCATCG	61	100
BMPR1B	AF312016	5'-GGTGGAGCAGTGACGAGTG 3'-TCTTGAGGCAGGATTGTGAGC	59	77
BMPR2	XM_617592	5'-AGGTGTTCTGGATCGTCTTGTG 3'-CATGGATTGCTGTTGTTGTTGTTG	60	87
Id2	NM_001034231	5'-CGCGGTCAGCATGAAAGCC 3'-GGTTTTGCTCCGGGAGATGC	61	91
Noggin	DQ152984	5'-TGCGGAGGAAGTTACAGATGTGG 3'-ACGGACTTGGACGGCTTGC	60	172
Activin β B (tissue)	FJ167874	5'-GACAGCAGGCACCGCATC 3'-CGCAGTAGTTCCCGTAGTAGC	59	148
Activin β B (cell)	FJ167874	5'-GCGGAAGGTGCGGGTAAAGG 3'-TGGGTTCCGTGAGTGGAAGG	61	122
Activin β A	L19218	5'-GTCACCATCCGTCTCTTTAAC 3'-CCCTTCAAGCCCACTTCTCTC	60	86
Inhibin α	L28815	5'-GACTGGACAGACAGGAGACC 3'-AGGACAACGCAGCAGGAG	59	197

threshold (C_t) in ordinate. The slope of the log-linear phase reflects the amplification efficiency derived from the formula $E = (10^{-\text{slope}} - 1) \times 100$. Amplification efficiency was included between 95 and 105%, and was equivalent for test gene and the reference gene. Amplification was followed by melting curve analysis for each primer pair to verify the presence of one gene-specific peak and the absence of primer dimers. PCR products obtained with each set of primers were run on an agarose gel, and visualized using ethidium bromide and u.v. illumination to confirm amplification of a single product of the correct size. This was followed by sequencing of each amplicon to confirm the identity of amplified product, and the results were matched with the National Center for Bioinformatics Computing (NCBI-BLAST) database. Quantification of specific mRNA levels was performed by the $\Delta\Delta C_t$ method with the internal reference gene *L19* (Bulletin #2; PE Biosystems, Foster City, CA, USA). The intra- and inter-assay CV for tissue analyses were ranged between 1.3 and 6.6% and between 1.9 and 10.8% respectively. The intra- and inter-assay CV for cell analyses were 1.5–6.9 and 3.4–9.7% respectively.

Immunocytochemistry

Affinity-purified polyclonal antisera raised in rabbits against synthetic fragments of porcine inhibin α (1-26)-Gly-Tyr, βA (81-113)-NH₂, or βB (80-112)-NH₂ were kindly provided by W Vale and characterized previously (Vaughan *et al.* 1989). Pituitary sections (7 μ m) were incubated for 20 min in 0.3% hydrogen peroxide, rinsed with 0.1 M PBS (NaHPO₄, Na₂PO₄, and 9 g NaCl/l), and exposed for 20 min to normal sheep serum (1:15 in PBS). Sections were then incubated for 2 h with α -, βA -, or βB antiserum diluted at 1/500, 1/500, or 1/1/2000 respectively in PBS containing 0.1% BSA. Washed sections were incubated for 1 h in biotinylated anti-rabbit gamma globulins, rinsed, and then exposed to avidin-biotin peroxidase complex (DAKO, Trappes, France) for 30 min. The sections were then treated for 8 min with 0.04% 3,3'-diaminobenzidine tetrahydrochloride dihydrate (Sigma) and 0.03% H₂O₂ in Tris-HCl (0.05 M, pH 7.8). Sections were coverslipped with Depex. All incubations were carried out in a humidified chamber at room temperature. Specificity of the staining was confirmed since immunostaining was abolished when non-immune rabbit serum was substituted for the primary antibody. Cells immunostained for α - or βB -subunit and cells that were immunonegative were counted at $\times 40$ objective after counterstaining with hematoxylin. Data were calculated by counting ~ 1000 total cells from three randomly selected sections per animal in an immunohistochemistry assay. Two immunohistochemistry assays were performed per animal. The percentage of immunoreactive cells within the total cells was calculated as an average of the two immunohistochemistry assays for each animal.

Table 2 Changes in plasma concentrations of FSH, LH, and estradiol (E_2) during the estrous cycle. Mean plasma of FSH, LH, and E_2 concentrations for each group was calculated by averaging values from the last blood sample taken before killing each animal in each group. Values are the means $\pm$ S.E.M.

	Late follicular	Before 2nd FSH surge	Luteal
Plasma E_2 (pg/ml)	4.7 $\pm$ 0.99	1.02 $\pm$ 0.26	0.16 $\pm$ 0.16
Plasma LH (ng/ml)	0.45 $\pm$ 0.10	3.02 $\pm$ 1.30	0.07 $\pm$ 0.03
Plasma FSH (ng/ml)	0.28 $\pm$ 0.12	0.64 $\pm$ 0.20	0.75 $\pm$ 0.42

Statistical analysis

The levels of hormones and mRNAs during the estrous cycle and in cultured pituitary cells were compared using one-way ANOVA followed by the Newman-Keuls test. For estrous cycle experiment, two repetitions of RT-PCR using different

Figure 1 Changes in mRNA levels for FSH β (A), BMP4 (B), BMPR1A (C), BMPR1B (D), BMPR2 (E), Id2 (F), and noggin (G) across the estrous cycle. Pituitaries were collected from ewes killed in late follicular phase ($n=5$), before the secondary FSH surge ($n=4$), and in luteal phase ($n=4$). Total RNA was extracted from the pituitaries of each animal, and 1 μ g total RNA was analyzed by real-time RT-PCR and standardized by the level of *L19* mRNA in each sample. Values are means $\pm$ S.E.M. a versus b, $P < 0.05$.

Figure 2 Changes in mRNA levels for activin β A- (A), activin β B- (B), and inhibin α - (C) subunits throughout the ewe estrous cycle. Pituitaries were collected from ewes killed in late follicular phase ($n=5$), before the secondary FSH surge ($n=4$), and in luteal phase ($n=4$). Total RNA was extracted from the pituitaries of each animal, and 1 μ g total RNA was analyzed by real-time RT-PCR and standardized by the level of L19 mRNA in each sample. Values are the mean $\pm$ S.E.M. a versus b, $P<0.05$.

RNA samples from different pieces of pituitary from the same animal of each group were performed separately. The mean of these two repetitions represented the value of each animal. For culture experiments, at least three experiments were performed in duplicate when RNA was analyzed or in triplicate when FSH concentrations were measured. All results are expressed as means $\pm$ S.E.M. Values of $P<0.05$ were considered statistically significant. The statistical analyses were performed using GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, USA).

Results

Plasma hormone concentrations during the estrous cycle

Plasma hormone concentrations and morphological analysis of ovaries allowed reclassification of animals into four groups. Thus, five ewes were killed before the preovulatory LH surge (group 1: late follicular, $n=5$), two ewes during the preovulatory LH surge (group 2: LH surge, $n=2$), four after ovulation (group 3: before the secondary FSH surge, $n=4$), and four during the luteal phase (group 4: luteal, $n=4$). Since there were only two animals in the LH surge group, we excluded this group from the following analyses. Plasma concentrations of E_2 in blood samples recovered at the time of killing gave further evidence for the correct timing (Table 2).

mRNA expression of FSH β during the estrous cycle

The mRNA levels for FSH β -subunit in the pituitaries were determined by real-time PCR (Fig. 1A). Mean FSH β -subunit mRNA levels varied during the estrous cycle. They increased before the secondary FSH surge compared to the late follicular phase ($P<0.05$).

mRNA expression of BMP4, BMP receptors, Id2, and noggin during the estrous cycle

As shown in Fig. 1B–E, BMP4 and BMP receptor mRNA levels did not significantly change in the different stages of the estrous cycle. Similarly, the levels of mRNA for the inhibitor of differentiation 2 (Id2) reflecting the BMP4 bioactivity *in vitro*, since they are stimulated by BMP4 (see later), were similar in all studied stages of the estrous cycle (Fig. 1F). Further, the mRNA level for the BMP4 antagonist, noggin, did not significantly change over the estrous cycle (Fig. 1G).

mRNA expression of activin β A-, activin β B-, and inhibin α -subunits during the estrous cycle

The mRNA levels for activin β A, activin β B, and inhibin α -subunits in the pituitaries were also evaluated by real-time PCR. The level of activin β B mRNA increased before the secondary FSH surge compared with the other stages of

Figure 3 Immunostaining for inhibin α -subunit (A) and activin β B-subunit (B) on section from ewe pituitary collected before the secondary surge of FSH. Scale bars represent 18 μ m. Percentages of immunoreactive cells for inhibin α -subunit (C) and activin β B-subunit (D) among the total pituitary cells during the estrous cycle. Pituitaries were collected from ewes killed in late follicular phase ($n=5$), before the secondary FSH surge ($n=4$), and in luteal phase ($n=4$). Values are the mean $\pm$ S.E.M. a versus b, $P<0.05$. Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-10-0035>.

the cycle (Fig. 2B). In contrast, the amounts of activin β A or inhibin α mRNA were similar in all studied stages of the estrous cycle (Fig. 2A and C).

Percentage of pituitary cells expressing activin β B- and inhibin α -subunits during the estrous cycle

The presence of α - and β B-, but not β A-, subunits was confirmed by immunocytochemistry (Fig. 3A and B). The percentage of α -subunit immunoreactive cells did not vary throughout the estrous cycle ($1.6 \pm 0.2\%$ of total pituitary cells; Fig. 3C). In contrast, the percentage of β B-subunit increased before the secondary FSH surge compared to the follicular phase (2.6 ± 0.2 vs $0.8 \pm 0.1\%$ of total pituitary cells, $P < 0.005$; Fig. 3D).

Effect of GnRH, activin, and E_2 on mRNA expression of BMP4, BMP receptors, and Id2 in cultured ovine pituitary cells

To determine whether major factors of FSH regulation, such as GnRH, activin, or E_2 , were capable of modifying mRNA expression of BMP4 and/or BMP receptors, ovine pituitary cells were incubated with 10^{-8} M GnRH for 6 h, or 10^{-9} M activin or 10^{-9} M E_2 for 48 h. Figure 4A shows that 6 h treatment with GnRH decreased the levels of FSH β mRNA by 47% compared to basal conditions. Similar treatments with GnRH did not affect the mRNA levels for BMP4, BMPR1A (ALK3), BMPR1B (ALK6), BMPR2, and Id2 (Fig. 4B–F). When cells were treated for 48 h with activin or E_2 , the levels of FSH β mRNA were increased by 29% ($P < 0.05$) or decreased by 63% ($P < 0.05$) respectively compared to basal conditions (Fig. 5A). There was no significant change in the mRNA levels for BMP4, BMPR1A (ALK3), BMPR1B (ALK6), BMPR2, and Id2 (Fig. 5B–F). However, in the presence of E_2 , the amount of mRNA for BMPR1B (ALK6) tended to increase compared to basal conditions ($P < 0.1$; Fig. 5B and D).

Effect of GnRH and E_2 on mRNA expression of activin β B in cultured ovine pituitary cells

Treatment of pituitary cells with GnRH for 6 h did not significantly modify the amount of mRNA for activin β B-subunit (Fig. 4G). In contrast, treatment with E_2 for 48 h increased the mRNA level ($P < 0.05$; Fig. 5G).

Noggin does not affect FSH release

If BMP4 acts on FSH synthesis through a paracrine or autocrine mechanism, we can expect that blocking its action with an antagonist leads to an increase in FSH secretion. We first checked that noggin, a BMP binding protein, which antagonizes BMP signaling, was able to prevent the action of exogenous BMP4 on FSH release. As shown in Fig. 6A, noggin (100 ng/ml for 24 h) blocked the inhibitory action of BMP4 (40 ng/ml) on FSH release in ovine pituitary cells.

Similarly, it prevented the increase of Id2 mRNA level induced by BMP4 (Fig. 6B). However, noggin (from 10 to 100 ng/ml) did not significantly modify FSH concentrations in the culture media (Fig. 6C). Moreover, noggin alone did not affect the level of Id2 mRNA (Fig. 6B).

Figure 4 Effect of GnRH on the levels of mRNA for FSH β (A), BMP4 (B), BMPR1A (C), BMPR1B (D), BMPR2 (E), Id2 (F), and activin β B-subunit (G) from ewe pituitary cells. Cells were cultured in serum-free medium for 6 h with GnRH (10^{-8} M) and harvested. Total RNA was extracted, and 1 μ g total RNA was analyzed by real-time RT-PCR and standardized by the level of L19 mRNA in each sample. Values are the mean $\pm$ s.e.m. from at least three culture experiments with duplicate determinations. a versus b, $P < 0.05$.

Figure 5 Effect of activin or E₂ on the levels of mRNA for FSHβ (A), BMP4 (B), BMPR1A (C), BMPR1B (D), BMPR2 (E), Id2 (F), and activin βB-subunit (G) from ewe pituitary cells. Cells were cultured in serum-free medium for 48 h with activin (10⁻⁹ M) or E₂ (10⁻⁹ M) and harvested. Total RNA was extracted, and 1 μg total RNA was analyzed by real-time RT-PCR and standardized by the level of L19 mRNA in each sample. Values are the mean ± S.E.M. from at least three culture experiments with duplicate determinations. a versus b, P < 0.05.

SB431542 affects FSH release

The activin receptor-like kinase (ALK) 4/5/7 inhibitor, SB431542, is able to prevent the action of exogenous activin on FSH release (Fig. 7A). Moreover, treatment with SB431542 from 0 to 10 μM for 24 h inhibited FSH secretion from pituitary cells in a dose-dependent manner (Fig. 7B). Preliminary analyses showed that the signaling capacity of BMP receptors assayed by the ability of BMP4 to induce Smad1 phosphorylation was not affected by SB431542 (data not shown).

Discussion

Several BMPs, in particular BMP4, are able to inhibit FSHβ mRNA expression and FSH release from ovine pituitary cells, whereas activin stimulates this secretion (Faure *et al.* 2005). The presence of BMP4 mRNA, as well as BMPR type IA (ALK3) and type II at the gonadotrope cell level in the adult pituitary, suggests that BMP4 can act within the pituitary and participate to control FSH synthesis and secretion in the ewe. A lot of studies raised the question of the mechanism of action of BMPs at the gonadotrope cell level, but no data are available concerning the possible changes of pituitary BMP system during the estrous cycle. To determine whether the variations in FSHβ mRNA and plasma FSH concentrations are associated with changes in the expression of BMP4 and BMP receptor mRNA as well as activin βB mRNA, three stages of the estrous cycle exhibiting variations of plasma FSH as previously shown (Leung *et al.* 1988, Fafioffe *et al.* 2004) were studied: late follicular phase, before the secondary FSH surge, and the luteal phase.

Our results showed that the levels of BMP4 mRNA in the pituitary did not significantly change between the studied stages of the estrous cycle when the level of FSHβ mRNA varied. In addition, the levels of BMPR1A (ALK3) and BMPR2 mRNAs, as well as those of BMPR1B (ALK6), were similar over the estrous cycle. Other receptors beside BMPR1A (ALK3), BMPR1B (ALK6), and BMPR2 can be involved in signal transduction. For example, ALK2

Figure 6 Effects of noggin (100 ng/ml) and BMP4 (40 ng/ml) on FSH release (A) and Id2 mRNA expression (B) in ewe pituitary cells. Effect of increasing doses of noggin (0–100 ng/ml) on FSH release in ewe pituitary cells (C). Cells were cultured in serum-free medium for 24 h in the presence of indicated concentrations of noggin and/or BMP4. Media were collected, and the concentrations of FSH were determined by ELISA (A and C). Cells were harvested and total RNA was extracted, and 1 μg total RNA was analyzed by real-time RT-PCR for Id2 mRNA and standardized by the level of L19 mRNA in each sample (B). Values are the mean ± S.E.M. from three culture experiments with triplicate determinations for FSH concentrations measured and with duplicate determinations for RNA analyzed. a versus b, P < 0.05.

Figure 7 Effects of SB431542 (10 μ M) and activin (40 ng/ml) on FSH release in ewe pituitary cells (A). Effect of increasing doses of SB431542 (0–10 μ M) on FSH release in ewe pituitary cells (B). Cells were cultured in serum-free medium for 24 h in the presence of indicated concentrations of SB431542 and/or activin. Media were collected, and the concentrations of FSH were determined by ELISA. Values are the mean $\pm$ S.E.M. from three culture experiments with triplicate determinations. a versus b versus c, $P < 0.05$.

(also known as Acvr1) is able to induce BMP signaling (ten Dijke *et al.* 1994, Macias-Silva *et al.* 1998, Lee *et al.* 2007) and was previously shown to increase at the secondary FSH surge in ewe (Fafioffe *et al.* 2004). Nonetheless, BMP4 and BMP2 are known to bind to BMPR1A (ALK3) and BMPR1B (ALK6), whereas other BMPs bind more strongly to ALK2 (ten Dijke *et al.* 1994, Miyazono *et al.* 2010). In the murine gonadotrope cell line L β T2, the endogenous signal-propagating type I receptor at least for BMP2, structurally close to BMP4, would be BMPR1A (Ho & Bernard 2009). BMPs also show promiscuity in their binding to type II receptor. For example, in the absence of BMPR2, BMP2 and BMP4 can use ActR2A (Yu *et al.* 2005). However, BMPR2 is present on the gonadotrope cells. Moreover, the level of ActR2A mRNA is similar throughout the estrous cycle (Fafioffe *et al.* 2004). All together, these data led us to study preferentially BMPR2, BMPR1A, and BMPR1B. The lack of variations in the expression of BMP4 and BMP receptor mRNA does not necessarily mean that pituitary BMP4 activity does not change. For example, in the rat, neither activin β B nor activin receptor mRNA expression changes at the time of the secondary FSH surge. Instead, the expression of follistatin, an activin binding protein, is modulated freeing up activins to stimulate FSH (Halvorson *et al.* 1994). Hence, we followed the mRNA encoding noggin, a BMP4 antagonist. No change for the level of this mRNA was observed between the studied stages. We can argue that antagonists other than noggin can be involved. However, the absence of variation in the mRNA encoding Id2 (Peng *et al.* 2004), one BMP target gene reflecting the bioactivity of BMP4 in ewe pituitary cells, does not fit with this idea. All together, the data suggest that pituitary BMP4 activity did not significantly vary over the estrous cycle.

Because the levels of pituitary mRNAs during the estrous cycle result from a complex interplay between local and peripheral molecules, we examined the specific action of major factors of FSH regulation on the expression of BMP4, BMP receptors, and Id2 in cultured pituitary cells. Chronic treatment of pituitary cells with GnRH for 6 h, mimicking a situation similar to the preovulatory surge, induced a decrease

in the level of FSH β mRNA. This is consistent with the reduction observed *in vivo* in pituitaries collected during the LH surge (Fafioffe *et al.* 2004). Moreover, this agrees with reports showing that chronic GnRH inhibits the activity of the oFSH β promoter and the rat FSH β expression *in vivo* and *in vitro* (Attardi *et al.* 1989, Lerrant *et al.* 1995, Shafiee-Kermani *et al.* 2007). This inhibition can result from a fall of FSH β mRNA stability and/or a decrease of the transcription. In contrast, chronic GnRH did not modify the amount of mRNA for BMP4, BMP receptors, and Id2. Concerning the effect of the treatment with E₂ for 48 h, it inhibits the FSH β mRNA and FSH release (data not shown) as expected (Miller *et al.* 1977, Baratta *et al.* 2001). However, E₂ does not affect the expression of the mRNA for BMPR1A (ALK3) and BMPR2, but tends to increase that of BMPR1B (ALK6) mRNA. At this point, one could hypothesize that the effect of E₂ on FSH β is partly mediated by BMPs through BMPR1B (ALK6). However, BMPR1B (ALK6) was not detected on gonadotrope cells (Faure *et al.* 2005, Young *et al.* 2008). Moreover, the observation that the BMPR1B (ALK6) mRNA expression did not change throughout the estrous cycle does not favor a role of this receptor in gonadotropin regulation. Rather, one might propose that the trend to increase BMPR1B (ALK6) mRNA stimulated by E₂ *in vitro* reflects the involvement of the BMP system in another pituitary function. For instance, Paez-Pereda *et al.* (2003) have shown that a BMP4/estrogen receptor regulatory mechanism exists in lactotroph cells and is critical for maintaining the balance between proliferation and differentiation. Also, previous studies demonstrated that BMP4 repressed proopiomelanocortin transcription in corticotroph cells (Nudi *et al.* 2005). Hence, the regulation of the pituitary BMP receptors *in vivo* may require factors other than those operating for the control of the estrous cycle. When cultured pituitary cells were treated with activin for 48 h, the amount of mRNA for FSH β increased as expected (Dupont *et al.* 2003). However, no change in the mRNAs for BMP4, BMP receptors, or Id2 was observed.

Another factor of the same TGF- β family, activin, is considered a major intrapituitary regulator (Corrigan *et al.* 1991, Baratta *et al.* 2001, Padmanabhan & McNeilly 2001). To compare the expression patterns of both BMP4 and activin, we analyzed the mRNA levels of activin subunits. Surprisingly, no study about the expression of pituitary activin across the ovine estrous cycle is available. Interestingly, our results showed that the level of the mRNA for activin β B-subunit is higher before the secondary FSH surge compared to the other studied stages of the cycle. Also, the percentage of pituitary cells immunodetected with antibodies raised against β B-subunit increased before the secondary FSH surge. In contrast, neither the levels of the mRNA for inhibin α - and activin β A-subunits nor the percentage of pituitary cells immunodetected with anti- α antibody varied significantly across the cycle. Together with the high levels of ActR2, ALK2, and ALK4 mRNA observed during the secondary surge of FSH (Fafioffe *et al.* 2004), these results

reinforce the role of pituitary activin B as a major stimulus of FSH synthesis. Further evidence comes from exposure of ewe pituitary cells in culture to SB431542, an ALK4/5/7 inhibitor, showing inhibition of FSH release in a dose-dependent manner. These data contrast with the lack of changes in the BMP4 system and underline the difference between pituitary BMP4 and activin.

In cultured ewe pituitary cells, treatment of cells with GnRH for 6 h did not modify the level of activin β B mRNA. Similarly, in rat pituitary cells, chronic GnRH or rapid pulse frequency of GnRH did not affect activin β B mRNA levels in contrast to low pulse frequency (Demura *et al.* 1996, Dalkin *et al.* 1999). Treatment of pituitary cells with E_2 for 48 h increased the level of activin β B mRNA. This result is in contradiction with the data obtained by Baratta *et al.* (2001) who reported a decrease in activin β B mRNA after E_2 treatment. Both studies used ewe pituitaries collected during the non-breeding season, and the reasons for this discrepancy are unclear. They may rely on differences in experimental conditions such as incubation medium or the duration of E_2 treatment. The fact that E_2 increases activin β B mRNA is surprising and does not fit with the negative effect of E_2 on FSH β mRNA and FSH release. However, the increase, although reproducible, is weak and does not necessarily lead to a rise in activin protein. Moreover, we did not observe any difference in the amount of activin β B mRNA *in vivo* between luteal and late follicular phases when plasma concentrations of E_2 increase. However, the percentage of immunoreactive cells for β B is decreased suggesting that E_2 can affect post-transcriptional events. Further study at the protein level is required. Furthermore, factors other than E_2 can contribute to the control of activin β B expression. For instance, the rise in activin β B mRNA occurring before the secondary surge of FSH could be attributed to several factors such as the drop in circulating inhibin concomitantly to plasma E_2 decrease and/or to the low pulse frequency of GnRH.

Collectively, our data showed that changes in FSH β expression were not associated with variations in the expression of BMP4, BMP receptors, Id2, or noggin. The constant levels may reflect a constitutive BMP signaling in the pituitary. Nonetheless, treatment of pituitary cells with noggin did not modify Id2 level, at least *in vitro*, suggesting the absence of endogenous signaling. Moreover, blocking the action of BMP4 potentially produced by pituitary cells with noggin does not affect the *in vitro* release of FSH. Hence, the results do not favor the hypothesis that local BMP4 would play a regulatory role on FSH secretion during the estrous cycle. However, we cannot exclude the possibility that other BMPs can affect FSH production. Previous studies demonstrated that pituitary exhibits at least BMP2 and BMP7 mRNA expression (Huang *et al.* 2001, Faure *et al.* 2005). Alternatively, one can argue that BMP receptors, and potentially BMP4, are widely expressed in the pituitary making it possible that changes in their expression in the gonadotrope cells are masked by stable expression in other cells. However, we previously reported that the vast majority

of cells bearing the BMPR1A and BMPR2 was identified as gonadotropes (Faure *et al.* 2005). To confirm the lack of variations, it will be interesting to examine the expression of mRNAs for BMP4, BMP receptors, or Id2 at the gonadotrope cell level using *in situ* hybridization or immunohistochemistry. In addition, minor changes in mRNA expression evading detection may be sufficient to exert a paracrine/autocrine action. Nevertheless, the fact that treatment of pituitary cells with noggin, an antagonist of BMP4 and also BMP2 and BMP7, did not modify FSH release does not support this idea. This does not rule out a role for BMP4 on FSH synthesis and release in physiological conditions other than the estrous cycle such as pituitary development and/or in pituitary tumor. In contrast to BMP4, the amount of activin β B mRNA as well as the β B-subunit in the pituitary was shown to increase before the secondary FSH surge strongly suggesting that activin is a major stimulus of FSH synthesis necessary for the secondary FSH surge. Moreover, blocking the action of activin potentially produced by pituitary cells with SB431542 *in vitro* inhibited FSH release demonstrating the importance of local activin. Further study is now needed to better identify the regulatory factors of activin synthesis related to FSH secretion.

In conclusion, this study expands current knowledge on local BMP4 and activin actions in the regulation of FSH synthesis and release. Our study presents a line of data that are not in favor of a paracrine/autocrine action of BMP4, in contrast to activin, on FSH production in adult pituitary during the estrous cycle. However, BMP4 or other BMPs may play a role as endocrine factors. Indeed, Herrera & Inman (2009) showed the presence of BMP4, BMP6, and BMP9 in bovine serum. Further experiments are required to determine the presence or absence of BMPs in ovine serum and their physiological involvement in the regulation of FSH synthesis and release.

Declaration of interest

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

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