UNIVERSITE FRANCOIS RABELAIS
TOURS
UNIVERSITY OF EDINBURGH

Ecole Doctorale : Santé, Sciences, Technologies

Année universitaire : 2005

THESE POUR OBTENIR UN TITRE
CONJOINT DOCTEUR/DOCTOR OF PHILOSOPHY

Discipline : Sciences de la Vie

Présentée et soutenue publiquement

par Marie-Odile FAURE

le 2 Décembre 2005

Bone Morphogenetic Proteins (BMPs): nouveaux modulateurs de la synthèse et de la libération de l'hormone folliculo-stimulante (FSH)

Bone Morphogenetic Proteins (BMPs): new modulators of the follicle stimulating hormone (FSH) synthesis and release

---

Directeurs de these :
Dr. Catherine Taragnat
Pr. Alan McNeilly

JURY :

BENAHMED Mohamed	Directeur de Recherche	Université Lyon I
ROYERE Dominique	Professeur	Université de Tours
DAVIS Julian	Professeur	University of Manchester
FOWKES Rob	Chargé de Recherche	Royal Veterinary College London
MCNEILLY Alan	Professeur	Medical Research Council Edinburgh
TARAGNAT Catherine	Chargée de Recherche	INRA Tours

Acknowledgements

Writing this thesis was the ending of years spending with people I discovered for their friendship, their precious help in several urgent situations, their good mood, and their sense of humour. I would like to thank all of you for everything you gave me along this experience together.

My first acknowledge is for my two supervisors, Catherine and Alan who spent such a time for helping me from the first step (to experiment) to the last step (to write and discuss about this project). Thank you for transmitting your "savoir-faire", your passion about science and giving me the opportunity to test my ability and desire to become a scientist between your two groups. Thank you for accepting this challenge of transforming your strong collaboration in a new experience of joint-PhD. Thank you too for being by my side on February 2004, showing me your friendship in difficult moments.

I would also like to thank the Région Centre who accepted to finance my PhD during three years, the Académie d'Agriculture de France and the French Ambassy in Great Britain for financing my travels between Tours and Edinburgh. Philippe Chemineau, Danielle Monniaux and Benoît Malpaux should receive all my gratitude for letting me realise my PhD in the Physiologie de la Reproduction et des Comportements unit at the Institut de la Recherche Agronomique (Nouzilly) and Robert Millar and Alan McNeilly in the Center for Reproductive Biology at Medical Research Council Human Reproductive Sciences Unit (Edinburgh). I thank the Vice-President, Mr. Vaillant from the Université François Rabelais de Tours and the Vice-Principal, Mr. Boulton from the University of Edinburgh for signing new diploma and collaborate in aim to give joint-PhD to the students. A special thank is for Mr. Chevalier, belonging to The Science and Technology Department of the French Embassy in the United-Kingdom who helped me in so many situations, by being the motor of the joint-PhD story, the catalyser of the meeting between universities and my advisor in several cases.

I would like to thank Mohamed Benahmed, Dominique Royère, Julian Davis and Rob Fowkes for accepting to read my thesis, to give me their advices and to judge this work. I would also acknowledge the postgraduate studies comities in France and in UK especially Danielle Gourdji, Alain Caraty, Mohamed Benahmed and Stéphane Fabre for helping me to choose between different ways for the thesis.

Special thanks for the members belonging to Catherine and Alan's group of work:

To Joël who taught me how to plan an experiment, all techniques I learned… Now, it is my turn to teach you some tips!

To Linda who spent such a time to teach me real time PCR (yes, we loved it) and to teach me Scottish words and accent. How many "urgent" emails we sent to each other? How many hills we can climb together?

To Yves who helped me from my DEA to my future post-doc, to choose my way.

To Judy, Xavier, Danielle and Claire and who were always available for giving me advices, sharing their competency and their trust.

To Florence, Jean-Claude and François, always ready to give a hand or a chat!

To Mhairi who was a wee bee in the lab, very Scottish and who helped me to discover quickly the parties in Edinburgh

To Natacha and Manue, for being side by side in so many experiments and for trusting my beginning experience

To Elaine, Elodie and Sébastien who shared the worst and the best of PhD years. Especially for Elodie who accepted me so many times in her flat when I was writing up...

I would also like to thank other group members like Philippe for simplifying statistics, Stéphane for helping me during the thesis, Nadine for the good advices, Thierry for the cookery tips, Eric for compliments everyday, Martine for being my dear radio in the car every week and being my spy when I was not around, Norma for being half Scottish half French (don't worry you will wear a hat!), Moira for assisting me, Rosy for helping me with the LightCycler machine and Forbes for being funny all the time.

I thank the staff of INRA and MRC slaughterhouse and experimental farm for technical assistance and being patient for my beginnings and always available at any time.

Thanks to Maurice, Gaël and Dany who always knew how to save me with the printer even if I forgot to be on the network, to the IT support in Tours and Edinburgh who sorted our computer problems so many times, to Patrick who always found my boxes from Edinburgh wherever and whenever they arrived.

A special thank to all PhD and post-doc students I met during these three years. The elders: Sandrine, Charlotte, Alice (many thanks for all three for being there by email or by phone), Marie, Caroline, Rustem, Sébastien L., Pascal, Vincent. The same year PhD students: Sylvain (the best driver ever!), Elodie K. (who stayed until late to help me for the printing), Véro (nearly the end!), Sophie, Sébastien, Maud. To the next ones: Sébastien T. (my wee brother), Eric (the party organizer), Clémence (the dynamite), Cynthia (the London lady), Sarah, Kirsty (the horse whisperer). The others: Dimitra, Frances, Lindsey, Hazel and Yvonne (who were more than flatmates), Audrey (the french girl who began to be Scottish without noticing it), Céline.

I also thank all people I have not mentioned, who I crossed in corridors, at centrifuges or the office, the coffee-room or the canteen, people who with a smile or a simple word helped me to go further.

My friends, Carole & Cyrille, Nathalie, Alexiane, Marie-O & Bertrand, Eric & Céline, Fred & Fany, Marie-Lu & Nicolas, Jean-Marie, Fraise & Bryan, thanks to you, I took such great break. I promise, I will not tell you to wait several months to see me again next year!

I would like to thank my family (especially Babeth) and Nicolas' family (especially Jocelyne) who were close to me by giving me support so many times.

I would finish this acknowledgement by dedicating this thesis to my late Mum, to my Dad and to Nicolas, all three very special persons to me.

Abreviations

BSA: Bovine Serum Albumin

CDK: cyclin-dependant kinase

CHX: cycloheximide

DNA: Desoxyribonucleic Acid

cDNA : complementary DNA

RNA : Ribonucleic Acid

mRNA : messager RNA

BMP : Bone Morphogenetic Protein

BMPR : Bone Morphogenetic Protein Receptor

CG: granulosa cells

DMEM : Dulbecco's Modified Eagle Medium

E_2: oestradiol

ERK: extracellular signal-regulated kinase

FCS: Fetal Calf Serum

FecB: Fecondity Booroola

FOS: Fetal Ovine Serum

FSH : Follicle Stimulating Hormone

GDF : Growth Differentiation Factor

GnRH : Gonadotrophin Releasing hormone

GnRH-R: GnRH receptor

JAK/STAT: Janus kinase/Signal Transducers and Activators of Transcription

LH : Luteinising Hormone

MAPK: mitogen-activated kinase

oFSHβ-luc: construct of the ovine FSHβ promoter followed by the reporter gene, luciferase

PBS : Phosphate Buffer Salt

PCR : Polymerase chain reaction

RT-PCR : reverse transcription-PCR

SDS-PAGE : Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis

TAK1 : TGFβ kinase 1

TGFβ : Transforming Growth Factor-β

TBST: Tris-buffered saline-0.1% Tween 20

WT: wild-type

Thesis plan

Mrs Judith SALTERS
Postgraduate administrator
College of Medicine and
Veterinary Medicine Office
the University of Edinburgh

I declare that I have written this thesis based on my own work. The contribution of others has been clearly indicated.

Marie-Odile FAURE

24 May 2006

Foreword

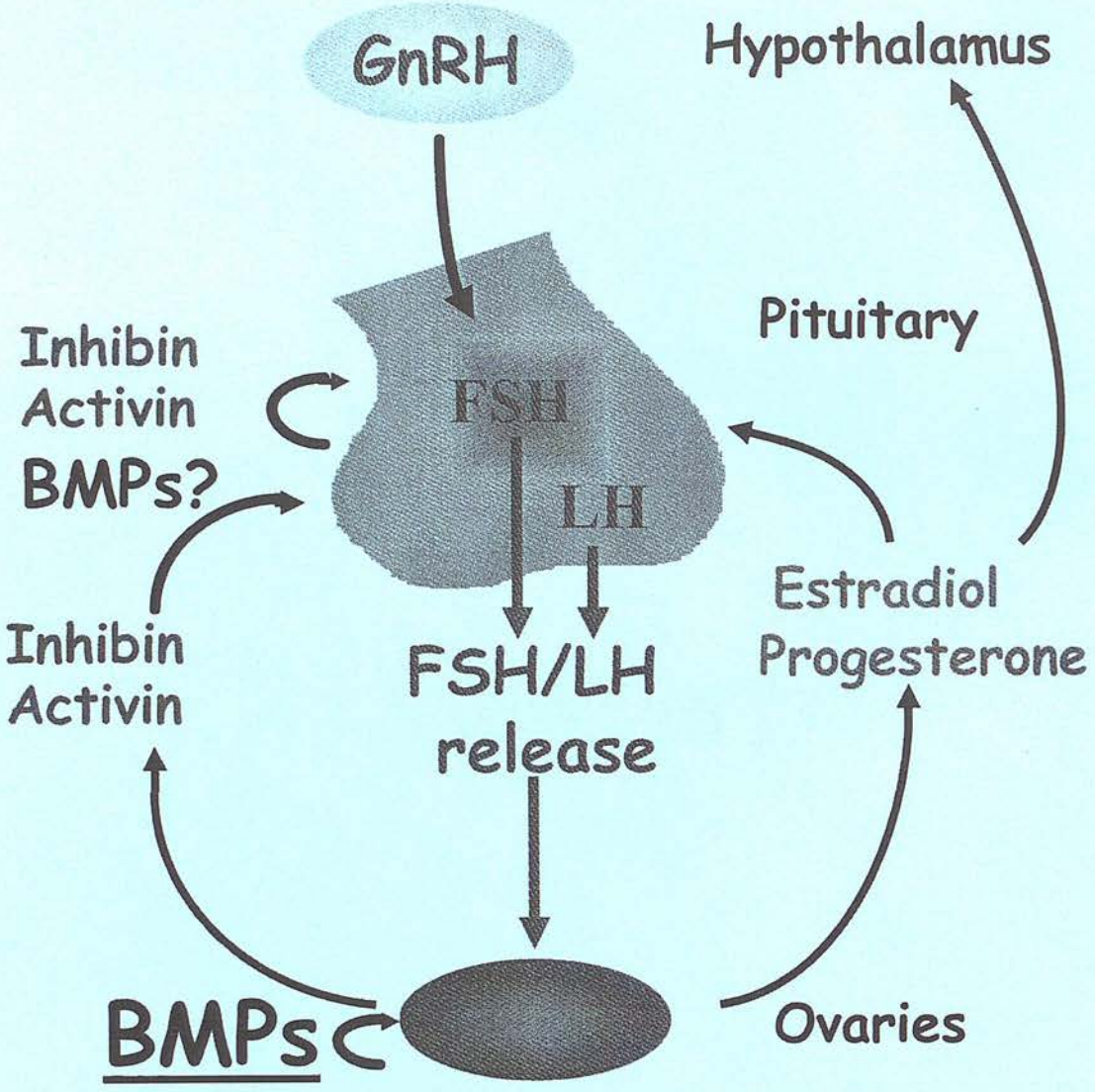


Fig.1. Schematical representation of differential regulation of gonadotropin secretion.

Foreword

Reproductive function requires the secretion of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) synthesized by the gonadotrope cells. A tight control of the production of these hormones is crucial, particularly in females, to coordinate the terminal follicular growth, the follicle selection as well as the timing and the number of ovulation. Synthesis and release of both gonadotropins are controlled by hypothalamic gonadotropin releasing hormone (GnRH) and gonadal steroids (progesterone and oestradiol). Despite these common regulators, divergences in the circulating patterns of LH and FSH are often encountered. Such divergences have suggested that other factors are specifically implicated in the control of FSH production. Inhibins and activins, members of the Transforming Growth Factor-Beta (TGF-β) superfamily, inhibit and stimulate respectively FSH synthesis and release. These gonadal factors are also produced in the pituitary where they act as paracrine factors. Other molecules of the TGF-β superfamily, the Bone Morphogenetic Proteins (BMPs) have recently been involved for their action in reproduction at ovary level. Whether BMPs act at pituitary level and participate with other factors to regulate FSH production in females is not elucidated. In order to extend our knowledge of the differential regulation of FSH and LH synthesis, this question was addressed in this study and developed around three purposes:

- Are BMPs and receptors expressed in pituitary? What are their effects on gonadotropin production? Does the natural mutation of BMPR-IB in ewes have an effect at pituitary level?
- What are the mechanisms of BMPs action at intracellular level?
- Is the mouse gonadotrope cell line $L\beta T_2$ a good model to analyse BMP action on FSH production?

Answering to these questions should help to better understand the mechanisms which control the fertility in females.

Before presenting the results, some data concerning the gonadotropins and the differential control of their synthesis and release are developed followed by a focus on BMP effects on reproduction. The mechanisms of action of BMPs, as well as activin are also detailed.

General introduction

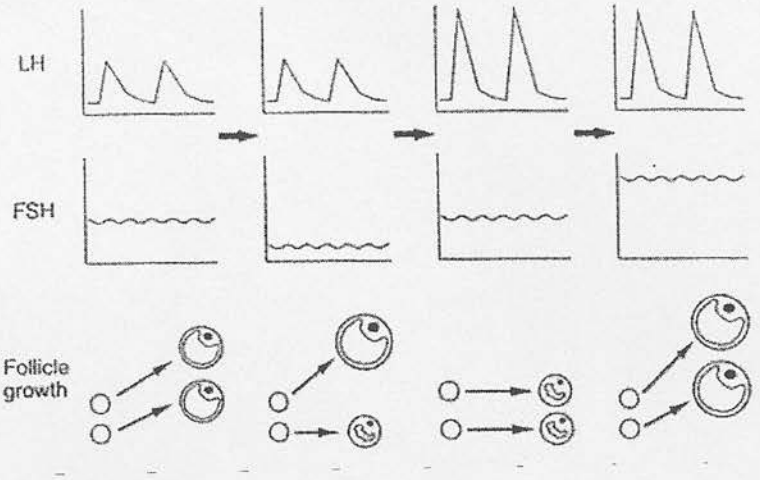


Fig.2. Schematical representation of the interaction between LH pulse amplitude and FSH on follicle growth. Small amplitude pulses of LH affect follicle growth if plasma concentrations of FSH decline. If LH pulse amplitude is increased then follicle growth is inhibited unless plasma concentrations of FSH increase (from McNeilly *et al.* 1992).

Introduction

The gonadotropins, luteinizing hormone (LH) and folliculo-stimulating hormone (FSH) are both synthesized in the pituitary by the gonadotrope cells (also called gonadotropes). Once released into the blood, these hormones target the gonads to regulate the gametogenetic and steroidogenetic functions. In females, they play critical and complementary roles during the terminal phase of the follicular growth. FSH stimulates follicular growth including cohort growth, selection and growth of dominant follicle, acting on ovary granulosa cells. LH induces the ovulation and the formation of the corpus luteum acting on granulosa cells and internal theca cells. An appropriate gonadotropin signal at the correct time at the ovary is a prerequisite to control the growth, the maturation and the number of follicles as well as the onset of ovulation (Fig. 2., McNeilly *et al.* 1992). To allow exquisite control of the ovarian function, a fine regulation of the secretion of both LH and FSH must exist and leads to variations in circulating gonadotropin concentrations throughout the oestrous cycle and divergent circulating patterns between LH and FSH are visible. For example, FSH concentration drops whereas LH pulses increase during the follicular phase, an important period for the selection of the preovulatory follicle. Such divergences in circulatory profiles of LH and FSH, necessaries for the appropriate development of the reproductive events arise from mechanisms and/or factors. Particularly, the regulation of the synthesis and release of FSH appears to be complex and is less well understood compared with LH. The purpose of this introduction is to review the current state of the knowledge of the mechanisms and the factors involved in the differential regulation of LH and FSH production.

I. Secretion profiles differ between gonadotropin hormones

A. The pulsatile nature of gonadotropin secretion

The release of LH mainly occurs in a pulsatile burst, characterised by a quick and punctual release in blood. Indeed, measurements of gonadotropins in the peripheral circulation show a clear pulsatile profile of LH secretion. A tight relationship between LH and the hypothalamic Gonadotropin Releasing Hormone (GnRH) was demonstrated using methods of collection of hypophyseal portal blood in sheep allowing measurements of GnRH concentrations close to its site of action simultaneously to the determination of LH

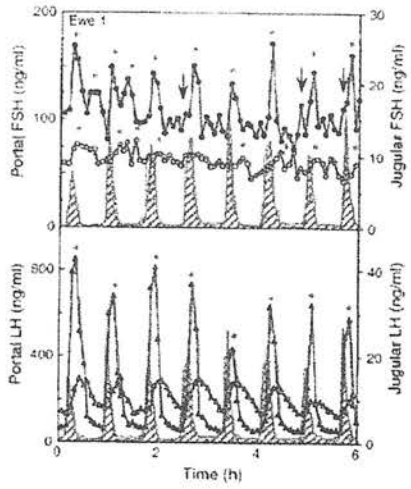


Fig.3. Patterns of hypophyseal portal (green) and jugular (blue) FSH patterns from one ovariectomized ewe sampled during the breeding season. Hypophyseal portal LH and peripheral LH patterns from a previous study are provided in the lower panels for comparison. To understand temporal relationships between FSH and GnRH, GnRH secretory patterns are overlaid (pink; scale not shown). Asterisks identify statistically identified pulses of FSH. Arrows indicate the GnRH-associated bursts of FSH that occur on top of a previously triggered episode of FSH release (Padmanabhan *et al.* 1997).

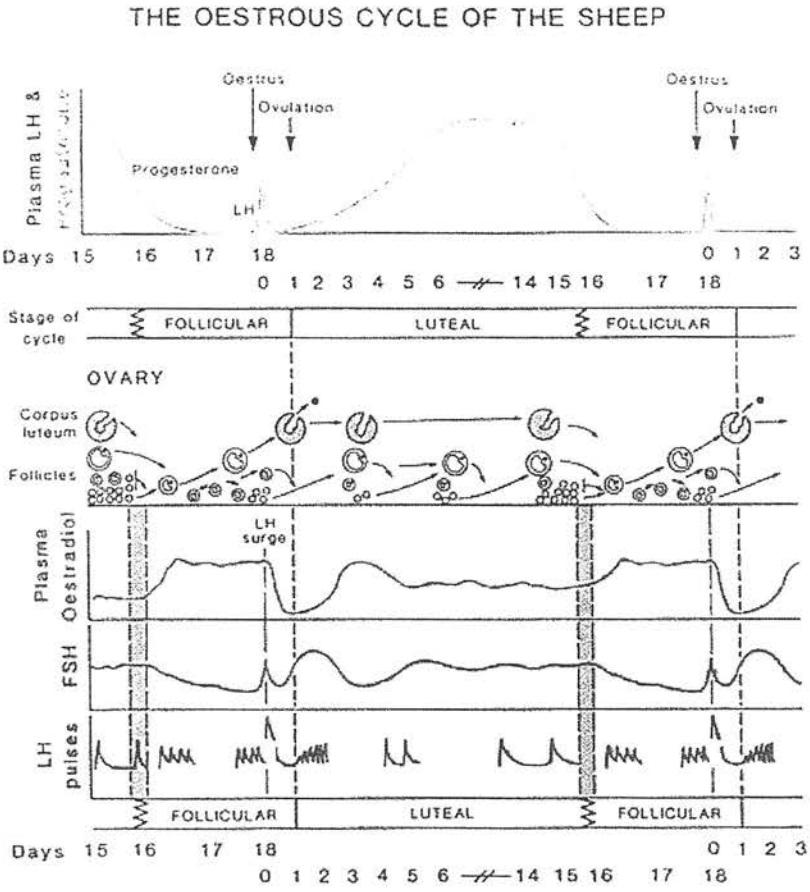


Fig.4. The oestrous cycle of the sheep (from McNeilly *et al.* 1992)

concentrations in the peripheral blood (Caraty *et al.* 1982, Clarke & Cummins 1982). These methods confirmed that each pulse of LH is a direct reflection of secretion of GnRH.

When FSH patterns are monitored in the peripheral circulation, the profile is not as regularly pulsatile as observed for LH and clear association between GnRH and FSH is not evident. One can argue that FSH has a longer half-life than LH and this can obscure the detection of FSH pulses in the peripheral circulation. Recent studies in sheep carried out to monitor FSH secretory pattern close to the site of release either in hypophyseal portal blood (Padmanabhan *et al.* 1997) or cavernous sinus (Clarke *et al.* 2002) have clearly shown that a synchrony exists between GnRH and FSH pulses but also that episodic secretion of FSH occurs independently of GnRH pulse (Fig. 3). Others factors and/or mechanisms would account for independent FSH pulses.

B. Throughout the oestrous cycle

In addition to the different nature of secretion observed between FSH and LH, circulating concentration patterns of these hormones can diverge throughout the oestrous cycle. In sheep, generally mono-ovulatory species with a long oestrous cycle characterised by a short follicular phase or in rat, pluri-ovulatory species, presenting a short cycle, the gonadotropin secretion profiles are very similar. Plasma LH concentrations are low throughout the cycle except during the preovulatory surge whereas FSH concentrations fluctuate at different stage of the oestrous cycle (Fig. 4). The initial portion of the follicular phase is characterised by a relatively high ratio of FSH to LH. This elevated FSH concentration is critical for the recruitment and maturation of ovarian follicles. By the middle of the follicular phase, LH pulse frequency increases whereas the rise in oestradiol and inhibin released from the growing follicle reduces FSH levels (Niswender *et al.* 1975). At the end of the follicular phase, the high oestradiol concentrations trigger the LH and FSH preovulatory surges. Just after ovulation, LH concentrations return to low levels while a second surge of FSH is noticeable attributed, at least partly, to the drop in estradiol and inhibin levels (Mann *et al.* 1990). During the luteal phase, LH concentrations remain low and FSH levels fluctuate episodically (Driancourt *et al.* 1991). Besides the large changes in LH concentrations at the preovulatory surge, there is little evidence for any change in levels of LHβ mRNA throughout the oestrous cycle (Currie and McNeilly 1995, Crawford *et al.* 2000, Fafioffe *et al.* 2004). In contrast, the release of FSH appears to be closely related to the levels of FSHβ mRNA (McNeilly *et al.* 2003, Fafioffe *et al.* 2004). Thus, these differences between FSH and LH

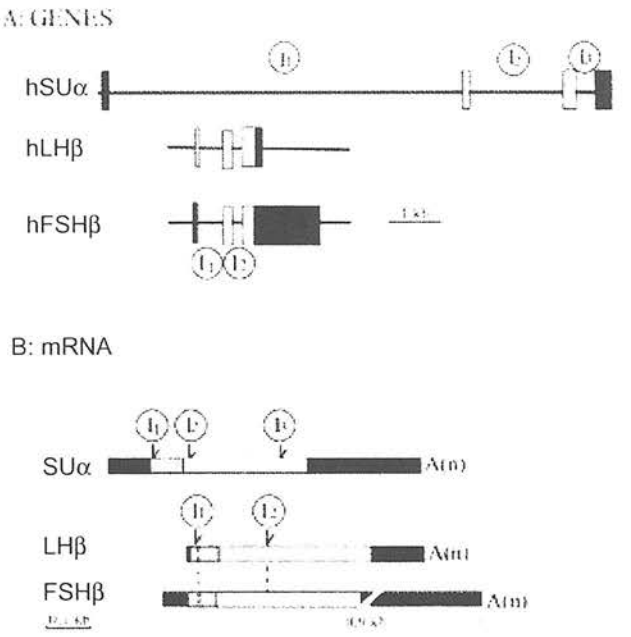


Fig.5. Structure of FSH and LH subunits genes and mRNA. i means introns. (from Counis *et al.* 2001).

profiles throughout the cycle underline the existence of specific mechanisms and/or factors of regulation. We propose to examine how the structure, the production site or/and the modulators of gonadotropins can participate to the differential regulation.

II. Levels of regulation implied into the differential secretion

A. The synthesis of gonadotropins

1. From DNA to mRNA

Gonadotropin hormones are constituted by an association of a common α subunit and a specific β subunit. Each subunit is encoded by a single gene. The α subunit gene comports 4 exons and 3 introns while LHβ and FSHβ subunit genes comport 3 exons and 2 introns. (Fig. 5). The maturation of the primary transcripts gives a single transcript for α and LHβ subunits whereas FSHβ gene generates various transcripts due to multiple polyadenylation sites (Jameson *et al.* 1988). Interestingly, the FSHβ mRNA major form has a shorter half life (for rat, 1h) compared to those of α and LHβ mRNA (for rat, α: 6h30; LHβ: 44h) (Bouamoud *et al.* 1992). This unstability of FSHβ mRNA is due to the presence of an untranslated long 3' poly-A tail (Bouamoud *et al.* 1992). This mechanism could represent a major post-transcriptional control means to regulate synthesis of FSHβ (Brown *et al.* 2001).

2. From mRNA to the protein

LH and FSH are both glycoprotein hormones. After the subunit gene transcription in the nucleus, the separated translation of the subunits follows into the rough endoplasmic reticulum. In this compartment, the signal peptide is excised and the immature sugar chains are transferred onto the α- and β-subunits. Then, the complete synthesis of the subunits and the non-covalent association of the α- and β-subunits are performed. Afterwards, the maturation of the oligosaccharidic chains is achieved throughout the transit from endoplasmic reticulum to the Golgi apparatus resulting in biologically active dimers LH or FSH. An ultimate achievement occurs within the secretory granules.

The composition of the oligosaccharidic chains provides the hormone complexity. The modifications of these chains occurring in the Golgi apparatus depend on the identity of the β-

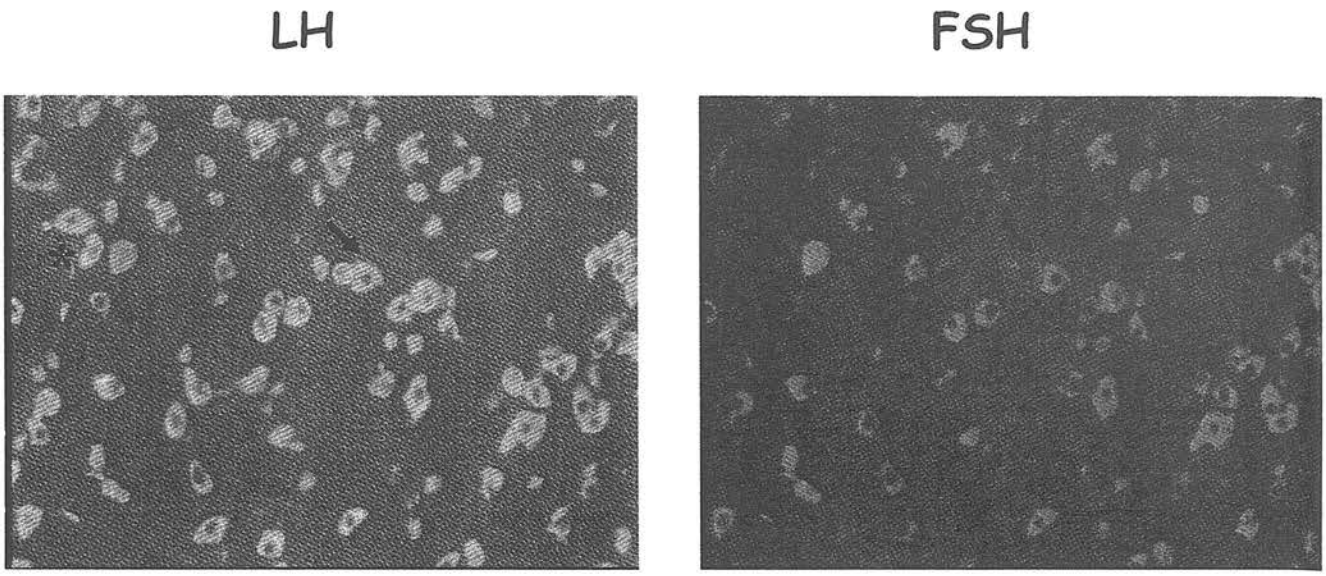


Fig.6. Double immunostaining LH (A) and FSH (B) of gonadotrope cells on the same section from ewe pituitary collected before gonadotrophin surge. Arrows indicate bihormonal LH/FSH and the star shows monohormonal LH (from Taragnat *et al.* 1998).

subunit. In ovine species, the α-subunit and the FSHβ-subunit have two sites of N-glycosylation whereas LHβ-subunit has one N-glycosylation site (Stockell-Hartree & Renvick 1992, Bousfield *et al.* 1994). Moreover, in spite of synthesis of LH and FSH takes place in the same pituitary cell, sulfated oligosaccharides predominate on LH while sialylated oligosaccharides predominate on FSH in human, bovine and ovine species (Baenziger & Green 1988). These differences in the oligosaccharide chains lead to different half-life of LH and FSH. Human LH protein injected in rat has a 15min half-life (Burgon *et al.* 1996) whereas human FSH protein half-life injected in monkey is about 1h30 (Klein *et al.* 2002). Adding to these characteristics, FSH has several isoforms that vary in their glycosylation status. These isoforms can be highly potent with a short half-life or less biological active with a longer half-life (for review: Ulloa-Aguirre *et al.* 1995). From a functional perspective, the control of FSH quality provides an exquisite fine-tuning system for precisely regulating the multiple functions of FSH (Padmanabhan *et al.* 2001).

B. The storage of gonadotropins

LH and FSH are both produced by the gonadotrope cells of the anterior pituitary. Gonadotropes constitute approximately 10% of the anterior pituitary cell population (Childs *et al.* 1987, Taragnat *et al.* 1998) that contains four other endocrine cell types, somatotropes, lactotropes, thyrotropes, corticotropes. Moreover, anterior pituitary also comprises folliculostellate cells and stem cells recently described (Chen *et al.* 2005).

1. The gonadotrope cell sub-populations

Ultrastructural studies have showed three types of gonadotrope cells in rat: the type I corresponds to large ovoid cells with small and large secretory granules, the medium-size type II is more angular with densely packed granules and the small size type III is angular with peripherally organised granules (Childs *et al.* 1980, Tougard & Tixier-Vidal 1988, 1994). In other species (porcine, ovine), one morphological cell type was observed with a homogenous distribution (Dacheux 1978, Currie & McNeilly 1995). However, immunohistochemical approaches or *in situ* hybridisation studies have shown gonadotropes containing both LH and FSH (bi-hormonal cells) or only LH or FSH (mono-hormonal cells) (Fig. 6). The pattern of storage seems to differ between species. In rat, bihormonal, mono-LH and mono-FSH cells have been described (Childs *et al.* 1987) whereas in cycling ewes, only bihormonal and

mono-LH were detected (Taragnat *et al.* 1998). These observations raised the following questions: Are these sub-populations independently regulated? Or are the cells intrinsically bihormonal with the capacity to direct the production of either hormone depending of the endocrine context? Interestingly, proportions of these different populations change throughout the oestrous cycle. Study of cycling female rats showed an increase in LH-containing cells during the LH surge by 2-fold although the percentage of total gonadotropes did not change suggesting that monohormonal FSH cells may have contributed to the surge by synthesizing LH (Childs *et al.* 1987). In cycling ewes, the proportion of gonadotrope cells falls at the end of the preovulatory gonadotropin surge, consequently to a specifically decrease of the bihormonal cells, strongly suggesting that the bihormonal gonadotropes preferentially participate to the LH surge (Taragnat *et al.* 1998). Moreover, the apparition of few mono-FSH cells at this phase of the cycle could reflect a specific loss of LH from bihormonal cells (Taragnat *et al.* 1998). After ovulation, the proportion of gonadotrope cells increases as result of a rise in the percentage of bihormonal cells. During the luteal phase of the cycle, the proportion of bihormonal cells continues to rise to detriment of mono-LH cells suggesting that mono-LH cells synthesize FSH to appear as bihormonal cells. Such fluctuations during the oestrous cycle suggest that regulatory factors as GnRH, steroids... play a role in the control of these sub-populations. Indeed, GnRH was shown to differentially affect these sub-populations. When gonadotrope cells from cycling female rats are stimulated with GnRH, the proportion of bihormonal cells increases (Childs 1985). This change is similar to that seen *in vivo*. Pulsatile injections of GnRH analogue at low frequency (1 pulse/6h) of ovariectomized ewes passively immunized against GnRH increased the percentage of bihormonal cells compared to high frequency (1 pulse/1h) by driving the synthesis of FSH in mono-LH cells (Molter-Gerard *et al.* 1999). Moreover, the stimulatory effect of estradiol induces a switch from bihormonal cells to mono-LH cells by depleting cells from FSH (Molter-Gerard *et al.* 2000).

Altogether, these data demonstrate that monohormonal cells can convert to multihormonal cells and vice-versa under the influence of regulatory factors that differentially drive LH and FSH synthesis and/or release. Hence, their intracellular storage must be different to allow selective release.

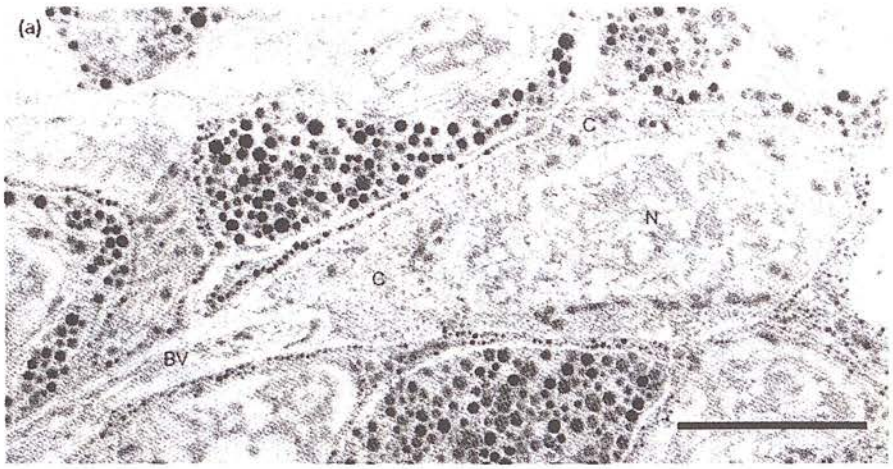


Fig.7. Transmission electron micrograph of a lightly stained, immunogold-identified (for LH) gonadotroph cell from an 80 nm section of male mouse pituitary gland (from Crawford *et al.* 2002).

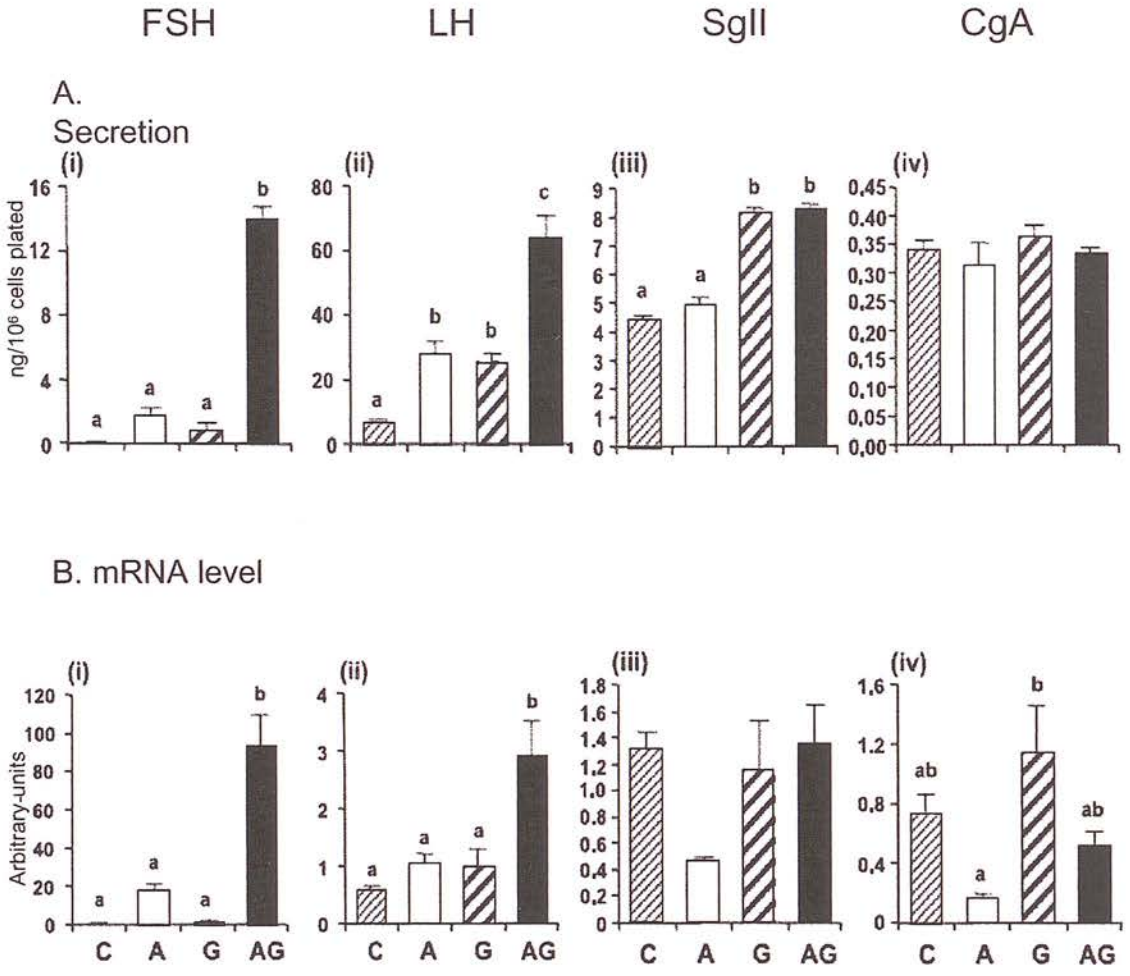


Fig.8. Effect of activin, GnRH, activin and GnRH on FSH, LH, SgII and CgA secretion and mRNA level in $L\beta T_2$ cells (from Nicol *et al.* 2004).

2. The subcellular level: the secretory granules

At the subcellular level, secretory granules within one cell have been found to contain either one or both gonadotropins (Inoue & Kurosumi 1984). In sheep, FSH appears to be localised in different regions of the cell compared with LH (Thomas & Clarke 1997). Whereas LH is packaged in electron-dense core granules (Currie & McNeilly 1995, Crawford *et al.* 2002, Fig. 7), FSH is present in less dense granules (Crawford *et al.* 2002). This differential packaging is associated to different patterns of trafficking. LH is released predominantly via a regulated pathway in response to GnRH stimulation and some FSH may be associated with LH. However, most FSH secretion is typical of a constitutive-like pathway in which freshly FSH synthesised is destined for release. The regulated secretion is mediated by dense core secretory granules containing highly concentrated stores of protein that are reserved for exocytose in response to a specific stimulus. In these granules, LH colocalises with secretogranin II (SgII) (Sion *et al.* 1988, Crawford *et al.* 2002) and SgII is released simultaneous to LH in response to GnRH (Nicol *et al.* 2002). The less dense granules containing FSH could mediate the constitutive-like secretion of FSH. These granules incorporate chromogranin A rather than secretogranin II (Watanabe *et al.* 1991). However, in the mouse gonadotrope cell line, LβT2, the release of FSH stimulated by both activin and GnRH is not correlated to the release of chromogranin A, suggesting that FSH release is granin-independent (Fig. 8, Nicol *et al.* 2004).

In addition to the different packaging of LH and FSH within the cell, differences in the trafficking of the LH and FSH granules to the cell membrane occur. This is illustrated by changes in secretory granule populations over the oestrous cycle.

In the luteal phase, the vast majority of cells (80%) contain LH secretory granules throughout the cytoplasm while 20% of cells present granules polarised to the region of the cell next to a vascular sinusoid (Currie & McNeilly 1995). The percentage of polarised cells increases during the follicular phase to 45% at oestrus, 75% at oestrus just before the LH surge and 90% in mid-LH surge. These results show that polarisation of secretory granules in gonadotrope cells is important for secretion of LH surge. Whether a similar polarisation exists for FSH-containing granules was not studied over the oestrous cycle. Nevertheless, oestradiol treatment of ovariectomized ewes causes the movement of LH secretory granules to the

periphery of the gonadotrope but does not influence the distribution of FSH-containing granules (Thomas & Clarke 1997).

These data are consistent with the notion that the trafficking of LH- and FSH-containing granules is differentially regulated. At present, the mechanisms that dictate and control the differential packaging and trafficking of LH and FSH are not known.

C. The modulation of LH and FSH secretion by common regulators: hypothalamic and gonadal factors

Gonadotropin Releasing Hormone (GnRH) is the hypothalamic factor that provides primary drive for the reproductive axis. Without GnRH, the gonadotropes and the gonads do not function. However, the secretion of GnRH as well its action on gonadotropes is modulated by a range of factors such as gonadal and pituitary factors.

1. GnRH, the hypothalamus modulator

a. GnRH structure and origin

GnRH is a decapeptide that was first characterised in mammals (GnRH-I). Using various molecular techniques, twenty-three structural forms have been identified in vertebrates but in the majority of species, two or three variants (GnRH-I, GnRH-II, GnRH-III) occur in anatomically and distinct neuronal populations (Millar *et al.* 2004). The GnRH-I is the hypothalamic form (called here as GnRH for the following sections) that provides the hypophysiotropic drive to the gonadotropes whereas the GnRH-II is more ubiquitous (review, Millar *et al.* 2004). The GnRH neurons originate from the olfactory placode (Schwanzel-Fukuda & Pfaff 1989) and migrate to the forebrain. In adult, the GnRH neuronal cell bodies localised in the hypothalamus project to the external zone of the median eminence, where terminals are found in close proximity to the primary capillary bed of the hypophyseal portal system (Page & Dovey-Hartman 1984). The peptide is released in synchronized pulses to stimulate the biosynthesis and secretion of LH and FSH (Fink 1988).

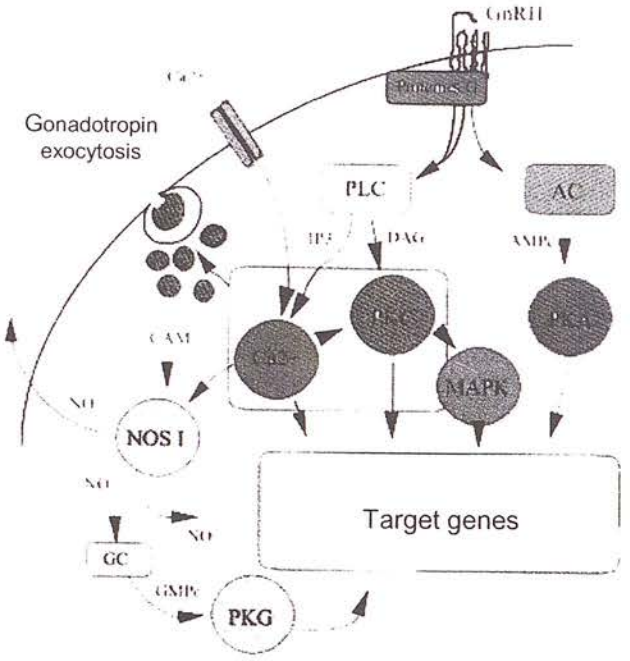


Fig.9. GnRH signaling pathways in gonadotrope cells (from Counis *et al.* 2001).

b. GnRH receptors and signaling pathways

GnRH binds with high affinity to its cognate type I receptors on the membranes of the pituitary gonadotropes to elicite its action. The presence of GnRH II in most vertebrates suggests the probable existence of type II receptors. However, although the type II receptor gene is present in the genome of different species, its expression is silencious at least in human, sheep, bovine and rat (Gault *et al.* 2004). Thus, it is reasonable to think that GnRH triggers its signaling pathway via type I GnRH receptor (GnRH-R). The GnRH receptor is a member of the rhodopsin-like G protein coupled receptor family (GPCR) which is characterised by seven transmembrane helices, linked by extracellular and intracellular loops (Tsusumi *et al.* 1992, Illing *et al.* 1993). The mammalian GnRH-R is unique in lacking an intracellular carboxyl-terminal tail, a feature that contributes to the lack of rapid desensitization (Davidson *et al.* 1994) and a slow internalization (Blomenrohr *et al.* 1999). In gonadotropes, GnRH-R interacts with $G_{q/11}$ protein through the second and third loops, activating the phospholipase Cβ (PLCβ), resulting in the hydrolysis of membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol 1,4,5-triphosphate (IP3) and diaglycerol. The IP3 bound to its receptor triggers the release of intracellular calcium and the diaglycerol activates the protein kinase C (PKC) (Naor *et al.* 1998, Fig. 9). Other couplings with Gs and Gi proteins have also been described but the predominant association between GnRH-R and G protein is with $G_{q/11}$ (review, Pawson & McNeilly 2005). Moreover, the activation of PKC-dependent MAP kinase (MAPK) subfamily as the extracellular signal regulated protein kinase (ERK), the c-junk N-terminal protein kinase (JNK), the p38 MAPK and the big MAPK (BMK) cascades have been described (Naor *et al.* 2000). For example, in murine gonadotrope cell line, LβT2, the activation of transfected rat LHβ promoter by a GnRH agonist was reduced by cotransfection with ERK or JNK dominant negative showing their implication on LHβ promoter activity (Harris *et al.* 2002, Nguyen *et al.* 2004).

The GnRH leads to a rise in intracellular calcium levels derived from IP3-activated intracellular storage pools and from the influx of calcium from extracellular fluid through L-type voltage sensitive calcium channels (Stojilkovic *et al.* 1994). In mouse gonadotropes $L\beta T_2$, GnRH stimulated calcium/calmodulin-dependent protein kinase type II (Ca/CaMK II) subunit activity by 3-fold. The administration of the Ca/CaMK II-specific inhibitor blocked the GnRH response and suppressed the α subunit and LHβ promoter responses to GnRH by 40-60% (Haisenleder *et al.* 2003).

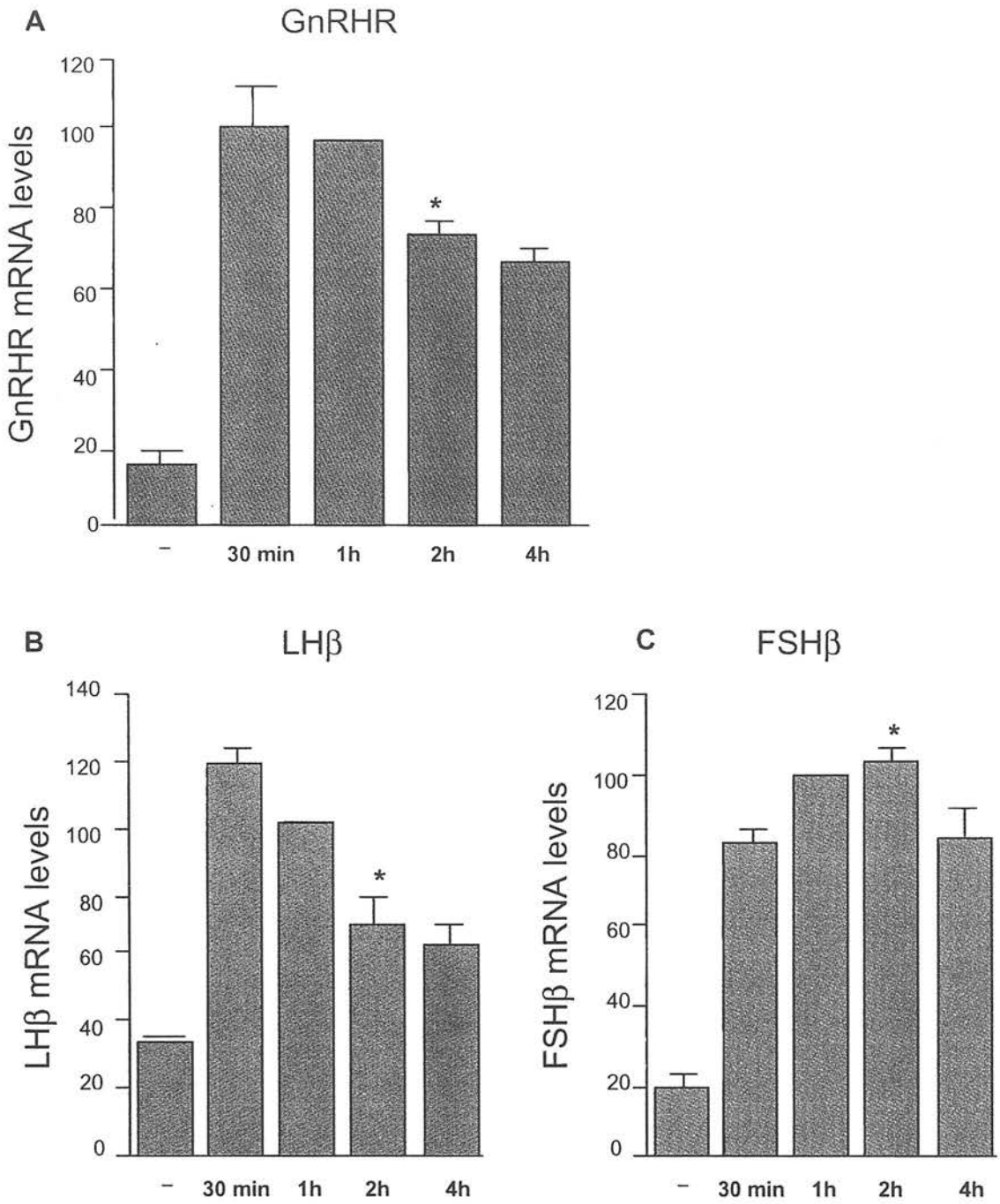


Fig.10. Effects of GnRH pulse frequency on GnRHR (A), LHβ (B) and FSHβ (C) subunits mRNA levels in primary rat pituitary cell cultures. The mRNA levels expressed as a percentage of mRNA levels at a GnRH pulse frequency of every hour, are shown for each frequency of pulsatile GnRH tested. *, $P < 0.05$ compared with pulsatile GnRH every 30min (from Kaiser *et al.* 1997)

c. GnRH effect on gonadotropin subunit expression and gonadotropin secretion

The indispensable role of GnRH to induce the synthesis and release of gonadotropins is demonstrated by several evidences. Genetic GnRH deficiency in rodents (Mason *et al.* 1986) or humans (Seminara *et al.* 1998) results in a decrease of the plasma gonadotropin concentrations associated with hypogonadism and infertility. The same phenotype is observed in patients with loss of function mutations in the type I receptor gene (Kottler *et al.* 1999). This role of GnRH is confirmed by experimental animal models where the blockade of GnRH action by hypothalamo-pituitary disconnection, passive immunization or GnRH antagonist provokes the decrease of FSH and LH release (Clarke *et al.* 1983, Caraty *et al.* 1984, Lalloz *et al.* 1988). This effect is accompanied by a reduction in the expression of the three gonadotropin subunit genes, α, LHβ and FSHβ. Moreover, GnRH response elements have been identified in the rat LHβ gene promoter (Kaiser *et al.* 1998) and in the ovine FSHβ gene promoter (Strahl *et al.* 1998, Miller *et al.* 2002, Coss *et al.* 2004). However, the degree of GnRH dependency differs between LH and FSH. When the action of GnRH is suppressed in hypothalamo-pituitary disconnected animals or in GnRH immunised animals, LH release is immediately blocked demonstrating the strictly GnRH dependency of LH. In contrast, FSH concentrations slowly decrease (Culler & Negro-Vilar 1986, Turzillo & Nett 1997, Molter-Gérard *et al.* 1999). GnRH-R mutations in patients with idiopathic hypogonadotropic hypogonadism affect differently the LHβ, FSHβ and αGSU gene promoters (Bédécarrats *et al.* 2003). Then, a question is how single hormone (GnRH) acting on a single cell can differentially regulate LH and FSH. It is well established that the pulsatile nature of GnRH release is crucial for stimulating LH and FSH production (Wildt *et al.* 1981). Only intermittent GnRH administration activates LH secretion while continuous GnRH leads to a decline in LH release after an initial stimulation. Furthermore, GnRH differentially regulates gonadotropin secretion via changes in the pattern of GnRH pulse release. *In vivo*, the patterns of GnRH pulses vary physiologically depending on the hormonal status and the reproductive cycle stage. It has been shown that high frequencies of GnRH inputs, similar to those observed in follicular phase are favourable to α, LHβ subunits expression and LH release while slow frequencies comparable to those occurring in luteal phase selectively increase FSHβ mRNA expression and FSH release (Dalkin *et al.* 1989, Molter-Gerard *et al.* 1999, Farnworth 2000, Burger *et al.* 2002, Fig. 10). These selective effects of GnRH pulse frequency are similar on subunit promoter activities (Bédécarrats & Kaiser 2003). Fast

frequencies stimulate expression of a rat LHβ promoter-reporter to a greater degree than slower frequencies. In contrast, a rat FSHβ promoter was preferentially stimulated by slower GnRH pulse frequencies.

How gonadotropes are able to differentiate GnRH pulse frequencies and activate appropriate signal transduction pathways to differentially stimulate α, LHβ and FSHβ gene expression and hormone release has yet to be elucidated. The response of gonadotropes to GnRH correlates with the number of GnRH-R on the cell surface. GnRH itself regulates this number. Administration to a GnRH antagonist in sheep showed a decrease in GnRH-R mRNA expression and GnRH-R numbers (Brooks & McNeilly 1994). Moreover, fast GnRH pulse frequencies induce high density of GnRH-R whereas lower frequencies are associated with a low density of GnRH-R (Clarke *et al.* 1987, Kaiser *et al.* 1997, Fig. 10). These results mimic the pattern of GnRH-R gene expression and receptor number observed throughout the oestrous cycle (Brooks *et al.* 1993, Fafioffe *et al.* 2004, Schirman-Hildesheim *et al.* 2005). Using cotransfection of increasing amounts of rat GnRH-R with gonadotropin gene reporter constructs in the heterologous rat somatolactotrope GH3 cell line, Kaiser *et al.* (1995) showed a differential regulation of LHβ and FSHβ by GnRH, with LHβ and FSHβ promoter activities being up-regulated to the greatest extent at high and low cell surface GnRH-R densities, respectively. In murine $L\beta T_2$ gonadotropes, the increase in GnRH number precedes the pulse frequency-dependent preferential stimulation of LHβ or FSHβ promoters and may be a mediator of the differential regulation of gonadotropin subunit expression by different frequencies of GnRH (Bédécarrats & Kaiser 2003). However, the mechanisms by which GnRH number contributes to these effects remain to be elucidated. Recent studies have shown that the pattern of MAP Kinase, particularly ERK is altered distinctly under high and slow GnRH pulse frequencies (Haisenleder *et al.* 1999, Kanasaki *et al.* 2005).

Although GnRH is an important factor of regulation of gonadotropin secretion capable of differentially modulate LH and FSH production via changes in its input patterns, evidence support the existence of non-GnRH-dependent mechanism regulating FSH.

Indeed, in the hypophyseal portal blood, some FSH pulses are not associated with GnRH pulses (Padmanabhan *et al.* 1997). These pulses are maintained after GnRH antagonist treatment (Padmanabhan *et al.* 2003) suggesting that other factors are involved in the

regulation of the FSH secretion (Padmanabhan & Sharma 2001, Pawson & McNeilly 2005). Then, the existence of a FSH-releasing factor (FSH-RF) has been suggested and supported by the following experiment. The radiofrequency lesions of the dorsal anterior hypothalamic area (DAHA) abolished FSH pulsatility without altering LH pulsatility suggesting the involvement of distinct neural region for controlling the FSH pulsatility (Lumpkin *et al.* 1989). Using biochemical separation methods, a preparation enriched with biologically active FSH-RF was obtained (Lumpkin *et al.* 1987). The identification of variant forms of GnRH opened up the possibility that one form was the putative FSH-RF. In the rat pituitary cells, the lamprey GnRH III which was shown to release FSH at lower doses than GnRH has been suggested (Yu *et al.* 1997, 2002). However, studies in ewe (unpublished data) and in cattle (Amstalden *et al.* 2004) showed no effect of the lamprey GnRH III on FSH secretion. Hence, to date, a physiologically relevant hypothalamic FSH-RF has not yet been characterized. Besides, several results suggested effects of gonadal and pituitary factors in the differential regulation of gonadotropin secretion.

2. The steroids, gonadal factors

In addition to the GnRH, steroid hormones, oestradiol and progesterone, have a large influence on the secretion of both gonadotropins by modifying the secretion of GnRH from the hypothalamus or acting directly at the pituitary level on gonadotrope cells. Their effects are either negative or positive on gonadotropin secretion. During the luteal phase of the ewe oestrous cycle, the GnRH pulse frequency is low due to the high circulating concentrations of progesterone secreted by the corpus luteum. Following the demise of the corpus luteum, the follicular phase is initiated, progesterone concentrations fall and plasma levels of oestrogen produced by the ovarian follicles inhibit FSH secretion. Later in the follicular phase, the plasma oestrogen concentrations increase and provoke a switch from negative effect to positive effect of oestrogen on GnRH/LH pulse frequencies which trigger the preovulatory LH surge.

a. Steroids signaling pathways

The steroids access to the pituitary via the blood and diffuse through the membrane to bind steroids hormone receptors (SHRs). These receptors are expressed in the gonadotropes (Childs *et al.* 2001). The effect of oestradiol is mediated by two types of oestrogen receptors (ERα and ERβ) while progesterone can bind the progesterone receptor (PR). In sheep

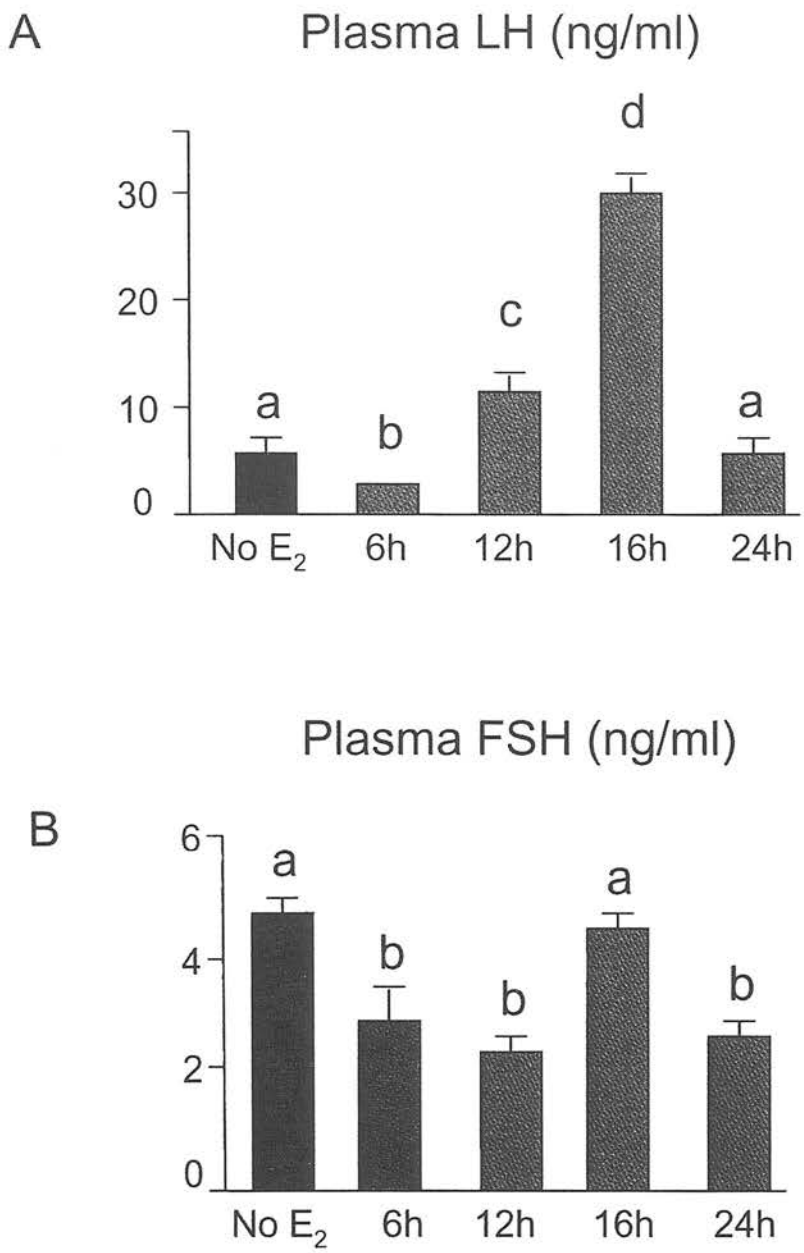


Fig.11. A biphasic effect on mean LH and FSH plasma levels in ovariectomized ewes was observed after oestradiol (E_2) treatment. Injection of E2 6h before slaughter induced a decrease in LH (A) and FSH (B) plasma levels compared with non-E2-treated ewes ($P< 0.01$). In contrast, an increase in LH plasma concentrations was observed in ewes killed 12 h after injection ($P< 0.01$ vs. non treated ewes) and FSH plasma reached the level of non treated ewes (from Molter-Gerard *et al.* 2000)

pituitary, there is a significant expression of the ERα while the expression of the ERβ is very low (Tobin *et al.* 2001). After the binding of the ligand, the conformation of the intracellular receptor complexed with conforming proteins (chaperones) changes. This phenomenon triggers the complex to bind specific regions of DNA of target genes called Steroid Response Element (SRE). SREs initiate chromatin remodelling and can be modulated by activator or repressor transcriptional factors, stimulating the transcription and protein synthesis (O'Malley & Tsai 1992, Beato & Klug 2000, Nilsson *et al.* 2001). Besides this classical steroid pathway, some data provide evidence for up-regulation of second messenger systems. For instance, oestradiol induces rapid effects via phosphorylation of MAPK and of cAMP response element binding protein (CREB) in the brain suggesting non genomic actions (Abraham *et al.* 2003).

b. Oestrogen effect on gonadotropin secretion

The responsiveness of gonadotropes to oestrogens varies throughout the oestrous cycle associated with an increase in the number of gonadotropes expressing ERα in the follicular phase compared to the luteal phase (Childs *et al.* 2001, Tobin *et al.* 2001). The biphasic effect of oestrogen is clearly demonstrated in ovariectomized ewes treated with oestradiol, in which a decrease in gonadotropin release occurs preceding an increase of gonadotropin secretion (Fig. 11).

* negative effects

The negative effect of oestrogens is demonstrated by gonadectomy. Indeed, after ovariectomy, LHβ and FSHβ mRNAs as well as LH and FSH concentrations increased. The addition of oestrogens suppresses this post-ovariectomy rise (Corbani *et al.* 1984, Shupnik *et al.* 1988). Concerning LH, this action reflects a hypothalamic action since a GnRH antagonist was able to block the post-ovariectomy increase (Dalkin *et al.* 1993, Shupnik & Fallest 1994). However, oestrogens also exert a direct action on the pituitary. To discern the hypothalamic effects of oestrogens from direct pituitary effects, animal models were developed in which the GnRH input was removed either by hypothalamo-pituitary disconnection (HPD) or by immunisation against GnRH. In such animals given with constant hourly pulses of GnRH or GnRH analogue to restore the gonadotropin secretion, injection of oestradiol induces a rapid suppression of LH release due to a reduction in LH pulse amplitude (Clarke & Cummins 1984, Molter-Gerard *et al.* 2000) without reduction in the level of LHβ mRNA (Mercer *et al.* 1993, Molter-Gerard *et al.* 2000). If oestradiol is administered chronically, the LH pulse amplitude is reduced by only 20% suggesting that the long term inhibitory feedback targets

mainly the hypothalamus (Clarke *et al.* 1988). To reinforce these data, it was observed that in ewe pituitary cells treated with oestradiol for 24h, LH release was not reduced. In contrast, an increase in LH concentrations was observed (Nett *et al.* 2002).

Concerning the oestrogen action on FSH synthesis and release, the post-ovariectomy increase in FSHβ mRNA was only partially reduced by oestradiol, suggesting the importance of another factor, such inhibin, in suppressing FSHβ expression (Condon *et al.* 1985, Gharib *et al.* 1987, Dalkin *et al.* 1990). In rat treated with a GnRH antagonist, oestradiol is not able to reduce the post-ovariectomy increase in FSHβ mRNA suggesting a pituitary site of action (Shupnik *et al.* 1988). In sheep, the negative effect of oestradiol on FSH production seems to target preferentially the pituitary. In GnRH deficient animals, oestradiol provokes an inhibitory effect on FSHβ mRNA and FSH release (Mercer *et al.* 1993, Molter-Gérard *et al.* 2000). This inhibitory effect is also observed when ewe pituitary cells are treated with oestradiol for 24h (Phillips *et al.* 1988, Nett *et al.* 2002, Faure *et al.* 2005). Moreover, treatment of ovine pituitary cells with oestradiol suppressed the activity of an ovine FSHβ promoter-luciferase transfected in ovine pituitary cells (Miller & Miller 1996). However, in contrast to LH promoter, no oestrogen response element (ERE) was present upstream of the coding region of the ovine FSHβ subunit gene. Other elements must confer the responsiveness to oestradiol.

The inhibitory effect of oestradiol in GnRH immunised ewes on LH and FSH release was accompanied with a specific decrease of bihormonal gonadotrope cells suggesting that both LH and FSH synthesis are reduced (Molter-Gérard *et al.* 2000).

* positive effects

The stimulatory effect of oestradiol on LH secretion is clearly illustrated by the preovulatory surge. A similar surge can be induced, after an initial inhibition of LH release, in ovariectomized animals treated with oestradiol (Clarke *et al.* 1989, Fig. 11). For this action, oestradiol alters at least two parameters. First, it increases the number of GnRH receptors present at the gonadotrope membrane in a rapid delay (Clarke *et al.* 1988, Gregg *et al.* 1990, Ghosh *et al.* 1996, Cowley *et al.* 1998). Second, it stimulates GnRH secretion (Moenter *et al.* 1990). Thus, oestradiol increases the sensitivity of gonadotropes to GnRH allowing a maximal response to massive release of GnRH. Exposure of pituitary cells to oestradiol for

24h leads to an increase in medium LH concentrations, consistent with the presence of an ERE upstream of the coding region for the LHβ subunit gene (Shupnik & Rosenzweig 1991).

Concomitantly to the LH surge, an FSH surge occurs after oestradiol treatment both in intact and GnRH immunized animals given with hourly GnRH pulse (Caraty *et al.* 1984, Molter-Gérard *et al.* 2000). In OVX-HPD ewes treated with oestradiol, no FSH surge is observed (Mercer *et al.* 1993). The stimulatory effect on FSH could be the reflection of the increase of the sensitivity of the gonadotropes to the enhanced release of GnRH rather than a direct pituitary effect. As a matter of fact, in superfused rat anterior pituitary gland, the administration of both oestradiol and pulses of GnRH induced the LH and FSH surges (Wun & Thorneycroft 1987).

c. Progesterone effect on gonadotropin secretion

Progesterone is an inhibitory factor of the gonadotropin synthesis and release. Similar to oestradiol, progesterone acts at hypothalamic level. This hormone decreases the frequency of the GnRH pulses (Karsch *et al.* 1997). In HPD ewes, progesterone does not modify the release of LH and FSH, suggesting that it does not act directly at the pituitary. However, following exposure of pituitary cells to progesterone, the release of GnRH-stimulated LH is blocked as well as the secretion of LH stimulated by oestradiol (Batra & Miller 1985a). The action of progesterone on LH should be modified by hormonal environment. Moreover, progesterone is able to inhibit the basal secretion of FSH and the expression of the FHSβ mRNA (Batra & Miller 1985b), demonstrating the differential effect of this steroid on the gonadotropin production.

Moreover, the association of oestradiol and progesterone increased gonadotropin secretion showing an indirect effect of progesterone on FSH and LH secretion (McPherson *et al.* 1975).

From these data, it is clear that steroids, particularly oestrogens alter differentially the ability of the gonadotropes to synthesize and release LH and FSH.

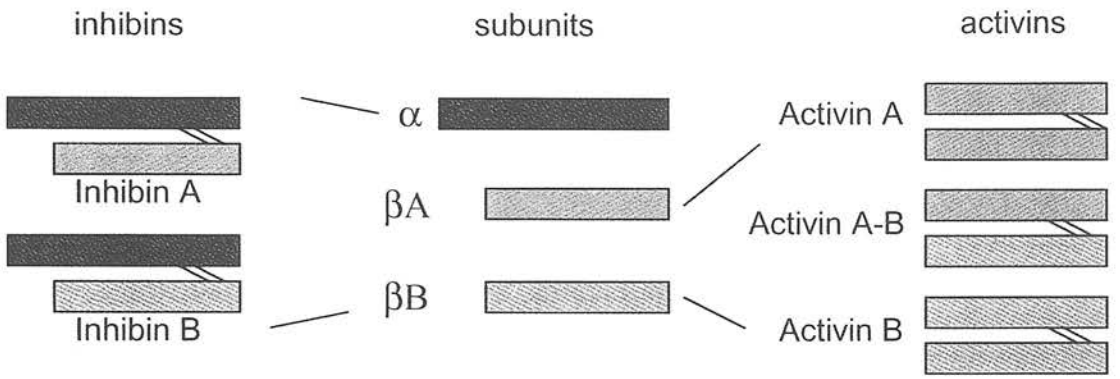


Fig.12. Activins and inhibins subunits (from Counis *et al.* 2001)

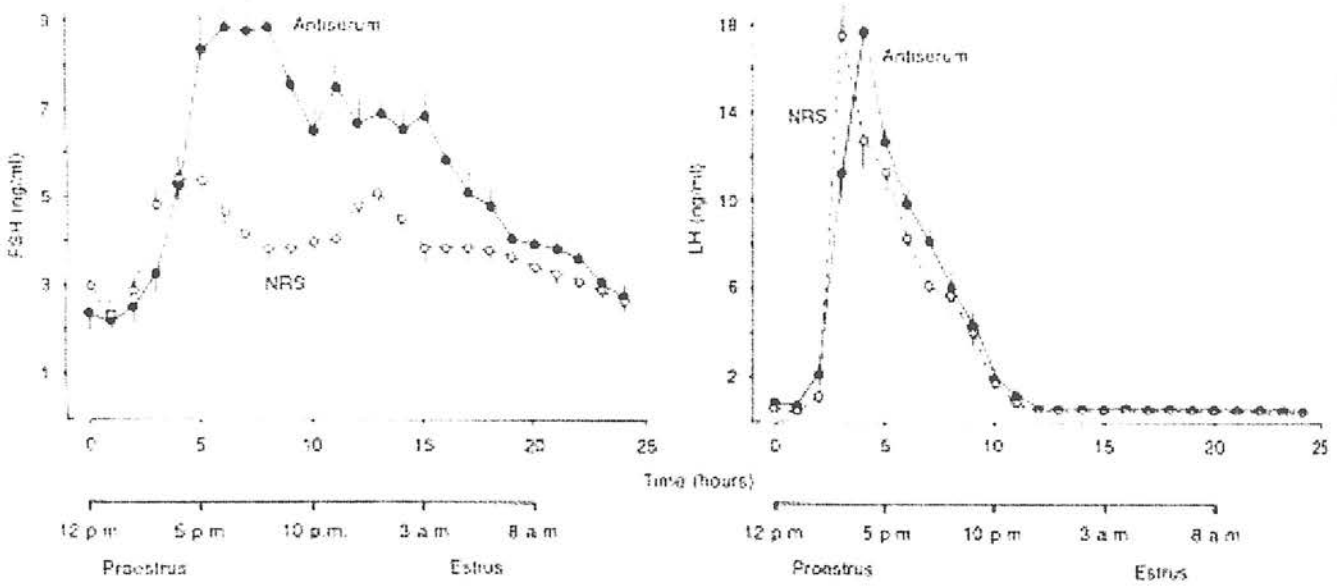


Fig.13. Effect of the intravenous infusion of normal rabbit serum or inhibin antiserum on plasma FSH and LH levels during proetrus and estrus (from Rivier *et al.* 1986).

D. Specific FSH regulatory factors: the gonadal and pituitary factors, activin, inhibin and follistatin

In addition to steroids, other proteins secreted by the gonads, activin, inhibin and follistatin have received considerable attention as specific regulators of FSH. These factors have been demonstrated to be also produced in the pituitary.

a. Structure of activin, inhibin and follistatin

Activin and inhibin, members of the transforming growth factor-β (TGFβ) superfamily, were initially discovered in ovarian follicular fluid. Inhibin is disulphide-linked heterodimers consisting of a α subunit and a β subunit (βA or βB) forming inhibin A or inhibin B. Both inhibin forms inhibit FSH secretion (De Kretser & Robertson 1989, Vale *et al.* 1990). Activin is the result of the dimerization of two β subunits giving activin A (βA-βA), activin B (βB -βB) and activin AB (βA-βB) (Fig. 12). Activin is a stimulating factor of FSH secretion (Ling *et al.* 1986, Vale *et al.* 1986). Follistatin, a glycoprotein hormone structurally unrelated to activin and inhibin, binds to activin and neutralizes its bioactivity (Ueno *et al.* 1987, Ying *et al.* 1987, Nakamura *et al.* 1990, Farnworth *et al.* 1995).

b. Activin and inhibin modulate preferentially FSH secretion

It is well established that activin and inhibin stimulate and inhibit respectively FSH production (reviews Knight 1996, Bilezikjian *et al.* 2004). For instance, the treatment of animals with charcoal-extracted follicular fluid (enriched in inhibin) reduces significantly the FSH secretion while LH concentrations are not affected (Martin *et al.* 1986, Brooks *et al.* 1992). The transcription rate for the FSHβ gene is inhibited of 50% in 6h after ewe treatment with inhibin (Clarke *et al.* 1993). Conversely, the injection of polyclonal antiserum to inhibin in the female rat causes an increase of FSH secretion without modification of LH concentrations (Fig. 13, Rivier *et al.* 1986). Such an injection increases FSHβ mRNA by 2 fold within 2h (Dalkin *et al.* 1993) and FSHβ-primary transcripts by 10 fold after 72h (Burger *et al.* 2001) showing an effect on FSHβ stability mRNA and gene transcription. Concerning activin, it stimulated FSH release and FSHβ mRNA expression level by stabilizing it, without affecting LH release or LHβ mRNA in rat cultured pituitary cells (Carroll *et al.* 1989, 1991). With the development of the murine $L\beta T_2$ gonadotrope cell line transfected with a luciferase-based reporter containing either 5.5 kb of ovine FSHβ regulatory sequence (Huang *et al.*

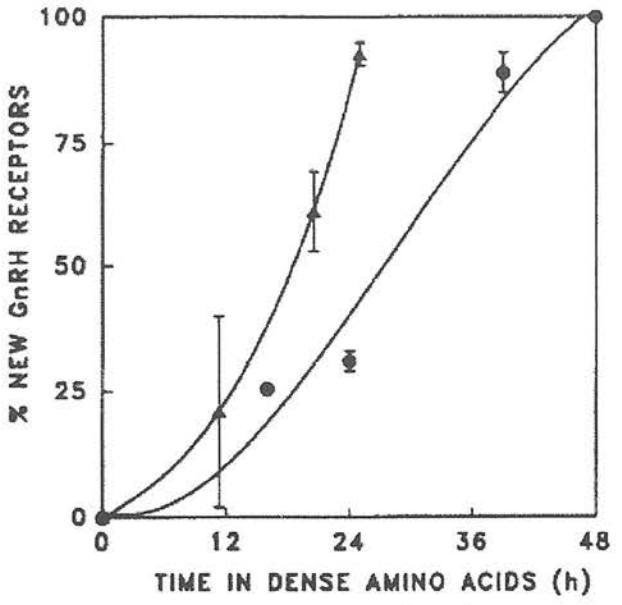


Fig.14. Effect of activin-A on incorporation of amino acids into GnRH receptors and control samples. Pituitary cells were exposed to vehicle (control) or 50 ng/ml activin then cultured for indicated time. Data are mean ±SEM, n = 3-12. ●, control; ▲, activin-A (from Braden & Conn 1992).

2001a) or rat FSHβ promoter (Suszko *et al.* 2003), it has been possible to demonstrate that activin stimulates promoter activity. Activin is generally described as a specific regulatory factor of FSH production without affecting LH secretion. Surprisingly, two recent studies have shown an effect of activin on LHβ promoter. In $L\beta T_2$ cell line and in primary mouse pituitary cells, activin A was able to increase both LHβ mRNA expression and LH secretion (Yamada *et al.* 2004, Coss *et al.* 2005). Moreover, activin stimulates the rat LHβ promoter activity transfected in $L\beta T_2$ through Smad-binding and homeobox elements (Yamada *et al.* 2004, Coss *et al.* 2005). The response to a combination of activin and GnRH was higher than that to activin A or GnRH alone.

Moreover, in rat activin and GnRH act synergistically on FSHβ promoter activity, FSHβ mRNA expression and FSH release (Nicol *et al.* 2004, Gregory *et al.* 2005). This effect could be mediated, at least partly, by the activin-induced increase in the GnRH receptors. Indeed, Braden & Conn (1992) demonstrated that, in rat pituitary cell culture, activin A was able to stimulate GnRH receptor synthesis by 2-fold compared to control (Fig. 14) while inhibin induced a decrease in GnRH receptors without change in GnRH affinity for its binding sites (Wang *et al.* 1988). Furthermore, addition of activin A on αT3 cell line transfected with a mouse GnRH-R promoter/luciferase gene (GnRH-Rluc) leads to increase about 2-fold the luciferase activity (Fernandez-Vazquez *et al.* 1996). The responsiveness to activin is conferred by an identified sequence on the GnRH receptor promoter containing two overlapping cis-regulatory elements of interest: the GnRH receptor activating sequence (GRAS) and a putative SMAD-binding element (SBE) (Duval *et al.* 1999, Norwitz *et al.* 2002). In contrast to rat, activin inhibits GnRH receptor expression and GnRH binding in sheep whereas inhibin has the opposite effect (Gregg *et al.* 1991). Further studies are required to elucidate the mechanism of action of activin on GnRH receptor expression.

c. Endocrine vs paracrine actions of inhibin and activin

The negative relationship existing between inhibin and FSH concentrations in the blood during the oestrous cycle supports an endocrine role for inhibin in the regulation of FSH secretion (Ying 1988). In contrast, similar correlation is not true for activin. Serum activin levels are unperturbed across the oestrous cycle (Knight *et al.* 1996) and circulating activin appears to be bound to follistatin virtually irreversibly (Schneyer *et al.* 1994). Rather, a paracrine/autocrine effect for activin has been suggested. Inhibin and activin subunits

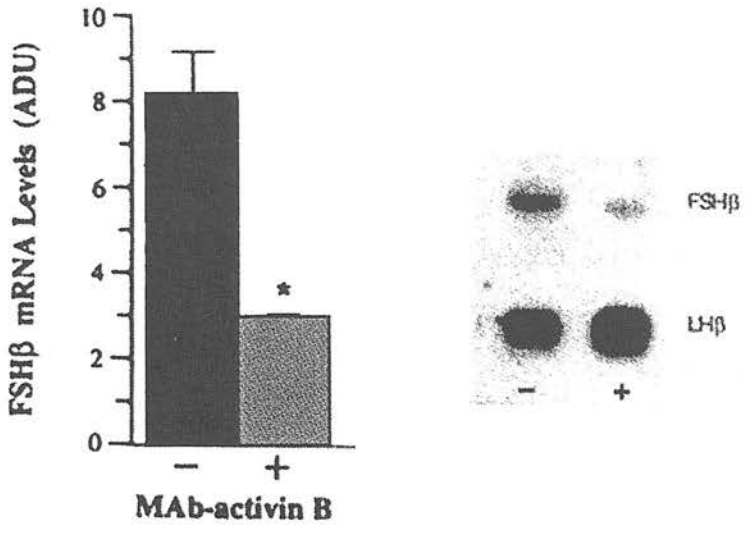


Fig.15. Effect of the incubation of mouse monoclonal antibody against activinB in rat pituitary cell culture on FSHb mRNA expression (from Corrigan *et al.* 1991).

(Meunier *et al.* 1988) as well as receptors for activin (Cameron *et al.* 1998, Fafioffe *et al.* 2004) were detected in rat in anterior pituitary, particularly in the gonadotrope cells (Mathoux *et al.* 2001). An activin bioactivity has been observed in supernatants from ewe pituitary cells (Mathoux *et al.* 2001). Moreover, the incubation of cultured rat pituitary cells with a monoclonal antibody specific for the activin B reduced the FSHβ mRNA expression and FSH concentration without affecting LH production (Fig. 15, Corrigan *et al.* 1991). Similarly, the treatment of pituitary cells with follistatin decreases FSH secretion (Robertson *et al.* 1990). In common with activin, follistatin does not appear to play an important role through gonadal feedback action. This is shown from animal studies which ovariectomy has no long-term effects on circulating follistatin concentrations (Klein *et al.* 1993). Collectively, these observations show that pituitary cells secrete activin which stimulates FSH synthesis and release. Therefore, pituitary activin must be considered as an important modulator of FSH biosynthesis and release.

To be considered as a physiological regulator, activin action has to be modulated. The expression of activin receptor type I (ALK-2 and ALK-4) and type II is modified throughout the oestrous cycle with higher levels before the preovulatory surge and during the secondary surge of FSH (Fafioffe *et al.* 2004). Although there is no data concerning the variations of pituitary activin subunits throughout the oestrous cycle, it has been showed that ovariectomy increases the level of activin βB-subunit mRNA as well as follistatin mRNA (Roberts *et al.* 1989, Halvorson *et al.* 1994). The increase in βB-subunit mRNA is prevented by treatment with oestradiol (Roberts *et al.* 1989). Furthermore, oestradiol inhibits the expression of activin βB-subunit in ewe pituitary cells (Baratta *et al.* 2001). This effect is independent of GnRH and represents a mechanism by which oestradiol modulates FSH secretion directly at the pituitary level. In addition to oestradiol, GnRH also regulates activin βB-subunit mRNA. Treatment of ewe pituitary cells with a high dose of GnRH decreases activin bioactivity (Mathoux *et al.* 2001). In rat, low frequencies of GnRH pulses, favourable to FSH synthesis and release, increases activin βB-subunit mRNA whereas faster frequencies have the opposite effect in rat (Dalkin *et al.* 1999). This effect appears to be mediated by follistatin whose concentrations or mRNA levels vary in opposite way to activin (Kirk *et al.* 1994, Besecke *et al.* 1997, Dalkin *et al.* 1999). Inhibin plays also a role in the regulation of activin bioactivity. Similarly to follistatin, inhibin antagonizes activin effect on FSH secretion. However, its mechanism of action is different from that of follistatin. After binding to the TGFβ type III

Ligands	Null mice phenotypes	References
BMP-2	Non viable, defect in cardiac development	Zhang & Bradley 1996
BMP-4	Non-viable, default in mesoderm differentiation	Winnier et al. 1995
BMP-6	Viable, no major defects except a consistent delay in ossification confined to the developing sternum	Solloway et al. 1998
BMP-7	Severe defects confined to the developing kidney and eye	Dudley et al. 1995
BMP-15		
GDF-9	Female infertility; block in folliculogenesis at primary stage	Matzuk 2000
BMP-15/GDF-9	Female infertility; oocyte loss	Yan et al. 2001

Table1. Knockout mice for selected BMPs/GDF.

receptor, also called betaglycan, inhibin acquires a high affinity for activin type II receptors and thereby prevents activin signaling and action (Lewis *et al.* 2000). In rat, betaglycan immunoreactivity on gonadotrope cell surface is regulated throughout the oestrous cycle and is highly correlated with serum pituitary levels (Chapman & Woodruff 2003).

Thus, activin and inhibin, members of the transforming growth factor-β (TGFβ) superfamily, are undoubtedly important modulators of FSH synthesis and release, and represent modifiers of hypothalamic or systemic endocrine signals.

III. Bone Morphogenetic Proteins (BMPs): newly identified modulators of FSH secretion

Recent studies have added a new wrinkle to the complexity of the differential regulation of LH and FSH secretion. It appears now that BMPs, other members of the TGFβ superfamily, can modulate FSH synthesis and release (Huang *et al.* 2001, Otsuka & Shimasaki 2002).

Over twenty bone morphogenetic proteins (BMP-1 to BMP-20) and nine growth differentiation factors (GDF-1 to GDF-9) considered as BMP sub-group have been identified (Knight & Glister 2003). Initially, BMPs were identified through their actions on bone morphogenesis. Subsequent studies showed that they are multifunctional proteins expressed in many tissues and involved in several functions as embryonic mesoderm induction (review: Harland 1994), genital duct differentiation (Durlinger *et al.* 2002), myogenesis (Massagué *et al.* 1986), bone formation (Centrella *et al.* 1987), erythropoiesis (Krystal *et al.* 1994), inflammation (Marek *et al.* 2002), wound repair (Montesano & Orci 1988) and ovarian folliculogenesis (Knight & Glister 2003). Knockout approaches to generate loss of function of BMP family members have demonstrated their critical role (Table 1).

Nevertheless, we should underline that multiple TGF-β superfamily members are often co-expressed in several tissues and they can compensate for each other. For example, BMP-6 null mice have no major effect whereas BMP-6/BMP-5 double mutant has exacerbated

defects (Solloway *et al.* 1998). Similarly, loss of either BMP-5 or BMP-7 has negligible effects on development while early embryonic lethality is observed for BMP-5/BMP-7 double mutant mice (Solloway & Robertson 1999).

A. Role of BMPs in reproduction

1. Natural mutations of BMP subgroup genes in ewes: examples of Inverdale and Booroola breeds

As reported in table 1, knockout mouse models for some BMPs have shown reproductive deficiencies, mainly due to abnormal ovarian function. Moreover, natural mutations of the BMP system in sheep have been identified that alter the ovulation rate. Recently, in different sheep families, namely Inverdale, Hanna, Belclare Cambridge and Booroola, mutations were identified in genes from the BMPs or their receptors.

a. Merino's Booroola ewes

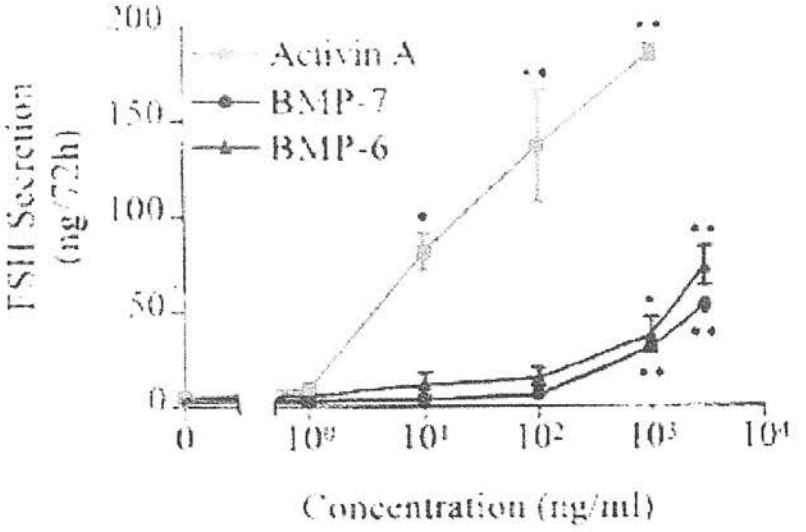
The Booroola phenotype is characterised by increased ovulation rate and litter size. The wild type ewes have one or two ovulations, the heterozygous ewes have 3-4 ovulations and the homozygotes for the *FecB* (*FecB*: Fec for fecundity and B for Booroola) gene have ovulation rate equal or more than 5.

The discovery of the *FecB* gene associated to the hyper-prolificacy revealed a single amino acid substitution (Q249R) in the intracellular serine/threonine kinase domain of the Bone Morphogenetic Receptor type IB, BMPR-IB (Mulsant *et al.* 2001, Souza *et al.* 2001, Wilson *et al.* 2001).

b. Inverdale ewes

In New Zealand, hyper-prolificacy in Romney breed was detected and associated with a gene localised on X chromosome: $FecX^{I}$ (Davis *et al.* 1991). The Inverdale homozygous ewes are infertile due to impairment of the follicular development beyond the primary stage while heterozygotes are hyperprolific. The gene mutation responsible for this phenotype is one substitution in amino-acid sequence in *BMP-15* gene (Galloway *et al.* 2000). Other sheep breeds as Belclare and Cambridge are also characterised by sterility in homozygotes and high

A



B

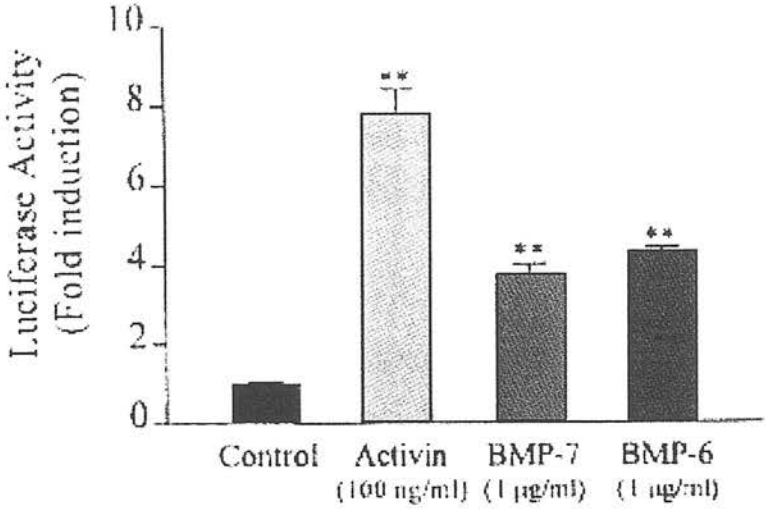


Fig.16. A. FSH secretion from LβT2 cells in response to activin-A, BMP-6 or BMP-7. B. Activation of oFSHβ promoter construct by activin-A, BMP-6 or BMP-7 in LβT2 cells (from Huang *et al.* 2001)

prolificacy in heterozygotes. The phenotype is similar to the Inverdale ewes and is associated with *GDF-9* and *BMP-15* mutations localised on X chromosome (Yan *et al.* 2001, Hanrahan *et al.* 2004).

In contrast to the findings in ewes, *BMP-15* knockout mice are fertile with minimal ovarian defects (Yan *et al.* 2001). This observation raises the possibility that members of the TGFβ superfamily behave differently in mono- versus polyovulatory species.

2. Do natural mutations act at ovary and/or at pituitary level?

The available evidence strongly indicates that the FecB mutation exerts its action at the ovary rather through altered amounts of gonadotropin stimulation (Souza *et al.* 2003). The most consistent changes associated with the mutations described for Booroola, Inverdale or Hanna ewes are the size and the number of the ovulatory follicles (Driancourt *et al.* 1986, McNatty *et al.* 1986). In homozygous FecB/FecB ewes, the increased ovulation rate is associated to the "precocious" development of a large number of antral follicles that are smaller than in wild-type follicles (McNatty *et al.* 1986). As Booroola ewes, Inverdale follicles are precociously differentiated in $FecX^{I}$ heterozygous ewes. The dynamics of follicle development in ewe with or without the Booroola mutation are similar during the follicular and early luteal phases of the oestrous cycle, but the follicles ovulate and achieve dominance at a smaller size. The presence of BMPs and their receptors in the ovaries has been reported (Souza *et al.* 2002). BMPRIB mRNA was detected by *in situ* hybridization on oocyte and granulosa cells, from the primordial follicle stage to the antrum follicle stage (Wilson *et al.* 2001). The treatment of cultured granulosa cells with GDF-5 and BMP-4 (preferential BMPR-IB ligands) induce an inhibition of progesterone secretion. These inhibitory effects are lower for granulosa cells from homozygous FecB/FecB ewes than from wild type ewes. In contrast, activin had similar inhibitory effect in both (Fabre *et al.* 2003). Altogether, these data provide evidence for the action of FecB mutation at the ovary level. Whereas in Inverdale and Hanna ewes all the effects appear to be within the ovary, it was suggested that some effects of the Booroola mutation may occur at the pituitary.

Some studies report higher FSH concentrations in presence of the FecB mutation during various stages of the oestrous cycle and anoestrus (Bindon *et al.* 1984, McNatty *et al.* 1987). However, other reports found no difference (Souza *et al.* 1997). Gonadotropin subunit mRNA expression was not significantly different between the FecB carrier and the non carrier

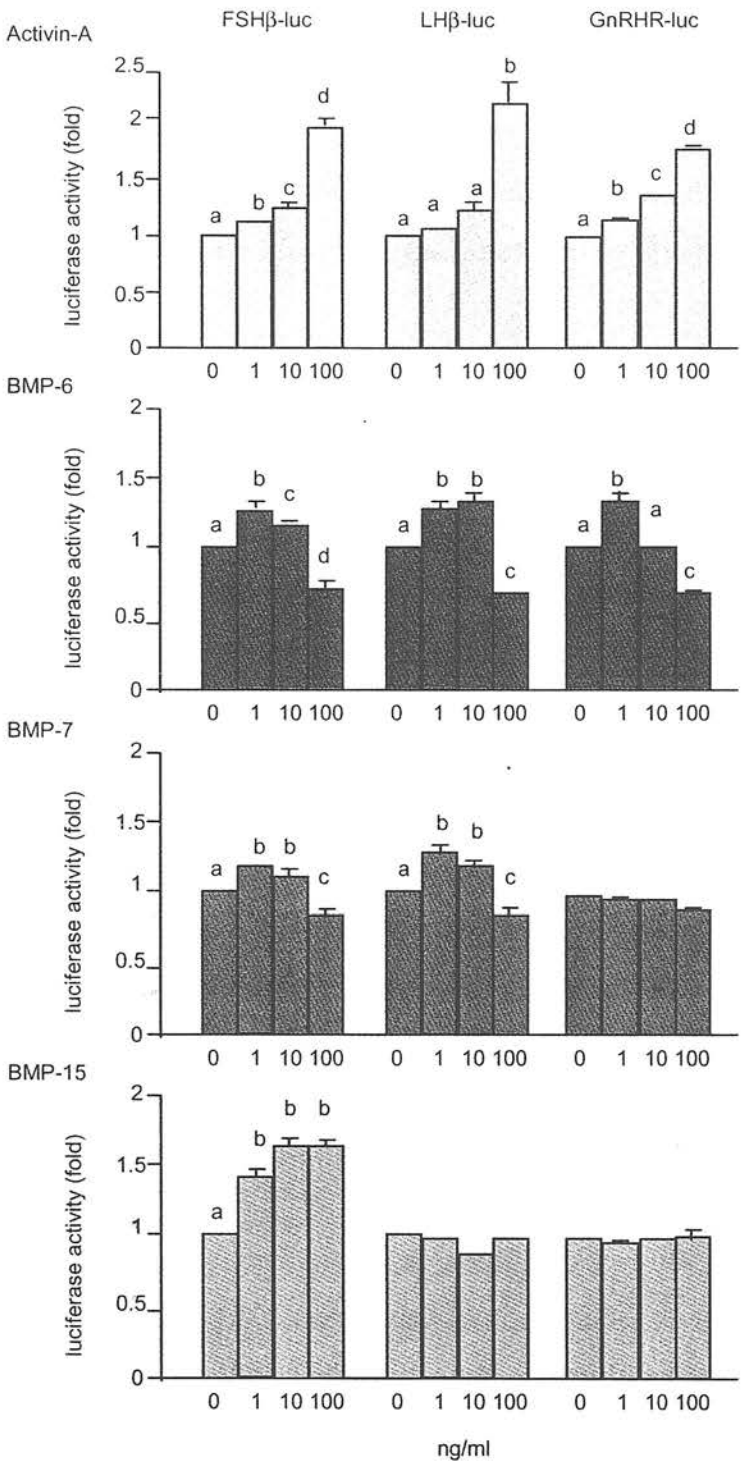


Fig.17. Effects of activin-A, BMP-6, BMP-7 and BMP-15 on transcriptional regulation of FSHβ, LHβ and GnRH-R in LβT2 cells (from Otsuka & Shimasaki 2002)

genotypes (Fleming *et al.* 1995). Moreover, the difference in ovulation rate can still be observed under similar gonadotropin environment. For instance, hypophysectomised ewes induced to ovulate by chorionic gonadotropin injections maintain the genotype difference in ovulation rate (Fry *et al.* 1988). These observations strongly suggest that the Booroola mutation does not act through pituitary function. Nevertheless, BMP mRNAs and BMP receptors are detected in the pituitaries from different species suggesting a role for BMPs in the pituitary (Wilson *et al.* 2001, Huang *et al.* 2001, Faure *et al.* 2005).

3. Which role for BMPs on gonadotropin synthesis and release?

The finding of BMP mRNAs and BMP receptors in the ewe pituitary in preliminary studies incited us to investigate the role of these molecules on gonadotropin synthesis and release. In the same time, Huang and coworkers (2001) showed that BMP-6 and BMP-7 were able to stimulate the ovine FSHβ promoter linked to a luciferase reporter gene (oFSHbetaLuc) in transgenic mice primary cell cultures or after transient expression in murine gonadotrope cell line, $L\beta T_2$, by 6-fold and 4-fold respectively (Fig. 16). The treatment with 1µg/ml of BMP-6 or BMP-7 of $L\beta T_2$ cells also increased the endogenous FSH secretion by 10-fold. Nevertheless, BMP-6 and BMP-7 doses are very high. Indeed, BMP-6 and BMP-7 at 100 µg/ml showed no effect on FSH secretion (Fig. 17, Otsuka & Shimasaki 2002). However, the bioneutralizing antibody to BMP-7, cross-reacting with BMP-6 but not with activin decreased the basal oFSHbetaLuc expression and FSH secretion from transgenic mouse pituitary cell cultures indicating endogenous BMPs role. Similar result was found in rat and sheep pituitary cell cultures (Huang *et al.* 2001). Another BMP, BMP-15 at 100 µg/ml was able to stimulate FSHβ subunit transcription in $L\beta T_2$ cells and in rat primary pituitary cells without affecting LHβ or GnRH receptor transcription (Otsuka & Shimasaki 2002).

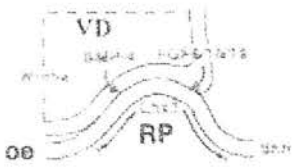
Taken together, these data underline the role of BMPs as stimulatory factors of FSH production, at least in rodents.

B. Other roles of BMPs in pituitary

1. Role in pituitary organogenesis

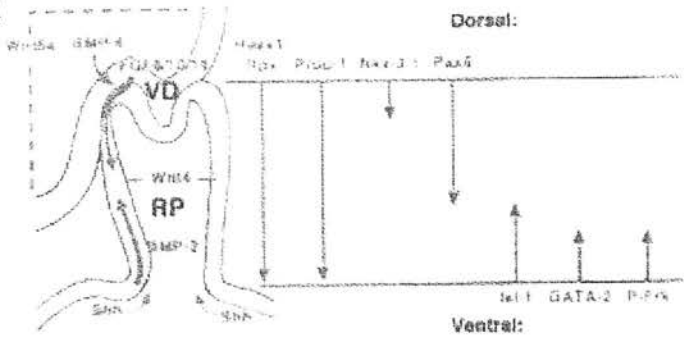
Members of BMP family are detectably expressed during mouse pituitary organogenesis and their role has been established, initially in an organ system culture and

E9



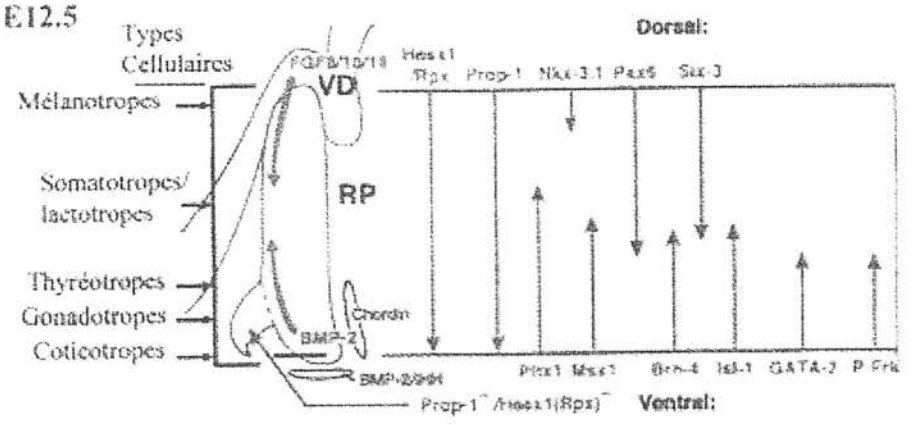
Signals from the ventral diencephalon (VD) (FGFs, BMPs, Wnt5a) and the oral ectoderm (oe) (Shh) determine the identity of Rathke's pouch cells (RP)

E10.5



Intrinsic signals (BMP-2 ventro-dorsal and FGF dorso-ventral gradients) induce expression of several transcription factors along the dorso-ventral axis of Rathke's pouch

E12.5



Signaling gradients induce transcription factors which determine the identity of different cell types

E17.5

Schematical representation of the spatial oragnization of the Rathke's pouch rudiment. Corticotrpes are in the post bourgeon and embryonic thyreotropes are locolized in rostral bourgeon. Gonadotropes (G) are more ventral cells then the tyreotropes (T), somatotropes (S) and lactotropes (L) and melanotropes (MSH). PL: Posterior lobe. VD: ventral diencephalon.

Fig.18 Organogenesis of pituitary (Dasen *et al.* 1999)

more critically *in vivo*. A three-phase signaling regulation of pituitary organogenesis is observed. Each stage is mediated by the actions of a series of intrinsic and extrinsic signaling molecules which include BMPs. These factors act to positionally determine cell types.

a. The Rathke pouch rudiment formation

The adenopituitary comes from an ectoderm primordium which involution forms the Rathke's pouch rudiment (Fig. 18). BMP-4, Wnt-5a and FGF-8 are expressed in distinct, overlapping patterns in the ventral diencephalons and BMP-4 plays a critical role for Rathke's pouch formation (Ericson *et al.* 1998, Treier *et al.* 1998).

b. Determination of progenitor cells in distinct pituitary cell types

The anterior pituitary presents distinct cell types which express different hormones. The appearance of specific cell types, beginning at e10.5 coincides with the onset of BMP-2 in the pituitary ectoderm in a ventral-dorsal gradient (Ericson *et al.* 1998, Treier *et al.* 1998). This gradient acts in concert with a FGF-8 dorsal-ventral gradient. These opposing gradients dictate overlapping patterns of transcription factor expression and are crucial for cell type determination (Fig. 18, Dasen *et al.* 1999). BMP-2, ventral factor, induces the expression of transcription factor, GATA-2, in the ventral region. This induction inhibits the expression of Pit-1 responsible for the differentiation of thyrotropes, somatotropes and lactotropes, allowing the gonadotropes to take place. Hence, BMP-2 is required to generate ventral pituitary cell types including gonadotropes, while FGF-8 determines dorsal cell types including corticotropes.

c. Terminal differentiation of ventral cell types

Finally, the temporary loss of BMP-2 signal is required for terminal differentiation of ventral cell types, the third phase of organogenesis (Treier *et al.* 1988).

Thus, BMPs exhibit sequential roles, first dorsally and then ventrally to progressively determine organogenesis and cell types within the pituitary.

2. Pituitary pathologies associated with BMPs

A report on human pathologies described three patients with deletion in the region q22-q23 of the chromosome 14 presenting bilateral anophthalmia and absent pituitary or

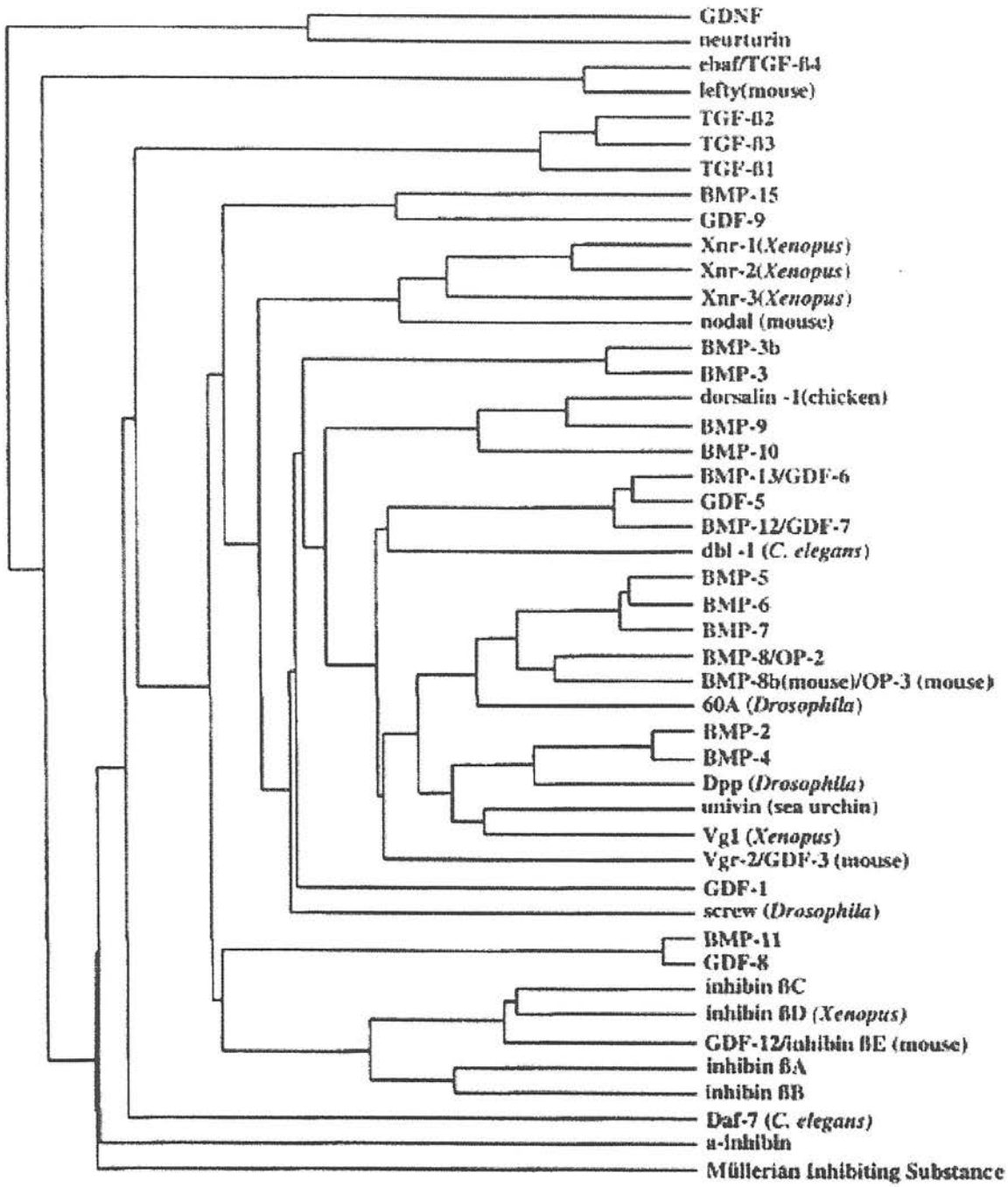


Fig.19. Members of the TGFβ superfamily (from Dube *et al.* 2000)

hypogonadism. Interestingly, the human BMP-4 gene maps to this region suggesting its implication in these pathologies through its role in pituitary and eye development (Lemyre *et al.* 1998). Moreover, the involvement of activin/BMP system was demonstrated in human pituitary adenomas. In the tumors, pituitary activin/BMP system was present and the levels of follistatin mRNA were reduced in FSH –producing adenomas compared with non-functioning pituitary adenomas, suggesting that endogenous follistatin is involved in FSH overproduction through inhibition of activin/BMP system (Takeda *et al.* 2003). In prolactinomas, the down regulation of noggin (a BMP-2/BMP-4 inhibitor) and the overexpression of BMP-4 responsible for the cell proliferation were detected (Paez-Pareda *et al.* 2003).

Collectively, these data show that BMPs can be involved in the tumorigenesis in pituitary.

IV. Mechanisms of action of Bone Morphogenetic Proteins (BMPs) and Activin

A Signaling

1. Ligands

TGFβ superfamily members are conserved proteins between species and classified in two subfamilies, TGFβ/Activin/Nodal and BMP/GDF (Growth differentiation factor) /MIS (Muellerian inhibiting substance), defined by their sequence similarity and their specific transduction signal (Fig 19). These proteins share a set of common sequence and structural features. They are synthesized as proprotein then clived to yield the C terminus. The active forms of these proteins are homo- or hetero-dimers linked by a disulfide bridge (Vitt *et al.* 2001). Each monomer comprises several extended β strands interlocked by disulfide bonds forming a cystine knot BMPs heterodimers association can enhance their biological activity. For example, co-expression of BMP-2 with BMP-7 in Chinese Hamster ovary cells forming heterodimeric BMP-2/7 showed a specific activity about 20-fold higher than BMP homodimers in an *in vitro* alkaline phosphatase induction assay. These heterodimers were also

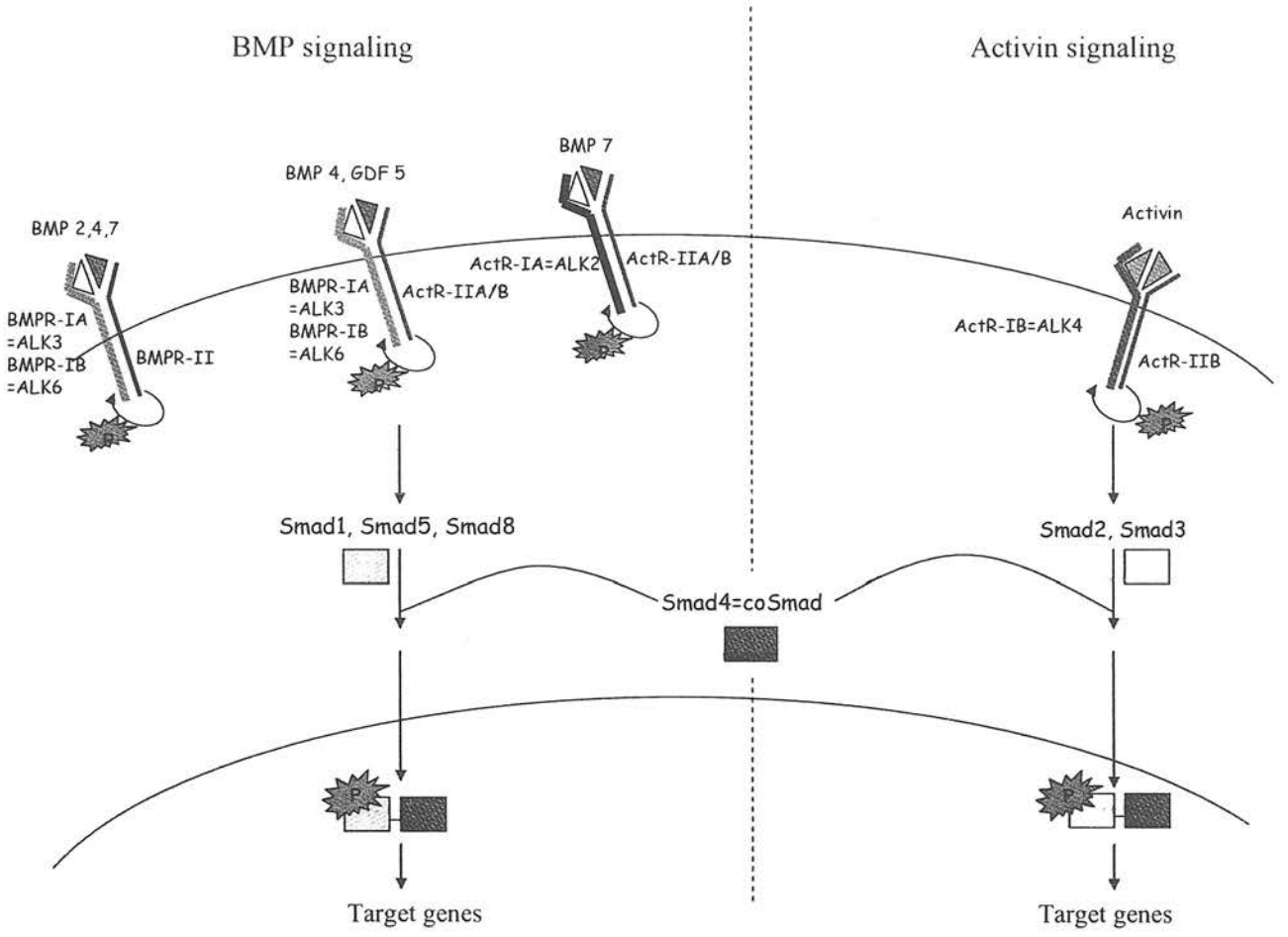


Fig.20. BMP and activin smads signaling patways (from Massagué & Wotton 2000).

5- to 10-fold more efficient than BMP-2 in inducing cartilage and bone in an *in vivo* assay. Similar results were obtained with BMP-2/6 heterodimer (Israel *et al.* 1996).

2. Receptors (Fig. 20)

a. Type I and II receptors

The TGFβ superfamily members act through two type of receptors, type I and type II. Seven type I receptors and five type II receptors are known (review: Manning *et al.* 2002). For example, type I and II receptors for activin are ALK-2/ActRI, ALK-4/ActRIB, ActRII and ActRIIB (Donaldson *et al.* 1999, Gray *et al.* 2000, Harisson *et al.* 2004). For BMPs, specific type I and type II receptors are ALK-3/BMPR-IA, ALK-6/BMPR-IB and BMPR-II (Takeda *et al.* 1994, Rosenzweig *et al.* 1995, Ikeda *et al.* 1996). These receptors have a short extracellular domain, a single transmembrane domain and an intracellular domain containing serine/threonine kinase region. Type I receptors, but not type II, have a characteristic repeat of gycines and serines also called GS region in the N terminal domain of the serine/threonine kinase region (Kawabata *et al.* 1998). The kinase domain contains a specific binding site to Smads, intracellular effectors (see next part): the L45 loop that determines the specificity of Smad binding to the receptor. When the L45 region was exchanged between TGFβ type I and BMP type I receptors, the Smad activation and signaling pathway depending to them was switched from one to another (Persson *et al.* 1998, Itoh *et al.* 2003). One characteristic of BMPR-II receptor is a long C terminal tail following the serine/threonine region, important for specific pathways (Rosenzweig *et al.* 1995).

b. Receptor activation

Proteins of the TGF-β superfamily bind to two different types of receptors termed as type II and type I receptors. Both types contain serine/threonine kinase domains in their intracellular portion and exist on cell surface in various oligomeric forms, ie type II-type I heterodimers, type II homodimers or type I homodimers (Gilboa *et al.* 2000). Activin and TGFβ ligands bind with a high affinity to a homodimeric complex of type II receptors which are constituvely active. Then, the complex recruits a homodimer of type I receptors before inducing the signaling pathway (ten Dijke *et al.* 1994a; Wrana *et al.* 1994). In contrast, a heterotetrameric complex of type I and type II receptors is required to be bound by BMPs. This cooperation between the two types of receptors is necessary for optimal binding and Smad signal transduction at least for BMP-2 and BMP-7 (Liu *et al.* 1995, Rosenzweig *et al.*

1995). Nevertheless, BMP-2 can bind to BMPR-II receptor and recruits the other receptor inducing another signaling pathway, p38 MAPK (Nohe *et al.* 2002).

Once the ligand associated with the receptor complex, the type II receptor trans-phosphorylates the type I receptor on its GS domain. Thus, type I receptor is activated and phosphorylates intracellular substrate.

c. Receptor specificity

Specific ligands can bind specific receptor. Nevertheless, ALK-2/ActRI acts as a type I receptor for activin and also for certain BMPs including BMP-6 and BMP-7 (ten Dijke *et al.* 1994b). Whereas BMPR-II specifically binds to BMPs, Act-RII and Act-RIIB can also bind BMPs (BMP-7 for example) and trigger the activin signaling pathway in some cells but not in all. For instance, BMP-7 was not able to stimulate FSH secretion in rat pituitary cells (Yamashita *et al.* 1995). Altogether, these studies show crosstalks between TGFβ superfamily members on their reciprocical signaling pathways (for review, Miyazono *et al.* 2001).

3. Smad, intracellular proteins and transcription factors

Smad proteins are major signaling molecules acting downstream of the serine/threonine kinase receptors (Heldin *et al.* 1997). There are eight smad proteins that are classified in three groups:

-RSmad -1, -2, -3, -5 and -8 (receptor regulated smad) which bind specifically to the receptor to transduce the signaling pathway

-coSmad-4 (common smad) which interacts with R-Smad, forming a complex in aim to translocate into the nucleus

-ISmad-6 and -7 (inhibitory smad) which modulate TGFβ members signaling pathway (see signaling regulation below)

a. Smad structure

Smad proteins, except Smad-6 and -7, have a conserved structure. The N terminal end containing Mad Homology domain-1 (MH1) has DNA-binding activity (Shi *et al.* 1998) whereas the C terminal end, through the MH2 domain (Attisano & Wrana 2000) is implicated into receptor binding, RSmad-coSmad interaction and has transcription regulatory activity. The RSmad have a specific pattern SSXS which is the type I receptor phosphorylated site

(Souchelnystskyi *et al.* 1997). More recently, a deletion analysis of human Smad-3 protein showed in mammalian cells that Smad-3 contains a middle transactivation domain, important for transcriptional activation and interaction with coactivators (Prokova *et al.* 2005).

b. Smad activation

Smad-1, Smad-5 and Smad-8 are specific to BMP signaling pathway (Tamaki *et al.* 1998, Yamamoto *et al.* 1997) whereas Smad-2, Smad-3 are activated by activin and TGFβ receptors (Macias-Silva *et al.* 1996, Candia *et al.* 1997, Nakao *et al.* 1997b). The activated type I receptor L45 loop (Persson *et al.* 1998) can bind to R-Smad via the L3 loop located near the C terminal domain. Even if this sequence is highly conserved between RSmads, two amino acids in the L3 loop are essential for the specificity of receptor/smad interaction. Swapping these can induce a switch between TGFβ and BMP subgroup signaling pathways (Chen *et al.* 1998, Lo *et al.* 1998). The receptor/smad interaction triggers the phosphorylation of R-Smads serine 465 and 467 in the C terminus (Souchelnystskyi *et al.* 1997). Moreover, the specificity of phosphorylation is leading by adjacent sequences as arginine 462 and cysteine 463 in the C terminus of Smad-2 for specific binding to the type I TGFβ receptor (Yakymovych *et al.* 2004). Once phosphorylated by receptors, these Smads bind to Smad-4, also called co-Smad forming a transcriptional complex which translocates into the nucleus to promote specific gene expression (Massagué & Wotton 2000, for review: Zwijsen *et al.* 2003).

c. Smad translocation into the nucleus, role on gene transcription

Once the complex Smad-4/RSmad is translocated into the nucleus, it interacts with various DNA-binding proteins which bind to promoter regions on Smad Binding Element (SBE) of target genes. In addition, Smad-4 helps R-Smad for the binding with cofactors like FAST-1, SMIF for Smad-2 (Liu *et al.* 1997, Bai *et al.* 2002) or p300/CBP for Smad-1 (Pouponnot *et al.* 1998) via Smad-4 activation domain (de Caestecker *et al.* 2000).

A wide range of genes can be modulated by Smad signaling pathways, corresponding to TGFβ superfamily effect (review: Miyazono *et al.* 2005). Concerning the reproductive function, for instance, BMP-4 has been shown to inhibit progesterone production in ovine granulosa cells. This inhibitory action was associated with a decrease in the expression of cAMP-regulated genes, steroidogenic acute regulatory protein (StAR) and P450 side-chain

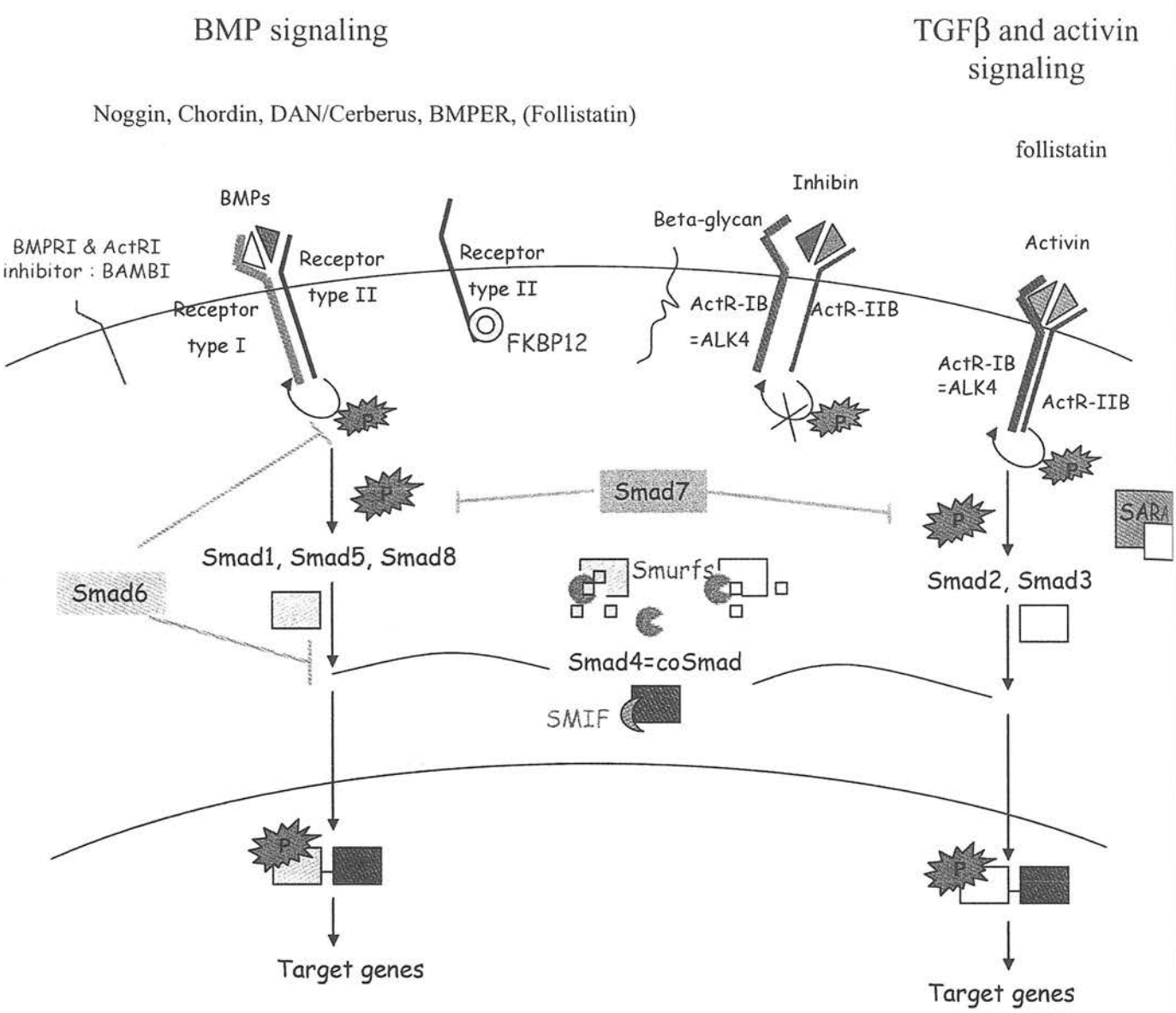


Fig.21. TGFβ superfamily modulating factors (from Massagué & Wotton 2000)

cleavage (P450 scc). This inhibitory effect is mediated by BMP-4-activated Smad-1 which inhibits the transcriptional activity of SF-1 on its responsive element (Pierre *et al.* 2004). In mouse gonadotrope cells, the activation of Smad-2 and Smad-3 mediates the stimulatory effect of activin on FSHβ promoter activity and FSH secretion (Dupont *et al.* 2003, Bernard 2004), potentially mediated by an activin response element (Huang *et al.* 2001, Suszko *et al.* 2003).

B Modulation of TGFβ superfamily signaling pathways (Fig. 21)

BMPs have diverse and often complementary effects. It is obvious that a tight regulation of different actions of these proteins is necessary for proper functioning. Hence, BMPs signaling is subjected to regulation at multiple levels: extracellular level, at the membrane site and intracellular levels.

1. At extracellular level : antagonists

At extracellular level, proteins can bind ligand proventing them to access to the receptor or to trigger the signal transduction (review: Massagué & Chen, 2000, Balemans & van Hul 2002).

a. Follistatin is preferentially an activin antagonist

Follistatin, a 35,000 Kda protein was isolated from porcine follicular fluid by chromatography and was responsible for specifically inhibiting FSH release, not LH, in the rat anterior pituitary monolayer culture (Ueno *et al.* 1987). Then, follistatin mRNA was detected in the ovary, the kidney and also the brain by Northern blot in rat (Shimasaki *et al.* 1989). Follistatin was described as an activin antagonist, interacting directly with activin (Nakamura *et al.* 1990), and preventing activin interaction with its type II receptor (de Winter *et al.* 1996). Follistatin (review Phillips & de Kretser 1998) is localized in several cell types including gonadotropes and folliculostellate cells in rat (Kaiser *et al.* 1992). In ovine pituitary cell cultures, follistatin was continuously secreted and increased in a dose dependence manner with exogenous recombinant activin A (Farnworth *et al.* 1995) and prevented activin action.

Follistatin was also described as BMP antagonist. For example, follistatin can bind to BMP-4 and BMP-7 forming a trimeric complex with the receptor, antagonizing their effect (Fainsod *et al.* 1997, Iemura *et al.* 1998).

b. BMPs antagonists

Noggin, a secreted polypeptide, can bind BMP-2, BMP-4 and BMP-7 with a higher affinity for BMP-2 and BMP-4 and abolishes BMP-4 activity by blocking binding to cognate cell-surface receptors, preventing for instance BMP-4 inhibition on neural induction and mesoderm dorsalization during the amphibian embryogenesis (Zimmerman *et al.* 1996). BMPs and Noggin can regulate each other as BMP-2 and BMP-6 can induce Noggin mRNA expression and can stimulate protein level in rat cultures of osteoblast-enriched cells (Gazzerro *et al.* 1998).

Chordin, was described as a protein secreted by Spemann organizer tissue during gastrulation in Xenopus. Chordin antagonizes specifically BMPs (BMP-4) but not activin by blocking binding to their receptors (Piccolo *et al.* 1996).

DAN/Cerberus protein family was described in Xenopus embryos, both proteins Cerberus (Piccolo *et al.* 1999) and Dan (Stanley *et al.* 1998) sharing structural homology and antagonizing BMP functions (Pearce *et al.* 1999). In this family, Gremlin was also described as specific BMP antagonist (Hsu *et al.* 1998) and its mRNA and protein level are induced by BMP-2 in osteoblasts (Pereira *et al.* 2000).

BMPER (bone morphogenetic protein [BMP]-binding endothelial cell precursor-derived regulator), more recently, has been described as a protein which can directly interact with BMP-2, BMP-4 and BMP-6 and implicated into endothelial cell differentiation in vasculogenesis (Moser *et al.* 2003)

We should underline that the system ligands/antagonists can modulate the accessibility of BMPs and activins to their receptors.

2. At receptor level

a. BAMBI

The protein BAMBI (BMPs and Activin receptor membrane bound inhibitor) is a transmembrane inhibitor which is related to TGFβ family type I receptors but lacks an intracellular kinase domain. BAMBI associates with TGFβ family type I receptors preventing receptor complexes, inhibiting TGFβ family signaling (Onichtchouk *et al.* 1999). Interestingly, BAMBI is co-expressed with BMP-4 during early Xenopus embryogenesis and induced by BMP-4 in mouse embryonic fibroblasts (Grotewold *et al.* 2001). Moreover,

BAMBI transcription is regulated by TGFβ signaling through direct binding of Smad-3 and Smad-4 to the BAMBI promoter (Sekiya *et al.* 2004).

b. FKBP-12

The immunophilin FKBP12 (12-kDa FK506-binding protein) is a common cytoplasmic interactor of TGFβ family type I receptors, hiding the phosphorylation sites of the type II receptor (Wang *et al.* 1996, Okadome *et al.* 1996). Mutations of binding sites of FKBP12 or type I TFGβ receptor prevent the interaction between the proteins, leading to receptor activation in absence of ligand (Chen *et al.* 1997, Huse *et al.* 1999). Another study showed that FKBP2 was a negative regulator of type I TGFβ receptor internalization (Yao *et al.* 2000).

c. Betaglycan/inhibin

The betaglycan, membrane-bound proteoglycans also referred to the type III TGF-beta receptor was purified and showed to bind to TGFβ 1 and 2 with high affinity (Andres *et al.* 1989). Moreover, the betaglycan is also able to bind inhibin A (Esparza-Lopez *et al.* 2001). The study of betaglycan mRNA and protein distribution showed its presence in brain, pituitary and gonads underlining an important role in reproduction (MacConell *et al.* 2002). Binding to inhibin, the betaglycan can functionally antagonize activin signaling by enhancing the binding of inhibin A to activin type II receptor (Lewis *et al.* 2000) and the TGFβ signaling by preventing type I/ type II complex formation (Eickelberg *et al.* 2002). Even if inhibin and betaglycan system antagonize preferentially activin signaling, a recent study showed an inhibitory effect on BMP signaling (Wiater & Vale 2003). In female rat, the betaglycan has been localized in pituitary cells (Chapman & Woodruff 2003) and its repartition varies along the ooestrous cycle (Fafioffe *et al.* 2004). For example, in rat pituitary, betaglycan has been localised in gonadotrope cell membranes, concentrated before and after the primary and secondary FSH surges (Chapman & Woodruff 2003). Modulation of inhibin binding for betaglycan by competition with TGFβ reversed the inhibitory effect of inhibin on GnRH-R and FSHβ subunit transfected promoters in the gonadotrope cell line LβT2, showing an interplay between activin, inhibin and TGFβ for the betaglycan accessibility (Ethier *et al.* 2002, review: Bilezikjian *et al.* 2004). More recently, the expression and the regulation of the betaglycan gene was showed to be increased by FSH and oestradiol in cultured rat granulosa cells (Omori *et al.* 2005).

3. At intracellular level

a. Smad anchor receptor activation (SARA)

SARA (for Smad anchor for receptor activation) was identified for interacting directly with Smad-2 and Smad-3 and with the TGFβ receptor. Phosphorylation of Smad-2 induces dissociation from SARA with concomitant formation of Smad-2/Smad-4 complexes and nuclear translocation (Tsukazaki *et al.* 1998). SARA was also described into endosomes and it required for the internalization of the activated receptors (Hayes *et al.* 2002).

b. Smad-4 interacting protein: SMIF

After TGFβ stimulation, receptor-associated Smads are phosphorylated and form a complex with the common mediator Smad-4. SMIF, a ubiquitously expressed protein, was recently described as a Smad-4-interacting transcriptional co-activator inducible by BMP-4 binding (Bai RY *et al.* 2002).

c. Inhibitory smads: Smad-6 and Smad-7

Smad-6 and Smad-7 were described as inhibitory Smads (Nakao *et al.* 1997, Topper *et al.* 1997). Both have MH2 domain like the RSmads and Smad-4 but have a long N terminal tail which are both important for their inhibition (Hanyu *et al.* 2001, Nakayama *et al.* 2001, Mochizuki *et al.* 2004). Smad-7 is described for binding BMP and TGFβ receptors to inhibit their signaling pathways (Souchelnytskyi *et al.* 1998). Even if Smad-6 and Smad-7 have common sequences, they play different roles on BMP and TGFβ signaling, depending also to the cell type. Both were demonstrated as antagonist of BMP-2 induced growth arrest and apoptosis in mouse B lineage cells. Although both can inhibit the Smad-1/Smad-5 phosphorylation (BMP signaling), only Smad-7 was able to antagonize activin effect inhibiting Smad-2 phosphorylation on B cells (Ishisaki *et al.* 1999). In another study, adenoviral overexpression of Smad-6 or Smad-7 in chondrocytes showed that Smad-7 totally inhibited important TGFβ mediated biological responses such as proliferation and PG synthesis while overexpressed Smad-6 had not or only a partial inhibitory effect on TGFβ activity (Scharstuhl *et al.* 2003). To summarize this part, BMP signaling can be inhibited by Smad-6 and Smad-7 whereas TGFβ signaling (also activin) can be specifically inhibited by Smad-7.

Although one study demonstrated Smad-6 role on TGFβ signaling at high dose through TβRI receptor, decreasing about 40% the phosphorylation of Smad-2 but not Smad-3 in presence of TβRI (Imamura *et al.* 1997), the common effect described of Smad-6 is to block specifically BMP signaling preventing the interaction between the phosphorylated form of Smad-1 (not Smad-2) and Smad-4 without interfering with the receptor-mediated phosphorylation of Smad-1 in Xenopus embryos and mammalian cells. Even if Smad-6 can nonselectively interact to type I receptors (TGFβ-R and BMP-R), Smad-6 and Smad-4 compete for receptor activated Smad-1 (not Smad-2). Smad-6 mutant proteins experiment confirmed these results (Hata *et al.* 1998). At nuclear level, Smad-6 repressed bone morphogenetic protein-induced gene transcription in mammalian cells recruiting transcriptional corepressor or acting itself as corepressor (Bai S *et al.* 2000, Lin X *et al.* 2003b). A feedback loop has been described as BMPs can induce Smad-6 and modulate its own signaling pathway (Takase *et al.* 1998, Li *et al.* 2003). Moreover, the transfection of mouse Smad-6 promoter demonstrated the importance of a sequence called the proximal BMP-responsive element (PBE) for the transcriptional activation by BMPs (Ishida *et al.* 2000).

Smad-7 is described as TGFβ and activin signaling pathways inhibitor, associated with type I/II receptors complex and inhibiting the phosphorylation of Smad-2 and Smad-3 (Nakao *et al.* 1997a, Casellas & Brivanlou 1998). Moroever, Smad-7 has been described as BMP signaling inhibitor interacting with type I BMP receptors inhibiting Smads phosphorylation (Souchelnytskyi *et al.* 1998). In rat pituitary cells, Smad-7 mRNA was increased by activin and transient transfection of Smad-7 inhibited an activin/TGF beta-responsive reporter as modulator of activin pathway in alpha T3-1 cells (Bilezikjian *et al.* 2001). Studies on Smad-7 promoter showed the direct association of activin or TGFβ specific R-Smads, Smad-3 on a consensus Smad-3-Smad-4 binding element (Nagarajan *et al.* 1999).

d. Smad degradation by ubiquitinylation

For ending the signal transduction, processes of dephosphorylation and degradation by ubiquitination of the R-Smads due to Smurf proteins (Smurf1 and Smurf2), members of the Hect family of E3 ubiquitin ligases, are important (Lo & Massagué 1999, Zhu *et al.* 1999, Lin X *et al.* 2002, Dato & Wang 2005). Smad-4 is regulated by SUMOylation in transfected HeLa cells. SUMO-1 overexpression increased Smad-4 level, growth inhibitory response, as well as

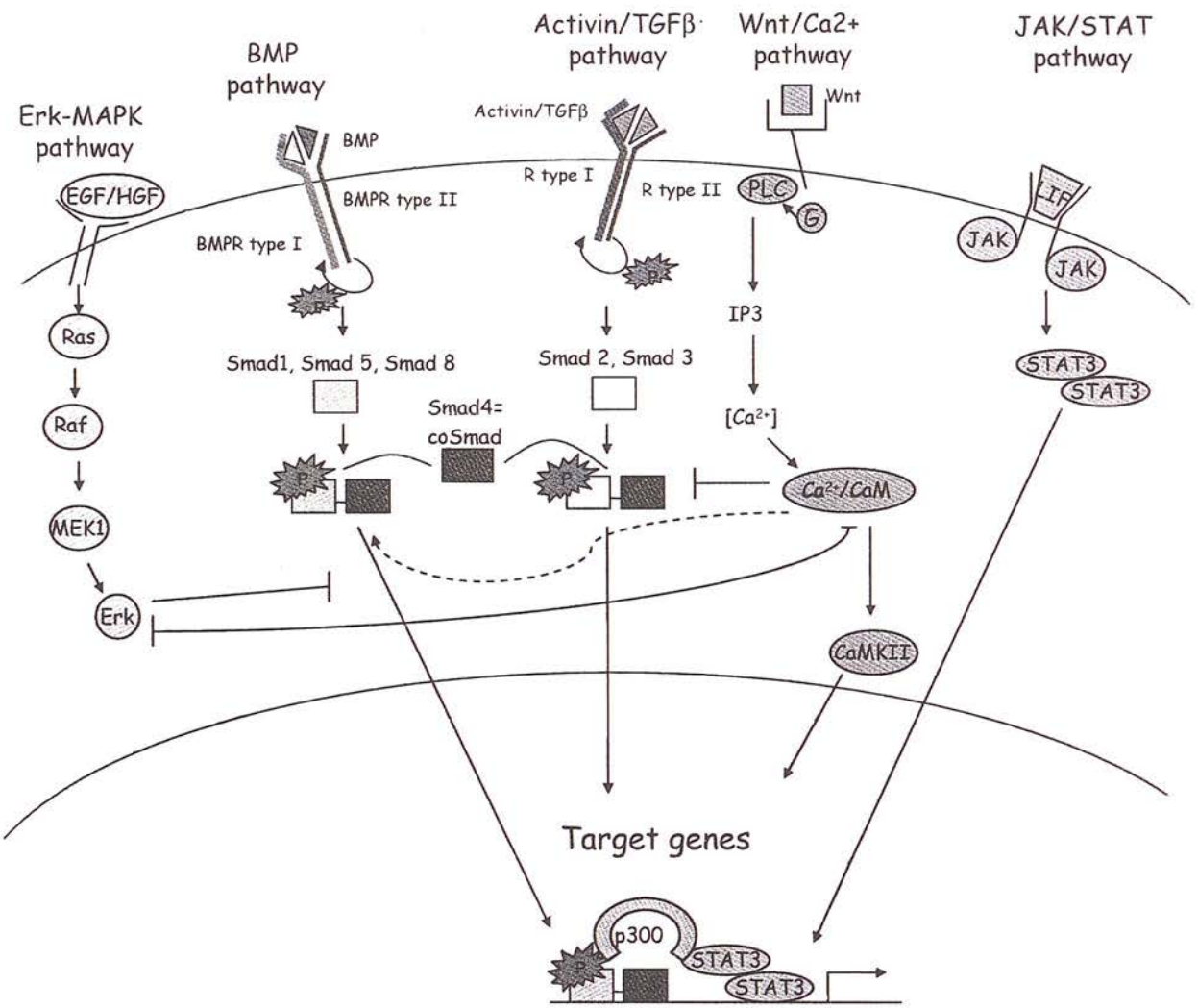


Fig.22. Interactions between Smad signaling pathway triggered by BMPs and other signaling pathways like TGFβ/Activin, Erk-MAPK, JAK-STAT and Wnt/Ca2+ pathways (for review, Von Bubnoff *et al.* 2001).

transcriptional responses to TGFβ, preventing Smad-4 from ubiquitin-dependent degradation (Lin X *et al.* 2003a).

Smad-6 and Smad-7, binding Smurfs proteins and the type I receptor, also play a role in this process, targeting the type I receptor degradation (Kavsak *et al.* 2000, Ebisawa *et al.* 2001, Murakami *et al.* 2003). Furthermore, Smad-7 mRNA stability is also regulated by a balance between ubiquitinylation and acetylation (Gronroos *et al.* 2002, Simonsson *et al.* 2005). We should also underline that the tight control of TGFβ signaling pathway to avoid amplification of the response is modulated by the induction of Smurf2 by TGFβ (Ohashi *et al.* 2005).

4. At nucleus level

At nucleus level, many transcription factors can interact with Smads, acting specifically as enhancer (STAT3) or repressor (Hox family) depending of the constitution of the cell (for review, Miyazono *et al.* 2001; Reguly & Wrana 2003).

C Interaction with other signaling pathways (Fig. 22)

Functional cross-talks between different signaling pathways have been documented. Thus, TGFβ pathways can interact with other pathways than Smads as mitogen-activated protein kinase (MAPK), Ca2+/calmoduline, CDK or JAK/STAT signaling pathways (for review: von Bubnoff & Cho 2001, Miyazono *et al.* 2001, Nohe *at al.* 2004).

1. MAPK/Erk

The Erk/MAP kinase pathway which mediates the effect of tyrosine kinases receptors can also interact with TGFβ signaling pathways. Smads can be phosphorylated in the linker region between their conserved Mad Homology 1 (MH1) and 2 (MH2) domains by MAPK (Massagué 2003). This phosphorylation inhibited their translocation to the nucleus thereby antagonizing TGFβ signaling (Kretzschmar *et al.* 1997). Recent studies analysed the phenotypes of Smad-1 mutants mice carrying mutations preventing phosphorylation required for BMP downstream activation (Smad-1(C)) or for MAPK binding (Smad-1(L)). Smad-1(C/C) mutants had same phenotype as Smad-1 null mice whereas Smad-1(L/L) survived embryogenesis in spite of defects in gastric epithelial homeostasis. Interestingly, allelic

complementation in mutants (C/L) restored the lack of primordial germ cells seen in Smad-1(L/L) mice (Aubin *et al.* 2004). These results showed the interaction between MAPK and BMP signaling pathways.

2. Ca^{2+}/calmodulin

The calmodulin is intracellular Ca2+ receptor which plays a role in the inositol-phospholipid pathway triggered by G protein coupled receptors. The calmodulin is able to bind Smad-1, 2, 3 and Smad-4 by interaction cloning strategy with calmodulin-agarose. Moreover, a calmodulin antagonist, W13, increased the expression of the activin inducible transcriptional reporter, 3TPlux whereas overexpression of calmodulin decreased the expression of the reporter (Zimmerman *et al.* 1998). A study showed the calmodulin inhibitor effect on activin/TGFβ signaling pathway via the down regulation of Smad-2 activity while calmodulin increased Smad-1 activity stimulating BMP signaling pathway (Scherer & Graff 2000). In the contrary, using the Xenopus embryo system, another work showed that dorsal injection of Smad-1 RNA into the 4-cell-stage embryos resulted in embryonic ventralization which was inhibited by calmodulin (Xu *et al.* 1999). Even if these results are contradictory, they suggested interaction between TGFβ signaling pathways and calmodulin.

3. JAK/STAT

The evidence of interaction between TGFβ and STAT (Signal Transducers and Activators of Transcription) has been studied. LIF (leukemia inhibitory factor) acting via STATs can act in synergy with BMP-2 on primary fetal neural progenitor cells to induce astrocytes. The formation of a complex between STAT3 and Smad-1, bridged by p300 which is a STAT3 coactivator, is involved in the cooperative signaling of LIF and BMP-2 (Nakashima *et al.* 1999).

4. CDK

More recently, cyclin-dependent kinases (CDKs) were shown to phosphorylated Smad-2 and Smad-3 *in vitro*. TGFβ signaling induces the transcription of growth inhibitory cell cycle regulators such as p15 or p21, and suppresses the expression of stimulatory growth factors as c-myc. Moreover, a mutant of Smad-3 enables to be phosphorylated by CDK enhanced p15 transcription. As CDK phosphorylated Smad-3 reducing its activity, CDK can tightly modulate TGFβ signaling to allow cell cycle progression (Matsuura *et al.* 2004).

Aims of the thesis

Aims of the thesis

GnRH, steroids, activins and inhibins are regulatory factors involved in the differential regulation of FSH and LH secretion. BMPs, members of TGFβ superfamily as activin and inhibin, were recently described as important modulators of reproductive function, particularly in the ewe. However, little is known about their role at pituitary level. In order to extend our knowledge of the regulation of gonadotropin synthesis and release, we investigated the potential role of BMPs in ewe pituitary. The first part of the study was addressed to determine whether BMPs, particularly BMP-4 and BMP-6, can affect gonadotropin synthesis and release in ewe pituitary cells. We detected the presence of several BMP mRNAs and BMP receptors type IA, IB and II in the pituitary. Further, BMP receptors type IA and II colocalised with gonadotrope cells indicating a putative role of BMPs on gonadotropin regulation. Pituitary cell treatment with different BMPs exerted an inhibitory effect specifically on FSH synthesis and release, without modifying LH production. This result incited us to investigate whether BMPs interact with modulators of gonadotropin secretion, mainly oestradiol and activin. Indeed, BMPs amplifies the inhibitory effect of oestradiol and block the stimulatory effect of exogenous activin. Since activin is also produced by pituitary cells and stimulates basal FSH secretion, we secondly questioned whether BMP-4 acts through its own signalling pathway or by inhibiting endogenous activin pathway. Lastly, we compared the effects of BMP-4 on gonadotropin production between ewe pituitary cells and mouse gonadotrope cells. Indeed, while BMP-4 has inhibitory effect on FSHβ expression and FSH release in ewe pituitary cells, studies in rodents have shown a stimulatory effect of other BMPs. We tempted to elucidate the effect of BMP-4 on gonadotropin secretion in complement to the main modulators (GnRH and activins) in the mouse gonadotrope cell line, $L\beta T_2$.

Chapter I:

BMP-4 inhibits the follicle-stimulating hormone secretion in ewe pituitary

Introduction

In females, the gonadotropins FSH and LH produced by pituitary gonadotropes are required for terminal follicular growth and regulation of ovulation number (McNeilly *et al.* 1992). The patterns of the synthesis and release of LH and FSH diverge in several physiological situations. However, the mechanisms involved in this differential regulation are still unclear. Gonadotropin synthesis and release are orchestrated by an interplay of hypothalamic GnRH, gonadal steroids (oestradiol and progesterone) and non-steroids gonadal and pituitary factors (activins and inhibins). Each factor acts differentially on FSH and LH synthesis and release. Particularly, activins and inhibins, members of the transforming growth factor beta (TGFβ) superfamily are involved in specific regulation of FSH production. Activins are known to increase FSHβ mRNA expression and FSH release (Carroll *et al.* 1989) and to act as paracrine factors (Knight 1996, Bilezikjian *et al.* 2001, Welt *et al.* 2002, Lin *et al.* 2003).

Recently, other molecules of the TGF-β superfamily, the bone morphogenetic proteins (BMPs) were shown to play a role in FSH regulation (Huang *et al.* 2001, Otsuka & Shimasaki 2002). It is now admitted that BMPs play a crucial role in reproduction. In sheep, natural mutations of the BMP system alter the ovulation rate. For instance, in the Booroola phenotype, hyperprolificacy is associated with a mutation in the intracellular serine/ threonine kinase domain of the BMP receptor IB (Mulsant *et al.* 2001, Souza *et al.* 2001, Wilson *et al.* 2001). Besides the regulatory role of BMPs in the ovary, little is known about the action of BMPs at the pituitary level, particularly in species other than rodents. In mouse embryos, the role of BMPs in pituitary organogenesis is well documented. BMP-4 is required for induction and formation of the Rathke's pouch rudiment (Takuma *et al.* 1998) while BMP-2 acting with FGF-8 in opposite gradients determines gonadotrope cell differentiation (Ericson *et al.* 1998, Dasen *et al.* 1999). In the adult mouse pituitary, high concentrations of BMP-6 and BMP-7 stimulate the activity of transfected ovine FSHβ promoter and the release of FSH (Huang *et al.* 2001). In the adult rat pituitary, BMP-15 stimulates specifically FSH release (Otsuka & Shimasaki 2002). Taken together, these data suggest that BMPs may act as regulatory factors in the pituitary, at least in rodents.

In order to extend our knowledge of the factors involved in the regulation of FSH synthesis and release in sheep, our study focuses on the role of BMPs. In this first part, three questions were addressed:

- Are BMPs and BMP receptors expressed in the pituitary?

- Does exogenous BMP-4 affect gonadotropin synthesis and release?
- Does BMP-4 modulate the effects of other FSH regulatory factors?
- Does the Booroola mutation on BMP receptor type IB modify the response of pituitary cells to BMP-4?

BMP-4 inhibits follicle-stimulating hormone secretion in ewe pituitary

M-O Faure, L Nicol[1], S Fabre, J Fontaine, N Mohoric, A McNeilly[1] and C Taragnat

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

[1]Medical Research Council, Human Reproductive Sciences Unit, Centre for Reproductive Biology, The University of Edinburgh Chancellor's Building, 49 Little France Crescent, Old Dalkeith Road, Edinburgh EH16 4SB, UK

(Requests for offprints should be addressed to C Taragnat; Email: taragnat@tours.inra.fr)

Abstract

Activins and inhibins, members of the transforming growth factor-beta family are able to stimulate and inhibit, respectively, FSH synthesis and release. Other members of this superfamily, the bone morphogenetic proteins (BMPs), may also affect FSH synthesis in the mouse. The aim of this work was to determine whether BMPs are expressed in the ovine pituitary and whether they play a role in the regulation of FSH release.

The mRNAs encoding BMP-2, BMP-4, BMP-7 and the oocyte-derived growth factor, growth differentiation factor (GDF)-9 were detected in the pituitaries of cyclic ewes by reverse-transcriptase PCR, as well as the mRNAs encoding the BMP type I receptors, BMPR-IA (activin-receptor-like kinase (ALK)-3) and BMPR-IB (ALK-6), and type II receptors (BMPR-II). Immunolabeling of pituitary sections revealed the presence of BMPR-IA (ALK-3) and BMPR-II in gonadotrope cells. To investigate the potential effects of BMPs on FSH secretion, ewe pituitary cell cultures were treated with BMP-4 (10^{-11} M to 10^{-9} M) for 48 h. Interestingly, FSH release was decreased in a dose-dependent manner. At 10^{-9} M BMP-4 both FSH concentration and FSHβ mRNA expression were reduced by 40% of control values. In contrast, there was no inhibitory effect on either LH or LHβ mRNA expression. A similar result was found with BMP-6. BMP-4 triggered the phosphorylation of Smad1, suggesting that the effect of BMP-4 on FSH secretion is due to the activation of the BMPs signaling pathway. Furthermore, BMP-4 blocked the stimulatory effect of activin on both FSH release and FSHβ mRNA and amplified the suppression of FSH release and FSHβ mRNA levels induced by 17β-estradiol. These results indicate that a functional BMP system operates within the sheep pituitary, at least *in vitro*, to decrease FSH release and to modulate the effect of activin.

Journal of Endocrinology (2005) **186,** 109–121

Introduction

In females the gonadotropins FSH and LH produced by pituitary gonadotropes are required for terminal follicular growth and regulation of ovulation number (McNeilly *et al.* 1992). The patterns of the synthesis and release of LH and FSH diverge in several physiological situations. However, the mechanisms involved in LH and FSH differential regulation are still unclear. Gonadotropin synthesis and release are orchestrated by an interplay of hypothalamic, gonadal and pituitary factors. The primary stimulus for synthesis and release of FSH and LH appears to be the hypothalamic gonadotropin-releasing hormone (GnRH). Nevertheless, depending on pulse frequency or amplitude, GnRH exerts a differential effect on both hormones. While a high frequency of GnRH pulses is favourable to LH secretion, a low frequency is associated with the production of FSH (Dalkin *et al.* 1989, Molter-Gerard *et al.* 1999). Other factors such as gonadal steroids, progesterone and estrogens can also differentially modulate the secretion of LH and FSH (Mercer *et al.* 1993, Molter-Gerard *et al.* 2000). In addition to GnRH and steroids, some members of the transforming growth factor-beta (TGF-β) superfamily, particularly activins and inhibins, are involved in specific regulation of FSH production. Activin is a dimer of two β subunits (A and B). The association of the subunits results in activin A, activin B or activin AB, each of which increases FSH β mRNA expression and FSH release (Carroll *et al.* 1989). Activins are produced by the gonads and the pituitary where they act as paracrine factors (Knight 1996, Bilezikjian *et al.* 2001, Welt *et al.* 2002, Lin *et al.* 2003). Inhibin is a dimer of an α subunit and either βA or βB subunits forming inhibin A or B respectively, each of which suppresses FSH synthesis and release (De Kretser & Robertson 1989). They are produced mainly in the gonads and act through an endocrine pathway. Recently other molecules of the TGF-β superfamily, the bone morphogenetic proteins (BMPs) were shown to play a role in FSH regulation (Huang *et al.* 2001, Otsuka & Shimasaki 2002).

Journal of Endocrinology (2005) **186,** 109–121
0022-0795/05/0186-109

DOI: 10.1677/joe.1.05988
Online version via http://www.endocrinology-journals.org

Initially, BMPs were identified through their actions on bone morphogenesis. They are now known to have effects on growth, differentiation, apoptosis and other phenomena in many cell types (for review, Shimasaki *et al.* 2004). BMP signaling occurs through a heteromeric complex with a type BMPR-IA (ALK-3) or BMPR-IB (ALK-6) and type BMPR-II serine/threonine kinase receptors (Kawabata *et al.* 1998, Miyazono *et al.* 2001). The ligand binding induces the trans-phosphorylation of the type I receptor by the type II receptor. Consequently, the activated BMPR-I phosphorylates cytoplasmic proteins called receptor-activated Smads (R-Smads) which interact with a co-Smad, Smad4. BMP signaling recruits the specific R-Smads, Smad1, Smad5 and Smad8 and the R-Smad-co-Smad complex translocates to the nucleus to promote specific gene expression (for review, Zwijsen *et al.* 2003).

It is now admitted that BMPs play a crucial role in reproduction. In sheep, natural mutations of the BMP system alter the ovulation rate. For instance, in the Inverdale, Hanna, Cambridge and Belclare phenotypes, mutations in the BMP-15 gene lead to hyperprolificity in heterozygous ewes, while homozygous ewes are sterile (Galloway *et al.* 2000). A mutation in GDF-9, associated with alterations in follicular development, has also been identified in Belclare and Cambridge sheep (Hanrahan *et al.* 2004). In the Booroola phenotype, hyperprolificity is associated with a mutation in the intracellular serine/threonine kinase domain of the BMP receptor IB (Mulsant *et al.* 2001, Souza *et al.* 2001, Wilson *et al.* 2001). Several BMPs have recently been implicated as autocrine/paracrine regulators of ovarian follicule development (Knight & Glister 2003, Shimasaki *et al.* 2004 (review)). For instance, BMP-15 produced by oocytes stimulates granulosa cell proliferation (Otsuka *et al.* 2000). BMP-4 and BMP-7 expressed by theca cells affect follicle development (Lee *et al.* 2001) and enhance FSH action on granulosa cells by stimulating estradiol (Shimasaki *et al.* 1999, Souza *et al.* 2002, Glister *et al.* 2004) and decreasing progesterone production (Shimasaki *et al.* 1999, Fabre *et al.* 2003). Besides the regulatory role of BMPs in the ovary, little is known about the action of BMPs at the pituitary level, particularly in species other than rodents.

In mouse embryos, the role of BMPs in pituitary organogenesis is well documented. BMP-4 is required for induction and formation of the Rathke's pouch rudiment (Takuma *et al.* 1998) while BMP-2 acting with FGF-8 in opposite gradients determines gonadotrope cell differentiation (Ericson *et al.* 1998, Dasen *et al.* 1999). In the adult mouse pituitary, high concentrations of BMP-6 and BMP-7 stimulate the activity of transfected ovine FSHβ promoter and the release of FSH (Huang *et al.* 2001). In the adult rat pituitary, BMP-15 stimulates specifically FSH release (Otsuka & Shimasaki 2002). Taken together, these data suggest that BMPs may act as regulatory factors in the pituitary, at least in rodents.

To extend our knowledge of FSH synthesis regulation, we investigated the potential role of BMPs in the ewe pituitary. Our data showed that BMP receptors and BMP-2, BMP-4, BMP-7 and GDF-9 mRNAs were present in the pituitary. Interestingly, BMP-4 and BMP-6 inhibited the FSH, but not the LH release from primary pituitary cells. In comparison with other physiological factors, we found that BMPs antagonized the activin effect known for stimulating FSH release and amplified the inhibitory action of 17β-estradiol. Thus, in the sheep pituitary BMP-4 and BMP-6 act to suppress FSH production.

Materials and Methods

Reagents

Cell culture reagents used were DMEM (Dulbecco's modified eagle's medium) and F12 (Nutrient mixture F-12 Ham) from Sigma (Saint Louis, MO, USA). Gentamicin, nistatine, L-ascorbic acid, apo-transferrin, FCS (fetal calf serum) and BSA (bovine serum albumin) were purchased from Sigma. Collagenase A and DNase I were from Roche Diagnostics Ltd (Meylan, France). Human recombinant Activin-A, BMP-6 and BMP-4 were obtained from R&D systems (Lille, France). 17-β estradiol was from Sigma. Rabbit antibodies against phosphorylated Smad1 and against Smad1 protein were from Upstate Biotechnology (Euromedex, Mundolsheim, France). Mouse monoclonal antibody directed against bovine LHβ (bLHβ 518B7) was obtained from J F Roser (Department of Animal Science, University of California, USA) (Matteri *et al.* 1987). Rabbit polyclonal antibody directed against BMPR-IA (amino acid 181–202; ten Dijke *et al.* 1994), BMPR-IB (amino acid 151–168; ten Dijke *et al.* 1994) and BMPR-II (amino acid 185–202; Rosenzweig *et al.* 1995) were kindly given by C H Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden).

Tissue collection

Pituitaries from Ile de France, Merinos d'Arles and Scottish Blackface ewes were collected throughout the year and dissociated for cell cultures or immersed in Bouin's Holland fixative containing $HgCl_2$ for immunohistochemistry. Some pituitaries and ovarian follicles were used for RNA extraction.

Reverse transcription-PCR on whole tissues

Total RNA from ewe pituitaries and ovarian follicles were extracted using TRI Reagent (Sigma). Complementary DNA was synthesized from 1 μg RNA in a volume of 20 μl containing 150 ng oligodT (Promega, Charbonnières, France),

Table 1 Oligonucleotide primer sequences used for PCR (A) and Real-time PCR (B).

A PCR

	Forward sequence	Reverse sequence	Annealing temperature (°C)	$MgCl_2$ concentration (mM)	Product size (bp)
Primer					
BMP-2	gTCACAgATAAggCCATTgC	CCTCCACAACAATgTCCTgA	58	1·5	403
BMP-4	ggTCACCTTTggCCATgATg	ACCTTgTCATACTCATCCAg	58	2	359
BMP-6	AAgCAgCCCTTCATggTggC	CATgTgTgCgTTgAgAgggA	58	1	296
BMP-7	AgAgCATCAACCCCAAgTTg	CgATgATCCAgTCCTgCCAg	62	1	284
BMP-15	ggATCACTggATCATTgCTC	gCAATCATACCCTCATACTC	58	1·5	250
GDF-9	TAgTCAgCTgAAgtgggACA	ACgACAggTACACTTAgTgg	58	1	275
BMP-RIA	AACACATgCATAACTAATgg	ACCATCTgAATCTgTTTggC	54	1·5	493
BMP-RIB	AgATTggAAAAggTCgCTATg	CgAgTgTTgggTggTATg	54	1·5	486
BMP-RII	ATggAACATgACAACATTgC	CCTgggAAgAggTCTgTACA	54	1·5	523

B Real time PCR

	Forward sequence	Reverse sequence	Probe
Primer			
FSHβ	CACgAgggTgCTTTTCCATT	TgggACCgTATCCCTAgCTTT	AATAATCTTAgAAATCCTCTCAggCAATCCCTTCCTC
LHβ	CCgCTCCCAgATATCCTCTTC	TgCTggCTTTgggAgTTAgg	AAggATgCCCCACTTCAATCTCCCAT

1 mM dNTPs, 20 U of RNasin, 1 × RT PCR buffer [from 5 × assay buffer B (250 mM Tris–HCl (pH 8·3), 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT)], 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 reverse transcription at 37 °C for 1 h. PCR was carried out in a volume of 25 µl containing 2·5 µl RT reaction mixture, 1 × PCR buffer [from 10 × PCR buffer without MgCl2 (500 mM KCl, 100 mM Tris HCl (pH 9) and 1·0% Triton X-100), 50 pmol of each primer, indicated concentrations of $MgCl_2$, 0·2 mM dNTPs and 10 U *Taq* polymerase (Promega). PCR reactions were performed for 30 cycles of 30 s at 94 °C, 2 min at indicated annealing temperature, and 3 min at 72 °C. For primer sequences and details, see Table 1A.

Immunohistochemistry

After fixation and dehydration, sheep pituitaries were embedded in paraffin wax. Sections (5 µm thickness) were dewaxed and re-hydrated in xylene then in decreasing concentrations of alcohol (100, 90 and 75%). Antigen retrieval was performed by steaming the sections in a pressure cooker in citrate buffer (0·01 M) pH 6·0 for 5 min, then cooled down for 20 min. After two 5 min washes in PBS a combined avidin-biotin block was performed according to the manufacturer's instructions (Vector Laboratories, Peterborough, UK). Sections were incubated at 4 °C overnight with the monoclonal anti-bLHβ diluted 1:250 in PBS containing 20% normal goat serum and 5% BSA (PBS/NGS/BSA). Negative controls were performed by replacing the first antibody with normal goat serum. After washes in PBS-Tween 20 (0·05%; PBST) and PBS, slides were incubated in goat anti-mouse Alexa 488 (Molecular Probes, Eugene, OR, USA) and diluted at 1:200 in PBS for 1 h. After washes in PBS, slides were blocked in PBS/NGS/BSA for 30 min. Sections were then incubated at 4 °C overnight with rabbit polyclonal antibody directed against BMPR-IA, BMPR-IB or BMPR-II diluted in PBS/NGS/BSA at 1:100, 1:100 and 1:25, respectively. Negative controls were performed by replacing either BMPR antibody with normal goat serum. After washes in PBST and PBS, slides were incubated with goat anti-rabbit biotinylated antibody (Dako, Cambridge, UK) and diluted 1:500 for 30 min. After three 5 min washes in PBS, sections were incubated with streptavidin Alexa 546 (Molecular Probes) diluted at 1:200 in PBS/NGS/BSA and counterstained for 2 min with propidium iodure at 1:2000 for 2 min. Sections from sheep ovaries were performed as positive controls (Souza *et al.* 2002). The slides were examined using LSN 510 meta confocal microscope.

Sheep pituitary cell cultures

Pituitaries were finely sliced and placed in F12 supplemented (3 µg/ml gentamicin, 2 µg/ml nistatine, 5% FCS, 0·4 mg/ml collagenase A and 0·025 mg/ml DNase I) and incubated for 1 h30 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 resuspended in culture medium (DMEM supplemented with 3 µg/ml gentamicin, 2 µg/ml nistatine,

100 μM L-ascorbic acid, 5 μg/ml apo-transferrin and 5% FCS). Cells were plated in 48-well plates at 200 000 cells/well, 12-well plates at 1×10^6 cells/well or 6-well plates at 2×10^6 cells/well in 500 μl, 1 ml or 2 ml respectively of culture medium (DMEM with 5% FCS), and allowed to attach for 2 days in a humidified atmosphere with 5% CO_2 in air at 37 °C. Media were replaced with serum-free DMEM containing 0·1% BSA. One hour later, the medium was changed and DMEM–0·1% BSA alone (control) or supplemented with test substances at different concentrations (see Results) was added. Media were collected 48 h later to assay for FSH and LH.

LH and FSH assays

The concentrations of FSH and LH were determined using double antibody ELISA immunoassays for all experiments, except for the estradiol experiments. For these latter, performed in Scotland, radioimmunological assay (RIA) for FSH was used.

ELISA LH and FSH were assayed in duplicate aliquots of pituitary cell culture supernatants. For LH, microtitration plates (Maxisorp C96, Nunc, France) were coated overnight at 4 °C with 100 μl/well of a monoclonal antibody to bovine LH β-subunit 518B7 (Matteri *et al.* 1987) and diluted 1:3200 in carbonate buffer. Plates were then washed and blocked with PBS pH 7·4 containing 0·09% Tween 20 and 12·5% Sea-Block (Pierce, Brebières, France). Purified ovine LH (oLH CY1083) used as standard, controls and supernatants were added at 20 μl/well along with 80 μl/well of dilution medium (PBS containing 0·09% Tween 20, 12·5% Sea-Block and 2% normal rat serum). Plates were incubated overnight at 4 °C. After removal of unbound material, biotinylated monoclonal antibody to human α-subunit (Dirnhofer *et al.* 1994) diluted at 23 ng/100 μl/well in PBS containing 0·1% Tween 20 and 1% rat serum was added for 1 h at 37 °C. Plates were washed and horseradish peroxidase-labelled neutravidin (Pierce) was added at 25 ng/100 μl/well. After 25 min at room temperature in obscurity and washing, peroxidase activity was developed with 100 μl tetramethylbenzidine/well (Dako) for 30 min at room temperature. The reaction was stopped with 50 μl Hcl/H2SO4/H2O (1 v/1 v/6 v) per well and the signal was measured at 450 nm and 620 nm. The minimum detectable concentration for LH was 0·1 ng/ml. The intra- and inter-assay coefficients of variation of the control averaged 4% and 10%, respectively. The cross-reaction with oFSH was 0·01%.

For FSH, concentrations were measured as described for LH assay with the following modifications. Plates were coated overnight at 4 °C with 100 μl/well of a monoclonal antibody to ovine FSH β subunit (Henderson *et al.* 1995) and diluted 1:100 in carbonate buffer. Purified ovine FSH (NIH RP2) used as standard, controls and supernatants were added at 50 μl/well along with 50 μl/well of dilution medium. The minimum detectable concentration for FSH was 0·2 ng/ml. The intra-and inter-assay coefficients of variation of the control were 2·6% and 2·1%, respectively. The cross-reaction with oLH was 0·07%.

RIA RIA assay on ovine FSH was previously described (McNeilly *et al.* 1976, 1986). The sensitivity of the FSH RIA was 0·1 ng/ml and the intra- and inter-assay coefficients of variation were less than 10%.

Quantitative RT-PCR from pituitary cell cultures

Ovine FSHβ and LHβ primers and probe were designed using Primer Express software (PE Biosystems, Warrington, Cheshire, UK) and synthesized by PE Biosystems (Table 1B). Ribosomal 18S primers and probe were from a Taqman RNA Control Reagents kit (VIC labeled probe; PE Biosystem).

Total RNA from 12-well plates were extracted using the Agilent Total RNA Isolation Mini Kit. RNA concentration was measured using RNA 6000 Nano Assay (Agilent Technologies, Stockport, UK). Aliquots of total RNA were treated with DNase I using DNA-free (Ambion Ltd, Huntingdon, Cambs, UK) according to manufacturer's protocol. Samples were then reverse transcribed, using oligod(T)16, with Taqman Reverse Transcription Reagents (PE Biosystems) according to the manufacturer's protocol, using a program of 10 min at 25 °C, 30 min at 48 °C and 5 min at 95 °C. DNase-treated RNA was approximatively added at 25–50 ng/μl. For PCR, a reaction mix was prepared consisting of Taqman Universal PCR Master Mix (1 ×), FSHβ or LHβ forward and reverse primers (300 nM each), FSHβ or LHβ probe (200 nM), ribosomal 18S forward and reverse primers (50 nM each) and ribosomal 18S probe (200 nM). This was aliquoted for each sample in separated tubes, then cDNA was added at 1 μl/25 μl reaction mix (equivalent to 25–50 ng total RNA/25 μl). Aliquots of 25 μl were dispatched into a 96-well PCR plate, with each sample added in duplicate, optical caps fixed onto the plates and the PCR reaction run on an ABI Prism 7900 PCR machine (PE Biosystems) using standard conditions. Controls including cDNA prepared without Multiscribe reverse transcriptase enzyme were done to check for efficiency of DNAse treatment. Prior to analysis, a validation assay was performed to demonstrate that amplification of FSHβ gene and the reference (18S) were approximately equal, whereas the amplification of LHβ gene and 18S were not equal. FSHβ and LHβ mRNA quantifications were performed by the ΔΔCt method and the standard curve method respectively (Bulletin number 2; PE Biosystems). A standard PCR reaction using the FSHβ and the LHβ primers was run on an agarose gel to confirm amplification of a single product of the correct size.

Western blotting analysis of Smad proteins

To investigate Smad-1 activation by BMP-4, ewe pituitary cells were cultured at 2×10^6 cells/well in 6-well plates for 48 h. Media were changed to remove non-attached cells and replaced by fresh media containing serum-free DMEM-0·1% BSA alone or supplemented with 10^{-9} M (50 ng/ml) of BMP-4 for 30, 60 and 90 min. Cells were then washed once on ice with cold PBS and lysed in lysis buffer (150 mM NaCl, 10 mM Tris pH 7·4, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0·5% Igepal CA-630, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 mM sodium orthovanadate, proteases inhibitor cocktail). The concentrations of the proteins were determined with BC Assay Protein Quantitation kit (Interchim, Montluçon, France). After 1 min at 95 °C in the reducing SDS-PAGE buffer containing β-mercaptoethanol, 15 μg proteins were separated in 10% SDS-PAGE gels and electroblotted onto Immobilon-P membranes (Millipore corporation, Bedford, MA, USA). Membranes were blocked for 1 h at room temperature in Tris-buffered saline–0·1% Tween containing 5% fat-free dry milk and incubated with the anti-phospho Smad1 or the anti-Smad1 protein diluted at 1:4000 and 1:1000, respectively, overnight at 4 °C. After washing, the membranes were incubated with the secondary antibody, a peroxidase-conjugated anti-IgG (Biorad, Marnes la Coquette, France) diluted 1:10000 for 1 h. Immunoreactive proteins were detected using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Orsay, France). Membranes were exposed to Hyperfilm ECL (Amersham Pharmacia Biotech) for 5 min.

Statistical analysis

Results are reported as mean ± S.E.M. All experiments were performed in triplicate in at least three different experiments. For each figure, the number of experiments shown is indicated in the legend. The effects of increasing doses of BMP-4 on FSH concentrations were analysed by one-way ANOVA followed by Newman-Keuls post-test. The time course experiment was analysed by two-way ANOVA followed by Bonferroni post-test in order to appreciate the 'time effect' as well as the 'ligand' effect. Comparison between BMP-4 or BMP-6 and control groups on FSH and LH hormone concentrations and mRNA expression levels were analysed by an unpaired *t*-test. Comparison between BMP-4, activin, BMP-4 plus activin, estradiol, BMP-4 plus estradiol and control groups on FSH hormone concentrations and FSHβ mRNA expression levels were analysed by one-way ANOVA followed by Newman-Keuls post-test. $P<0·05$ was considered significant. The statistical analysis was performed using the GraphPad Prism version 4·00 program for Windows (GraphPad Software, San Diego, CA, USA: www.graphpad.com).

Results

BMPs and BMP receptors are expressed in the ewe pituitary

To test whether ewe pituitary cells express BMP receptors and BMPs, RT-PCR was performed on Ile de France and Merinos d'Arles ewe pituitaries using primers described in Table 1A. After 30 cycles, DNA fragments were detected at expected sizes for BMP-2, BMP-4, BMP-7, GDF-9, BMP-RIA (ALK-3), BMP-RIB (ALK-6) and BMP-RII (Fig. 1A, B). No PCR product was detected for BMP-6 and BMP-15 in the pituitary. Positive controls were done on sheep ovarian follicles. In follicles, DNA fragments were observed for BMP-6 and BMP-15 (Fig. 1C, D). When negative controls were performed by omitting reverse transcriptase in the RT reaction, no PCR products were detected (not shown). Sequencing of all fragments confirmed product identity.

BMP receptors IA and II are present in gonadotrope cells

Immunohistochemistry analysis revealed BMPR-IA, BMPR-IB and BMPR-II positive cells in the pituitary. The vast majority of cells bearing the BMPR-IA and BMPR-II are identified as gonadotropes by colocalization of BMPR-IA or BMPR-II and LH immunoreactivity (Fig. 2A, C). In contrast, BMPR-IB did not colocalize with LH (Fig. 2B).

BMP-4 and BMP-6 inhibit FSH secretion and mRNA levels in the ewe pituitary

To determine whether BMPs were capable of modifying gonadotropin release from the ewe pituitary, cells were incubated with BMP-4. A dose–response study showed that treatment of cells with BMP-4 for 48 h inhibited FSH secretion from pituitary cells in a dose-dependent manner (Fig. 3A). The maximal inhibition of BMP-4 on FSH release was obtained with concentrations from 5×10^{-10} M (25 ng/ml). A time course experiment showed that significant effect with BMP-4 at 10^{-9} M (50 ng/ml) was observed from 24 h (Fig. 3B). When 10^{-9} M BMP-4 were used to treat cells for 48 h in additional experiments, we confirmed that BMP-4 inhibited FSH release by 40% ($P<0·01$) compared with control (Fig. 4A). LH release was not decreased (Fig. 4B). Similarly, FSHβ mRNA expression was decreased by 30–40% ($P<0·05$) but not LHβ mRNA expression (Fig. 4C, D). When cells were treated with 2×10^{-9} M (100 ng/ml) BMP-6 for 48 h, a similar inhibitory effect on FSH release was observed (Fig. 4E, F). These inhibitory effects of BMPs were found for Ile de France, Merinos d'Arles and Scottish Blackface ewes.

BMP-4 stimulates the BMP signaling pathway

To determine whether BMP-4 could activate the BMP signaling pathway, proteins were extracted after 30, 60 or

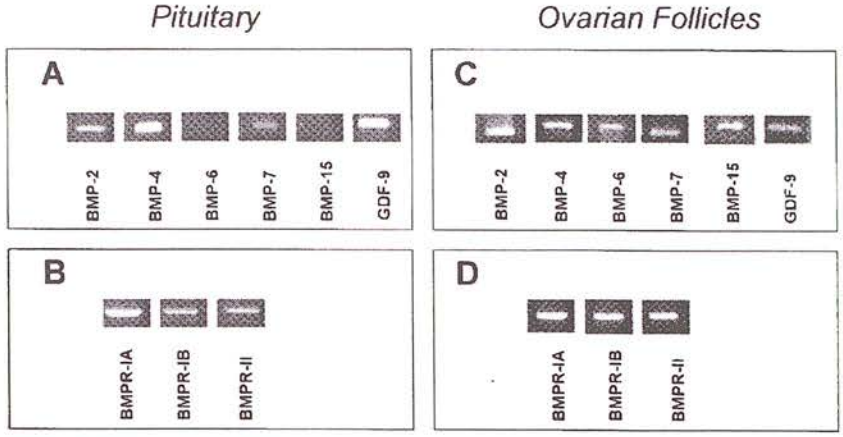


Figure 1 Expression of BMP ligands and receptors in sheep pituitary and ovarian follicles. Total RNA was extracted from sheep pituitaries (A,B) and ovarian follicles (C,D). The mRNAs encoding BMP ligands (A,C) and receptors (B,D) were analysed by RT-PCR at 30 cycles. DNA fragments were electrophoresed in 1% agarose gel and visualized by ethidium bromide staining.

90 min treatment with 10^{-9} M BMP-4. The activation of Smad-1 was followed by western blot analysis using an antibody which recognizes the dual serine phosphorylated Smad-1 (Ser 463/465) with an apparent molecular weight of 65–66 kDa in the phosphorylated state. Figure 5 shows that BMP-4 induced the apparition of a 66 kDa band within 30 min. The lower band observed on the blot corresponded to a non-specific band. In control samples harvested at the same time points, no phospho Smad1 immunoreactivity was detected. For Smad-1 protein, the intensity of the band did not change between control and BMP treatment (Fig. 5).

BMP-4 antagonises activin effects

To test whether BMP-4 was able to counteract the stimulatory effect of activin on FSH secretion and FSHβ mRNA expression, cells were incubated with both 2×10^{-9} M activin (50 ng/ml) and 10^{-9} M BMP-4. BMP-4 antagonised the activin effect on FSH secretion (Fig. 6A) and on FSHβ mRNA levels (Fig. 6B).

BMP-4 amplifies the effect of 17-β estradiol

To test whether BMP-4 was able to amplify the inhibitory action of estradiol on FSH production, the effects of simultaneous treatment with BMP-4 and 17-β estradiol were determined. A dose–response study showed that 17-β estradiol inhibited the FSH secretion from pituitary cells in a dose-dependent manner (Fig. 7A). At 10^{-12} M, 17-β estradiol inhibited by 30–40% FSH release ($P<0{\cdot}05$ vs control) and maximal inhibition (around 70%) was obtained with 10^{-11} M ($P<0{\cdot}001$ vs control; $P<0{\cdot}05$ vs 10^{-12} M). FSHβ mRNA expression was decreased by 80% ($P<0{\cdot}001$ vs control) with 10^{-11} M 17-β estradiol (Fig. 7B). When cells were incubated with both BMP-4 (10^{-9} M) and 17-β estradiol (10^{-12} M or 10^{-11} M), a stronger inhibition of FSH release was observed compared with either factor alone (Fig. 7A). This was also observed on FSHβ mRNA levels when cells were treated with both BMP-4 (10^{-9} M) and 17-β estradiol (10^{-11} M) (Fig. 7B).

Discussion

The synthesis and the secretion of the gonadotropins are differentially regulated by GnRH, ovarian hormones and pituitary factors. Control of FSH production includes specific factors belonging to the TGF-β superfamily, activin and inhibin. Recent data in rodents emphasize the potential role of other members of the same family, the BMPs, in the regulation of FSH synthesis and release (Huang *et al.* 2001, Otsuka & Shimazaki 2002). We investigated here whether BMPs could participate to control FSH synthesis and secretion in the ewe. We first demonstrated that ewe pituitary does express a set of BMP mRNAs, BMP-2, BMP-4, BMP-7 and GDF-9. Furthermore, BMP type IA (ALK-3) and II receptors are present in the gonadotrope cells suggesting that a functional BMP system can act within the pituitary. Indeed, we observed that BMP-4 and BMP-6 decreased FSHβ mRNA expression and FSH release from ewe pituitary cells, whereas similar inhibitory effects were not observed on LH secretion.

The presence of BMP-2, BMP-4 and BMP-7 mRNA in the adult ovine pituitary corroborates the results of Souza *et al.* (2003). Moreover, for the first time, GDF-9 mRNA, known to be specifically expressed in oocyte (Yan *et al.* 2001) was detected in the pituitary. However,

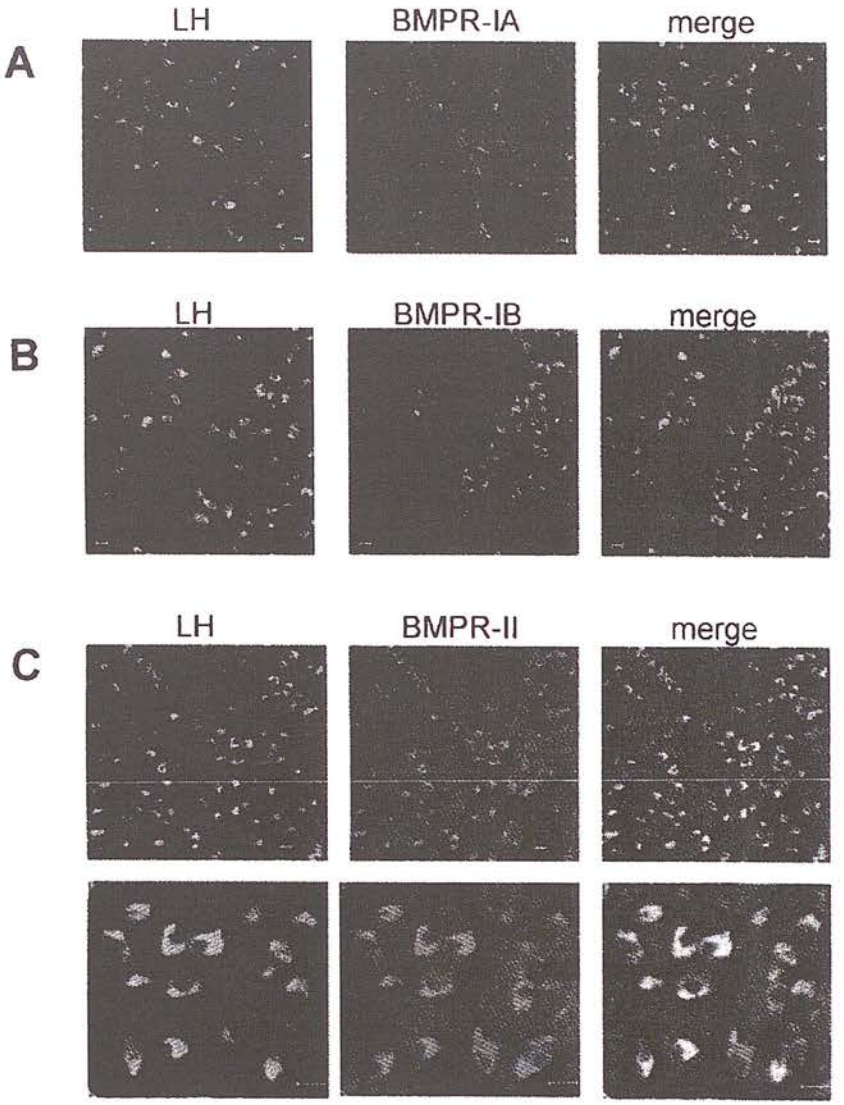


Figure 2 Immunohistochemical double staining for BMPR and LH. BMPR-IA, BMPR-IB and BMPR-II proteins were detected in the pituitary by immunohistochemistry. Ewe pituitary sections were incubated with mouse monoclonal antibody directed against LH (1:250) to detect gonadotrope cells followed by goat Alexa 488-conjugated anti-mouse IgG. In a second step, the same sections were incubated with rabbit polyclonal antibodies directed against BMPR-IA (1:100) (A), BMPR-IB (1:100) (B) or BMPR-II (1:25) (C) followed by goat anti-rabbit biotinylated antibody and then Alexa 546 labelled streptavidin. Nuclei were counterstained with propidium iodure. Most BMPR-IA and BMPR-II positive cells are also positive for LH. BMPR-IB positive cells are not positive for LH (B). Bar=10μm.

we did not detect BMP-6 and BMP-15 mRNA in ewe pituitary after 35 PCR cycles, whereas they are clearly observed in the ovarian follicles. In the mouse, these mRNAs appeared in the pituitary after more than 30–35 cycles (Huang *et al.* 2001, Otsuka & Shimazaki 2002). Hence, the pattern of BMP expression appears to be different among species. In the ewe, our results suggest than BMP-2, BMP-4, BMP-7 and GDF-9 could have important actions at the pituitary level. Because BMP-4 mRNA was strongly expressed in pituitary, we examined its effect on FSHβ expression and FSH release in the ewe pituitary cells in primary culture. The effect of BMP-6 reported as an FSH stimulator in mouse pituitary by Huang *et al.* (2001) was also examined. The findings of inhibitory effects of both BMP-4 and BMP-6, at low concentrations, on FSHβ mRNA expression and FSH release from ewe pituitary cells were unexpected. Indeed, similar doses of BMP-6 and BMP-7 had no effect on FSH

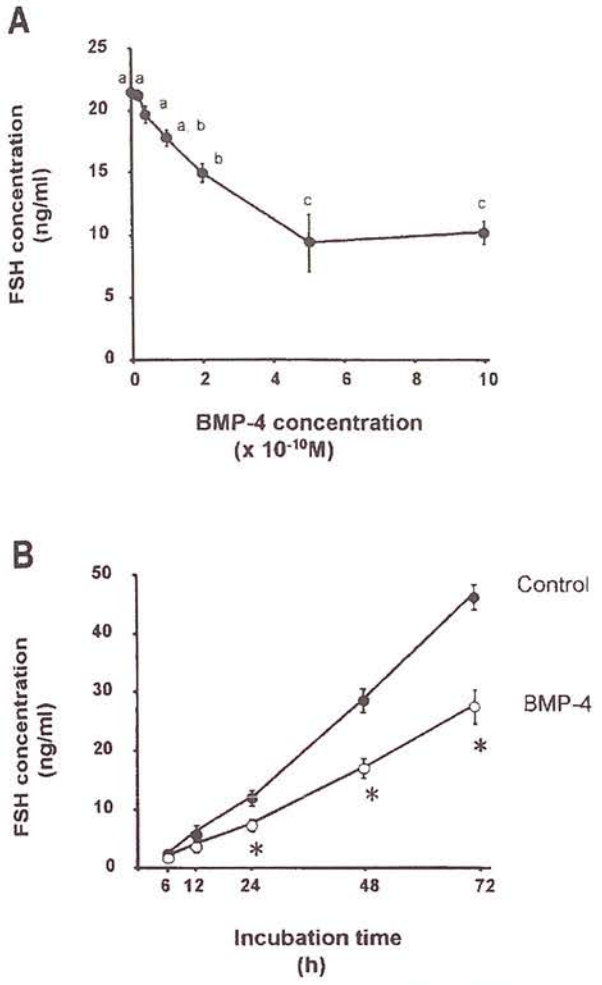


Figure 3 Dose response (A) and time course (B) of BMP-4 on FSH release from ewe pituitary cells. For the dose response, cells were cultured in serum-free medium for 48 h in the presence of indicated concentrations of BMP-4. For the time course, cells were cultured in serum-free medium in the presence or absence (control) of 10^{-9} M of BMP-4 for indicated times. Media were collected and the concentrations of FSH were determined by ELISA. Values are the mean ± S.E.M. from one representative experiment with triplicate determinations. Three experiments were performed with similar results. a vs b, $P<0.05$; a vs c, $P<0.0001$; b vs c, $P<0.01$: *, $P<0.05$ vs respective control.

synthesis and secretion in mouse pituitary cells or the gonadotrope cell line, LβT2 (Yamashita *et al.* 1995, Huang *et al.* 2001, Otsuka & Shimasaki 2002) while a much higher concentration (1 μg/ml, i.e. 2×10^{-8} M) increased FSH transcription and secretion. Another BMP, BMP-15 exhibited a stimulatory effect in rodents (Otsuka & Shimasaki 2002). The inhibitory effect of BMP-4 on ovine FSH production was not accompanied with an effect on pituitary cell proliferation as judged by the absence of changes in pituitary cell number after BMP treatment (data not shown). In mouse experiments, it has been suggested that BMP-6 and BMP-7 could transduce an activin-like signal since certain BMPs can bind to activin type II receptor present on mouse gonadotropes (Yamashita *et al.* 1995). Moreover, follistatin, known to neutralize activin, has been observed to bind several BMPs (Iemura *et al.* 1998) and local concentrations of follistatin may be sufficient to complex with BMP and compete with activin. However, in the ewe pituitary BMPs do not exert an activin-like signal although, activin type II and type I (ALK-2 and ALK-4) receptors are expressed (Dupont *et al.* 2003) as well as follistatin (Farnworth *et al.* 1995). Rather, BMPs can stimulate their own receptors. Activation of BMP type I receptors (ALK-3 and ALK-6) is known to induce the phosphorylation of intracellular receptor-specific Smad proteins (R-Smad), Smad1, Smad5 and Smad8 (Miyazono *et al.* 2001). In ewe pituitary cells, we observed that phosphorylation of Smad1 occurred within 30 min of BMP addition. This result indicates that BMPs induce their own signaling pathway. Whether the activation of Smad1 is responsible for the decrease of FSHβ mRNA levels and FSH release has yet to be confirmed.

Upon phosphorylation of the R-Smad proteins, these latter associate with a common partner, Smad-4 and translocate to the nucleus. The complexes R-Smad-Smad4 are then capable of binding DNA on a Smad binding element (SBE). The regulation of target gene requires additional interaction with transcription factors, either coactivators or corepressors (for review, Massague & Wotton 2000). Both SBEs and transcription factors may be cell type specific. For instance, a SBE was recently identified in the rat FSHβ subunit gene that is required for full activin responsiveness (Suszko *et al.* 2003). Interestingly, whereas this site is conserved in the mouse gene, it is not present in the ovine, bovine, porcine or human genes, suggesting that important species-specific differences exist in activin regulation of FSHβ gene expression. Similar differences probably exist in BMP regulation of target genes. Given that BMP-15 was found to stimulate the activity of ovine FSHβ promoter transfected in mouse LβT2 gonadotrope cells, the presence of specific repressors rather than particular SBE in ovine gonadotropes could be favoured. Alternatively, because we did not determine whether BMPs act directly on FSHβ promoter, we cannot exclude the possibility that BMPs affect FSH production via another target gene in gonadotropes or other cell type. Further experiments will help to discern between these hypotheses.

Considering the inhibitory effect of BMPs on FSH secretion from ewe pituitary cells, we questioned whether BMPs can affect the stimulatory effect of activin, an essential regulator of FSH. Activin is produced by the pituitary and exerts a paracrine action on FSH secretion (for review, Padmanabhan & McNeilly 2001). In the ewe, activin βB-subunit and activin receptors are expressed by the gonadotropes themselves (Dupont *et al.* 2003,

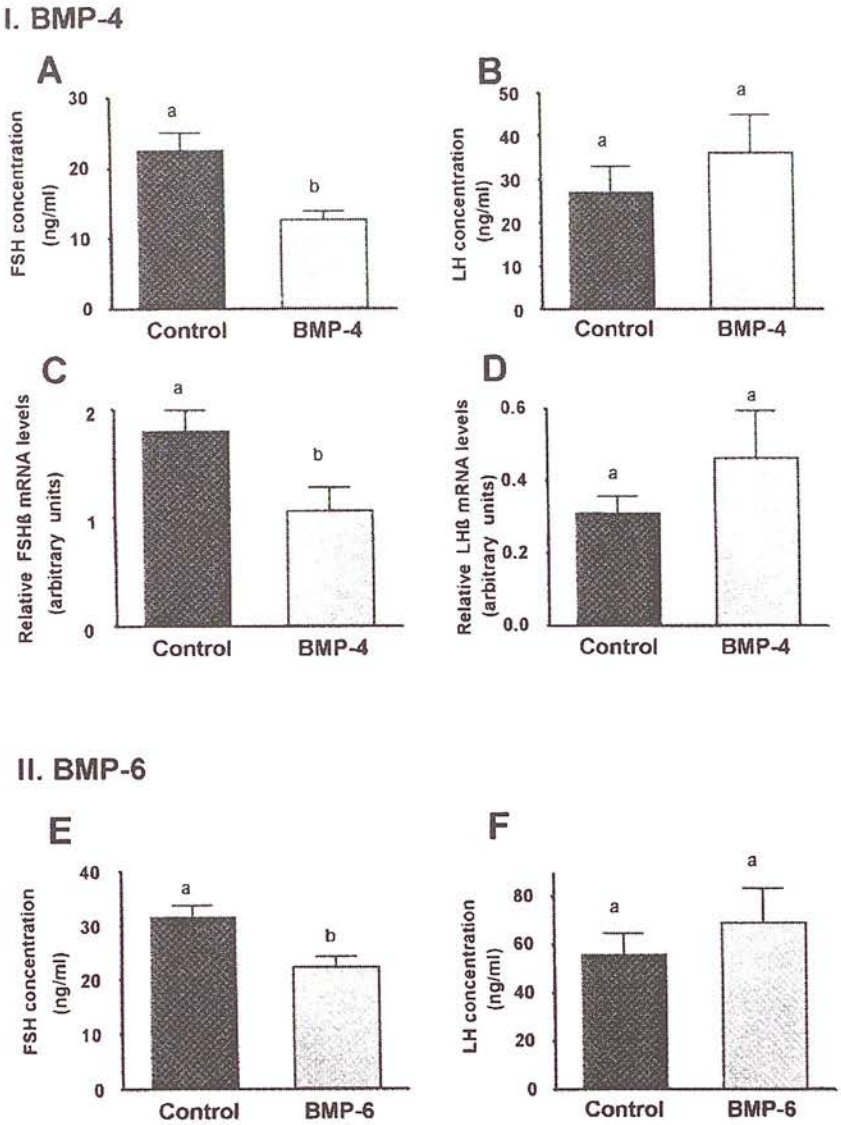


Figure 4 (I) Effect of BMP-4 on FSH (A) and LH (B) release and on FSHβ (C) and LHβ (D) mRNA expression from ewe pituitary cells. Cells were cultured in serum-free medium for 48 h with 10^{-9} M of BMP-4. Media were collected and the concentrations of FSH and LH were determined by ELISA. Values are the mean ± S.E.M. from ten experiments. a vs b, $P<0{\cdot}01$. Cells were harvested and total mRNAs were extracted. A real time PCR was performed to detect the relative level of FSHβ or LHβ mRNA expression compared with the 18S mRNA. Values are the mean ± S.E.M. from one representative experiment with triplicate determinations. Three experiments were performed with similar results. a vs b, $P<0{\cdot}05$. (II) Effect of BMP-6 on FSH (E) and LH (F) release from ewe pituitary cells. Cells were cultured in serum-free medium for 48 h with $2{\cdot}10^{-9}$ M of BMP-6. Media were collected and the concentrations of FSH and LH were determined by ELISA. Values are the mean ± S.E.M. from five experiments. a vs b, $P<0{\cdot}05$.

McNeilly *et al.* 2003) and the production of activin was suggested by the findings of an activin bioactivity from pituitary cell conditioned media (F Mathoux, D J Phillips and C Taragnat, unpublished observations). The action of activin is modulated by antagonists, mainly follistatin and inhibin which abolish the effect of activin. In our study, BMP-4 and BMP-6 (not shown) were found to antagonize the activin effect, suggesting that they can participate with other activin antagonists to regulate FSH synthesis and secretion. The precise mechanisms by which BMP and activin interact are under investigation.

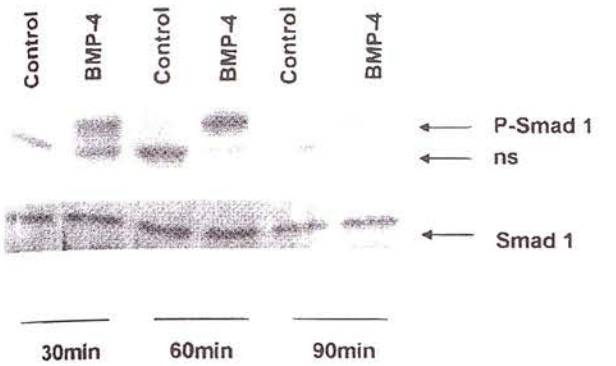


Figure 5 BMP-4 effect on Smad-1 phosphorylation. Pituitary cells were cultured in serum-free medium in presence or absence (control) of 10^{-9} M BMP-4 for indicated times. Total proteins (15µg) were separated on 10% SDS-PAGE and transferred on PVDF membrane. Blots were probed with phospho-Smad1 antibody (upper) or with a Smad1 antibody (lower) and bands were revealed by ECL. ns refers to non specific.

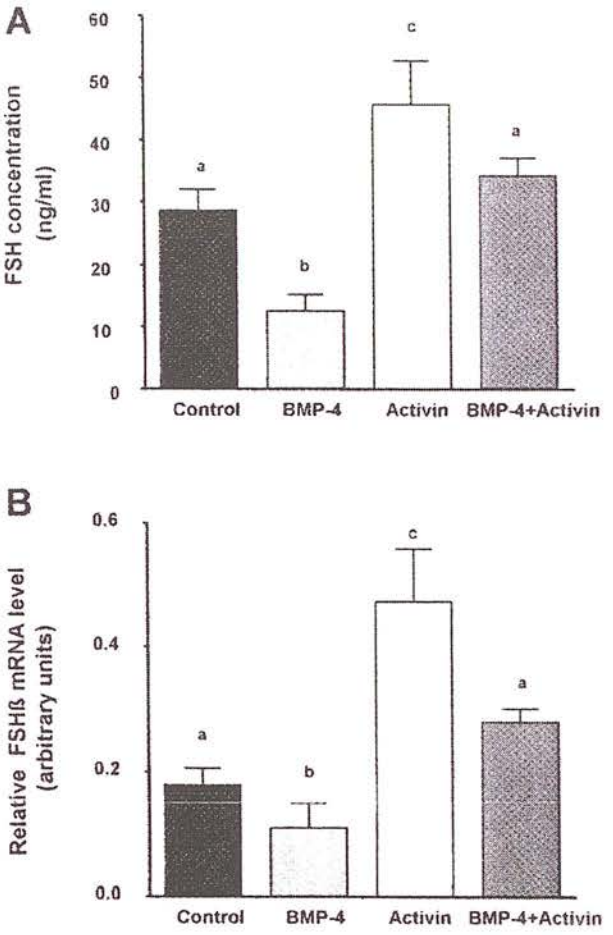


Figure 6 Interaction between BMP-4 and activin effects on FSH release (A) and FSHβ mRNA expression (B) in ewe pituitary cells. Cells were cultured in serum-free medium for 48 h in presence of 10^{-9} M BMP-4 and/or 2×10^{-9} M activin. Media were collected and the concentrations of FSH were determined by ELISA. Cells were harvested and total mRNAs were extracted. A real time PCR was performed to detect the relative level of FSHβ mRNA expression compared with the 18S mRNA (B). Values are the mean ± S.E.M. from one representative experiment with triplicate determinations. Three experiments were performed with similar results. Bars with different letters indicate that group means are significantly different at $P<0.05$.

Besides pituitary factors, another main regulator of FSH is estradiol. Estrogen receptors are expressed in gonadotropes in sheep (Sheng *et al.* 1998, Tobin *et al.* 2001) and injections of 17-β estradiol in ewes reduced FSHβ mRNA concentrations and FSH release directly at the pituitary level (Mercer *et al.* 1993, Phillips *et al.* 1998, Molter-Gérard *et al.* 2000). Moreover, the treatment of ovine pituitary cells with 17-β estradiol showed a direct negative effect on FSH promoter (Miller & Miller 1996). In our study, estradiol also exerted a strong inhibition on FSHβ mRNA expression and FSH release. Moreover, BMP-4 amplified this effect of estradiol. In other tissues, it was shown that estrogens and BMPs could interact through overlapping intracellular signaling mechanisms (Yamamoto *et al.* 2002). In pituitary prolactinomas in rodent and human where BMP-4 as well as 17-β estradiol induces cell proliferation, an additive effect of both BMP-4 and 17-β estradiol was observed (Paez-Pereda *et al.* 2003). In that model, Smad1 and Smad4 physically interacted with the estrogen receptor under BMP-4 stimulation (Paez-Pereda *et al.* 2003, Wu *et al.* 2003). A similar mechanism can occur in gonadotrope cells.

Whether BMP-4 exerts an inhibitory effect on FSH synthesis and release *in vivo* is not known at this date. In sheep, mutations have been identified either in BMPR-IB (ALK-6) in Booroola ewes (Mulsant *et al.* 2001, Souza *et al.* 2001, Wilson *et al.* 2001) or in BMP-15 in Inverdale and Hanna ewes (Galloway *et al.* 2000). Homozygous mutants for BMPR-IB and heterozygous mutants for BMP-15 exhibit a greater ovulation rate than wild type animals. Whether these mutants present changes in plasma FSH concentrations is a matter of controversy (McNatty *et al.* 1987, McNatty *et al.* 1991). However, we did not detect BMPR-IB in the gonadotrope cells. Hence, it is not surprising that the mutation does not significantly affect FSH release. We did not detect BMP-15 mRNA in the pituitary, excluding the effect of the mutation at the pituitary level. Therefore, these mutation models are not suitable for understanding further the role of BMP-4 in FSH control.

In conclusion, BMP-4 and BMP-6 were shown to decrease FSHβ mRNA expression and FSH release from ewe pituitary cells and are able to antagonize the effects of activin, at least *in vitro*. These effects were not found on LH secretion. Moreover, the detection of BMP-2, BMP-4, BMP-7 and GDF-9 mRNAs in the pituitary as well as the colocalisation of both types of BMP receptors on gonadotrope cells suggest that these BMPs can exert

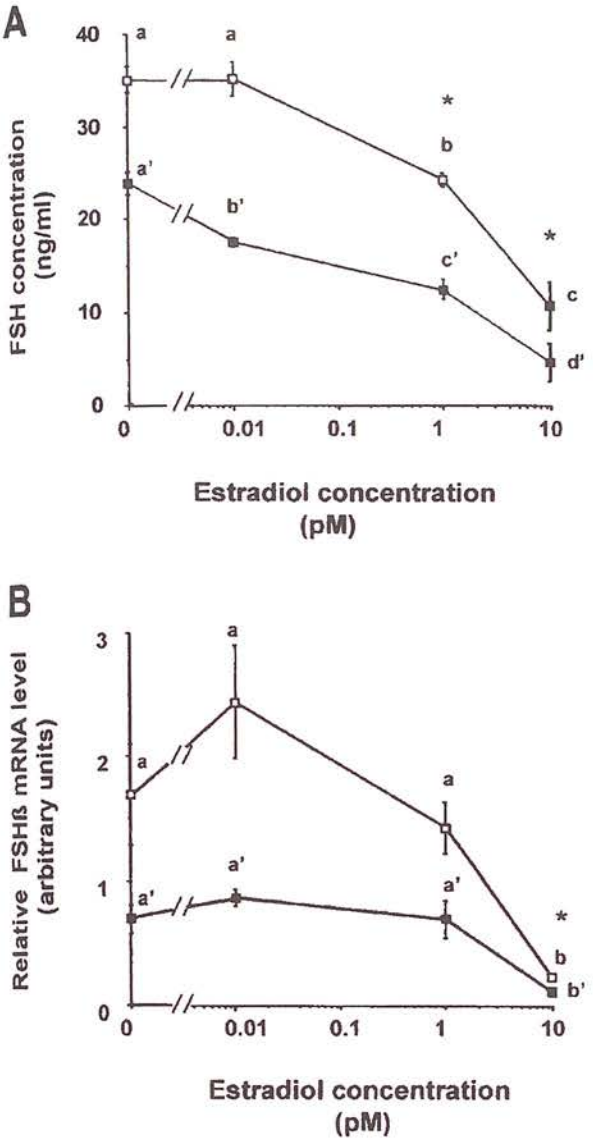


Figure 7 Effect of 17-β estradiol in presence of BMP-4 on FSH release (A) and FSHβ mRNA expression (B) in ewe pituitary cells. Cells were cultured in serum-free medium for 48 h in the presence of indicated concentrations of 17-β estradiol with or without 10^{-9} M of BMP-4. Media were collected and the concentrations of FSH were determined by RIA (A). Cells were harvested and total mRNAs were extracted. A real time PCR was performed to detect the relative level of FSHβ mRNA expression compared with the 18S mRNA (B). Values are the mean ± S.E.M. from one representative experiment with triplicate determinations and three experiments were performed with similar results. Different letters indicate that group means are significantly different at $P<0.05$. *, $P<0.05$ vs 17-β estradiol or BMP individually.

paracrine actions on FSH production modulating activin and/or estradiol action. Therefore, the findings of a functional BMP system in the ewe pituitary emphasize the role of BMPs in FSH control. Further experiments are required to establish the physiological importance of BMPs within the pituitary in the overall regulation of FSH synthesis and release.

Acknowledgements

We would like to thank Keith Henderson from Ag Research, Wallaceville Research Centre, Upper Hutt, New Zealand and Carl-Henrik Heldin from the Ludwig Institute for Cancer Research, Uppsala, Sweden for the gift of the BMP receptor antibodies. We thank Sylvie Canépa and Fabrice Mathoux for setting the oFSH ELISA assay, Ian Swanston and George Johnston for oFSH RIA technical assistance and Mhairi Laird and Sheila McPherson for immunohistochemistry technical assistance. We also thank the staff of INRA and MRC slaughterhouse and experimental farm for technical assistance. We acknowledge Judy McNeilly and Xavier Cayla for discussion.

Funding

This work was supported by Institut National de Recherches Agronomiques and by the Medical Research Council (Human Reproductive Sciences Unit, EH16 4SB, UK). MF was supported by a PhD fellowship from the Région Centre and by a fellowship for traveling from the Académie d'Agriculture de France and the Science and Technology Department of the French Embassy in the United Kingdom.

References

Bilezikjian LM, Blount AL, Corrigan AZ, Leal A, Chen Y & Vale WW 2001 Actions of activins, inhibins and follistatins: implications in anterior pituitary function. *Clinical and Experimental Pharmacology and Physiology* **28** 244–248.

Carroll RS, Corrigan AZ, Gharib SD, Vale W & Chin WW 1989 Inhibin, activin, and follistatin: regulation of follicle-stimulating hormone messenger ribonucleic acid levels. *Molecular Endocrinology* **3** 1969–1976.

Dalkin AC, Haisenleder DJ, Ortolano GA, Ellis TR & Marshall JC 1989 The frequency of gonadotropin-releasing-hormone stimulation differentially regulates gonadotropin subunit messenger ribonucleic acid expression. *Endocrinology* **12** 917–924.

Dasen JS, O'Connell SM, Flynn SE, Treier M, Gleiberman AS, Szeto DP, Hooshmand F, Aggarwal AK & Rosenfeld MG 1999 Reciprocal interactions of Pit1 and GATA2 mediate signaling gradient-induced determination of pituitary cell types. *Cell* **97** 587–598.

De Kretser DM & Robertson DM 1989 The isolation and physiology of inhibin and related proteins. *Biology of reproduction* **40** 33–47.

Dimhofer S, Madersbacher S, Bidart JM, Ten Kortenaar PB, Spottl G, Mann K, Wick G & Berger P 1994 The molecular basis for epitopes on the free beta-subunit of human chorionic gonadotrophin (hCG), its carboxyl-terminal peptide and the hCG beta-core fragment. *Journal of Endocrinology* **141** 153–162.

Dupont J, McNeilly J, Vaiman A, Canepa S, Combarnous Y & Taragnat C 2003 Activin signaling pathways in ovine pituitary and LbetaT2 gonadotrope cells. *Biology of reproduction* **68** 1877–1887.

Ericson J, Norlin S, Jessel T & Edlund T 1998 Integrated FGF and BMP signaling controls; the progression of progenitor cell differentiation and the emergence of pattern in the embryonic anterior pituitary. *Development* **125** 1005–1015.

Fabre S, Pierre A, Pisselet C, Mulsant P, Lecerf F, Pohl J, Monget P & Monniaux D 2003 The Booroola mutation in sheep is associated with an alteration of the bone morphogenetic protein receptor-IB functonality. *Journal of Endocrinology* **177** 435–444.

Farnworth PG, Thean E, Robertson DM & Schwartz J 1995 Ovine anterior pituitary production of follistatin *in vitro*. *Endocrinology* **136** 4397–4406.

Galloway SM, McNatty KP, Cambridge LM, Laitinen MP, Juengel JL, Jokiranta TS, McLaren RJ, Luiro K, Dodds KG, Montgomery GW, Beattie AE, Davis GH & Ritvos O 2000 Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner. *Nature Genetics* **25** 279–283.

Glister C, Kemp CF & Knight PG 2004 Bone morphogenetic protein (BMP) ligands and receptors in bovine ovarian follicle cells: actions of BMP-4, -6 and -7 on granulosa cells and differential modulation of Smad-1 phosphorylation by follistatin. *Reproduction* **127** 239–254.

Hanrahan JP, Gregan SM, Mulsant P, Mullen M, Davis GH, Powell R & Galloway SM 2004 Mutations in the genes for oocyte-derived growth factors GDF9 and BMP15 are associated with both increased ovulation rate and sterility in Cambridge and Belclare sheep (Ovis aries). *Biology of Reproduction* **70** 900–909.

Henderson KM, Camberis M & Hardie AH 1995 Generation of monoclonal antibody to ovine FSH and its application in immunoneutralization and enzymeimmunoassay. *Journal of Reproduction and Fertility* Suppl **49** 511–515.

Huang HJ, Wu JC, Su P, Zhirnov O & Miller WL 2001 A novel role for bone morphogenetic proteins in the synthesis of follicle-stimulating hormone. *Endocrinology* **142** 2275–2283.

Iemura S, Yamamoto TS, Takagi C, Uchiyama H, Natsume T, Shimasaki S, Sugino H & Ueno N 1998 Direct binding of follistatin to a complex of bone-morphogenetic protein and its receptor inhibits ventral and epidermal cell fates in early Xenopus embryo. *PNAS* **95** 9337–9342.

Kawabata M, Imamura T & Miyazono K 1998 Signal transduction by bone morphogenetic proteins. *Cytokine and Growth Factor Reviews* **9** 49–61.

Knight PG 1996 Roles of inhibins, activins, and follistatin in the female reproductive system. *Frontiers of Neuroendocrinology* **17** 476–509.

Knight PG & Glister C 2003 Local roles of TGF-b superfamily members in the control of ovarian follicle development. *Animal Reproduction Sciences* **78** 165–183.

Lee WS, Otsuka F, Moore RK & Shimasaki S 2001 Effect of bone morphogenetic protein-7 on folliculogenesis and ovulation in the rat. *Biology of reproduction* **65** 994–999.

Lin SY, Morrison JR, Phillips DJ & de Kretser DM 2003 Regulation of ovarian function by the TGF-β superfamily and follistatin. *Reproduction* **126** 133–148.

McNatty KP, Hudson N, Henderson KM, Gibb M, Morrison L, Ball K & Smith P 1987 Differences in gonadotrophin concentrations and pituitary responsiveness to GnRH between Booroola ewes which were homozygous (FF), heterozygous (F+) and non-carriers (++) of a major gene influencing their ovulation rate. *Journal of Reproduction and Fertility* **80** 577–588.

McNatty KP, Hudson NL, Shaw L, Condell LA, Ball K, Seah SL & Clarke IJ 1991 GnRH-induced gonadotrophin secretion in ovariectomized Booroola ewes with hypothalamic-pituitary disconnection. *Journal of Reproduction and Fertility* **91** 583–592.

McNeilly JR, McNeilly AS, Walton JS & Cunningham FJ 1976 Development and application of a heterologous radioimmunoassay for ovine follicle-stimulating hormone. *Journal of Endocrinology* **70** 69–79.

McNeilly AS, Jonassen JA & Fraser HM 1986 Suppression of follicular development after chronic LHRH immunoneutralization in the ewe. *Journal of Reproduction and Fertility* **76** 481–490.

McNeilly AS, Crow W, Brooks J & Evans G 1992 Luteinizing hormone pulses, follicle stimulating hormone and the control of follicle selection. *Journal of Reproduction and Fertility* Suppl **45** 5–18.

McNeilly AS, Crawford JL, Taragnat C, Nicol L & McNeilly JR 2003 The differential secretion of FSH and LH: regulation through genes, feedback and packaging. *Reproduction* Suppl **61** 463–476.

Massague J & Wotton D 2000 Transcriptional control by the TGF-beta/Smad signaling system. *The EMBO journal* **19** 1745–1754.

Matteri RL, Roser JF, Baldwin DM, Lipovetsky V & Papkoff H 1987 Characterization of a monoclonal antibody which detects luteinizing hormone from diverse mammalian species. *Domestic Animal Endocrinology* **4** 157–165.

Mercer JE, Phillips DJ & Clarke IJ 1993 Short-term regulation of gonadotropin subunit mRNA levels by estrogen: studies in the hypothalamo-pituitary intact and hypothalamo-pituitary disconnected ewe. *Journal of Neuroendocrinology* **5** 591–596.

Miller CD & Miller WL 1996 Transcriptional repression of the ovine follicle-stimulating hormone- beta gene by 17 beta-estradiol. *Endocrinology* **137** 3437–3446.

Miyazono K, Kusanagi K & Inoue H 2001 Divergence and convergence of TGF-beta/BMP signaling. *Journal of Cellular Physiology* **187** 265–276.

Molter-Gerard C, Fontaine J, Guerin S & Taragnat C 1999 Differential regulation of the gonadotropin storage pattern by gonadotropin-releasing hormone pulse frequency in the ewe. *Biology of Reproduction* **60** 1224–1230.

Molter-Gerard C, Caraty A, Guerin S, Fontaine J & Taragnat C 2000 Dynamic changes in the gonadotrope cell subpopulations during an estradiol-induced surge in the ewe. *Biology of Reproduction* **63** 1084–1091.

Mulsant P, Lecerf F, Fabre S, Schibler L, Monget P, Lanneluc I, Pisselet C, Riquet J, Monniaux D, Callebaut I, Cribiu E, Thimonier J, Teyssier J, Bodin L, Cognie Y, Chitour N & Elsen JM 2001 Mutation in bone morphogenetic protein receptor-IB is associated with increased ovulation rate in Booroola Merino ewes. *PNAS* **98** 5104–5109.

Otsuka F & Shimasaki S 2002 A novel function of bone morphogenetic protein-15 in the pituitary: selective synthesis and secretion of FSH by gonadotropes. *Endocrinology* **143** 4938–4941.

Otsuka F, Yao Z, Lee T, Yamamoto S, Erickson GF & Shimasaki S 2000 Bone morphogenetic protein-15. Identification of target cells and biological functions. *The Journal of Biological Chemistry* **275** 39523–39528.

Padmanabhan V & McNeilly AS 2001 Is there an FSH-releasing factor? *Reproduction* **121** 21–30.

Paez-Pereda M, Giacomini D, Refojo D, Nagashima AC, Hopfner U, Grubler Y, Chervin A, Goldberg V, Goya R, Hentges ST, Low MJ, Holsboer F, Stalla GK & Arzt E 2003 Involvement of bone morphogenetic protein 4 (BMP-4) in pituitary prolactinoma pathogenesis through a Smad/estrogen receptor crosstalk. *PNAS* **100** 1034–1039.

Phillips CL, Lin LW, Wu JC, Guzman K, Miloted A & Miller WL 1998 17β-estradiol and progesterone inhibit transcription of the genes encoding the subunits of ovine follicle-stimulating hormone. *Molecular Endocrinology* **2** 641–649.

Rosenzweig BL, Imamura T, Okadome T, Cox GN, Yamashita H, ten Dijke P, Heldin CH & Miyazono K 1995 Cloning and characterization of a human type II receptor for bone morphogenetic proteins. *PNAS* **92** 7632–7636.

Sheng C, McNeilly AS & Brooks AN 1998 Distribution of estrogen receptor a and luteinizing hormone β subunit in the ovine pituitary gland during fetal development. *Journal of Neuroendocrinology* **10** 713–718.

Shimasaki S, Zachow RJ, Li D, Kim H, Iemura S, Ueno N, Sampath K, Chang RJ & Erickson GF 1999 A functional bone morphogenetic protein system in the ovary. *PNAS* **96** 7282–7289.

Shimasaki S, Moore RK, Otsuka F & Erickson GF 2004 The bone morphogenetic protein system in mammalian reproduction. *Endocrine Reviews* **25** 72–101.

Souza CJ, MacDougall C, MacDougall C, Campbell BK, McNeilly AS & Baird DT 2001 The Booroola (FecB) phenotype is associated with a mutation in the bone morphogenetic receptor type 1 B (BMPR1B) gene. *Journal of Endocrinology* **169** R1–R6.

Souza CJ, Campbell BK, McNeilly AS & Baird DT 2002 Effect of bone morphogenetic protein 2 (BMP2) on oestradiol and inhibin A production by sheep granulosa cells, and localization of BMP receptors in the ovary by immunohistochemistry. *Reproduction* **123** 363–369.

Souza CJ, Campbell BK, McNeilly AS & Baird DT 2003 Bone morphogenetic proteins and folliculogenesis: lessons from the Booroola mutation. *Reproduction* Suppl **61** 361–370.

Suszko MI, Lo DJ, Suh H, Camper SA & Woodruff TK 2003 Regulation of the rat follicle-stimulating hormone beta-subunit promoter by activin. *Molecular Endocrinology* **17** 318–332.

Takuma N, Sheng HZ, Furuta Y, Ward JM, Sharma K, Hogan BLM, Pfaff SL, Westphal H, Kimura S & Mahon KA 1998 Formation of Rathke's pouch requires dual induction from the diencephalons. *Development* **125** 4835–4840.

ten Dijke P, Yamashita H, Ichijo H, Franzen P, Laiho M, Miyazono K & Heldin CH 1994 Characterization of type I receptors for transforming growth factor-beta and activin. *Science* **264** 101–104.

Tobin VA, Pompolo S & Clarke IJ 2001 The percentage of pituitary gonadotropes with immunoreactive oestradiol receptors increases in the follicular phase of the ovine oestrous cycle. *Journal of Neuroendocrinology* **13** 846–854.

Welt C, Sidis Y, Keutmann H & Schneyer A 2002 Activins, Inhibins, and Follistatins: from endocrinology to signaling. A paradigm for the new millennium. *Experimental Biology and Medecine (Maywood)* **227** 724–752.

Wilson T, Wu XY, Juengel JL, Ross IK, Lumsden JM, Lord EA, Dodds KG, Walling GA, McEwan JC, O'Connell AR, McNatty KP & Montgomery GW 2001 Highly prolific Booroola sheep have a mutation in the intracellular kinase domain of bone morphogenetic protein IB receptor (ALK-6) that is expressed in both oocytes and granulosa cells. *Biology of Reproduction* **64** 1225–1235.

Wu L, Wu Y, Gathings B, Wan M, Li X, Grizzle W, Liu Z, Lu C, Mao Z & Cao X 2003 Smad4 as a transcription corepressor for estrogen receptor α. *Journal of Biological Chemistry* **278** 15192–15200.

Yamamoto T, Saatcioglu F & Matsuda T 2002 Cross-talk between bone morphogenetic proteins and estrogen receptor signaling. *Endocrinology* **143** 2635–2642.

Yamashita H, ten Dijke P, Huylebroeck D, Sampath TK, Andries M, Smith JC, Heldin CH & Miyazono K 1995 Osteogenic protein-1 binds to activin type II receptors and induces certain activin-like effects. *The Journal of Cell Biology* **130** 217–226.

Yan C, Wang P, DeMayo J, DeMayo FJ, Elvin JA, Carino C, Prasad SV, Skinner SS, Dunbar BS, Dube JL, Celeste AJ & Matzuk MM 2001 Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. *Molecular Endocrinology* **15** 854–866.

Zwijsen A, Verschueren K & Huylebroeck D 2003 New intracellular components of bone morphogenetic protein/Smad signaling cascades. *FEBS letters* **546** 133–139.

Received 29 March 2005
Accepted 8 April 2005
Made available online as an Accepted Preprint
21 April 2005

Discussion

Our results showed for the first time that BMP-4 and BMP-6 were able to inhibit FSH, but not LH, synthesis and release in ewes. These data contrast with those obtained in the mouse gonadotrope cell line $L\beta T_2$ in which BMP-6, BMP-7 and BMP-15 exert stimulatory effect on FSH synthesis and release (Huang *et al.* 2001, Otsuka & Shimasaki 2002).This difference of BMP effect could be due to the species or to the BMP chosen. Therefore, we tested two other available BMPs. BMP-15 was chosen for its known action in murine $L\beta T_2$ gonadotropes and rat primary cell culture. GDF-9 represents a potential functional factor since it is expressed in ewe pituitary. BMP-15 and GDF-9 inhibited FSH release compared to control by a 40-50% at maximal doses (10^{-9} M (50 ng/ml) and $6x10^{-9}$ M (200ng/ml) respectively (Fig. 23)). Hence, all BMPs tested in ewe pituitary cell cultures have a similar inhibitory effect. The difference observed between ewe pituitary cells and mouse gonadotropes can rely on species difference and/or on culture conditions. In our study, BMPs were added to the serum-free medium for 48h whereas in $L\beta T_2$, medium contained 1% FCS potentially rich in growth factors. Further experiments comparing both rodent and ovine species will help to understand the opposite effect on FSH release between both species.

To test whether other hormone secretion could be affected by BMPs, we assayed prolactin hormone concentrations in ewe pituitary cells. Indeed, in human prolactinomas, BMP-4 was shown to stimulate cell proliferation (Paez-Pareda *et al.* 2003). After treatment with 10^{-9}M BMP-4 (50ng/ml) for 48h, no significant difference was found on prolactin secretion comparing to the control conditions (Fig. 24).

Our results also showed that certain BMPs, including BMP-4, are expressed in the pituitary. Together with the presence of BMP receptors on gonadotrope cells, these data suggest a paracrine role of BMPs. To test this hypothesis, ewe pituitary cells were treated for 48h with noggin, a BMP-2/BMP-4 inhibitor which physically interacts with them (Zimmerman *et al.* 1996). Very variable results were obtained but the overall effect is the absence of modification of FSH release. This suggests that BMP-2/BMP-4 would not act as paracrine factors on FSH production (Fig. 25). Nevertheless, the loss of BMP-2/BMP-4 could be compensated by other BMPs as BMP-6, BMP-7 and GDF-9 also expressed in the ewe pituitary and able to inhibit FSH release. These BMPs do not seem to act synergically since the addition of both 10^{-9}M BMP-4 and 2.10^{-9}M BMP-6 in the pituitary cell culture showed the same inhibition than either factor alone (Fig. 26). Rather, compensating effects are possible.

Various examples illustrate BMP compensation. For instance, BMP-5 or BMP-7 null mice present no major defect whereas BMP-5/BMP-7 double mutant embryos do not survive (Solloway & Robertson 1999).

BMP-4 was described to play a role with other factors modulating specifically FSH secretion. Activin, ovarian and pituitary factor stimulating FSH secretion, was antagonized by BMP-4 effect and the gonadal steroid oestradiol inhibitory effect was amplified by BMP-4. Besides these factors, the hypothalamic GnRH is known to be an important modulator of gonadotropins secretion as hypothalamo-pituitary disconnection or passive immunization against GnRH provokes the decrease of FSH and LH release (Clarke *et al.* 1983, Caraty *et al.* 1984). Considering BMP-4 inhibitory effect on FSH secretion, we tried to determine its putative interaction with GnRH effect. Nevertheless, GnRH stimulatory effect was difficult to find in our conditions. Only two experiments showing both GnRH and BMP-4 effects were available. In these experiments, the addition of 10^{-8}M GnRH the last three hours of 48h of treatment by 10^{-9}M BMP-4 showed that BMP-4 effect was able to antagonise GnRH effect (Fig. 27). GnRH and BMP pathways could interact as they both can transduce via Ca2+ and MAPK/Erk signaling pathways. Whether BMPs affect GnRH-R mRNA expression in ewe pituitary is not known. In rodent, BMP-15 does not affect GnRH-R mRNA expression (Otsuka & Shimasaki 2002). However, as said previously, rodent and ovine species respond differentially to BMPs. Further studies could explore these fields after having ameliorated our protocol to obtain consistent stimulatory effect of GnRH. We can suggest using a GnRH pulsatile treatment. To favour FSH secretion (Dalkin *et al.* 1989, Molter-Gerard *et al.* 1999, Farnworth 2000, Burger *et al.* 2002), low pulse frequencies could be applied by pituitary cell perifusion system. Alternatively, plated cells could be treated by one GnRH pulse per day following the same protocol as described in Nicol and coworkers (2002).

The inhibitory effect of BMPs on FSH secretion was found on three breeds of sheep: Ile de France, Scottish black face and Merinos. In Merino's Booroola family, a mutation in the BMP receptor type IB is associated with the hyperprolificacy of these ewes (Mulsant *et al.* 2001, Souza *et al.* 2001, Wilson *et al.* 2001). At pituitary level, the effect of the mutation is not elucidated. In some studies, higher FSH concentrations were found in presence of the mutation compared to the wild type (WT) animals (Bindon *et al.* 1984, McNatty *et al.* 1987) while in others no difference was detected between the two genotypes (Souza *et al.* 1997). In our study, BMP-4 inhibited FSH release in both genotypes (WT and FecB/FecB) with the

same 40-50% inhibition (Fig. 28). The lack of difference between both genotypes is consistent with the absence of BMPR-IB on gonadotrope cells (Faure *et al.* 2005). However, it is reasonable to think that the BMPR-IB mutation could affect another cell type since the BMPR-IB mRNA was detected in the ovine pituitary. On going experiments try to colocalise this receptor with another pituitary cell type and to determine whether other hormone levels are affected.

In conclusion, BMP-4, BMP-6, GDF-9 and BMP-15 inhibited FSH release but not LH nor prolactin from ewe pituitary cells. BMP-4 was described as a new modulator of FSH secretion and capable to interact with activin, oestradiol and GnRH. Moreover, consistent with the absence of BMPR-IB on gonadotrope cells, no difference was observed in the FSH response to BMP-4 between Booroola genotypes.

Altogether, these findings provide evidence for a role of BMP system in the regulation of FSH production in the ewe and their ability to interplay with main FSH modulators.

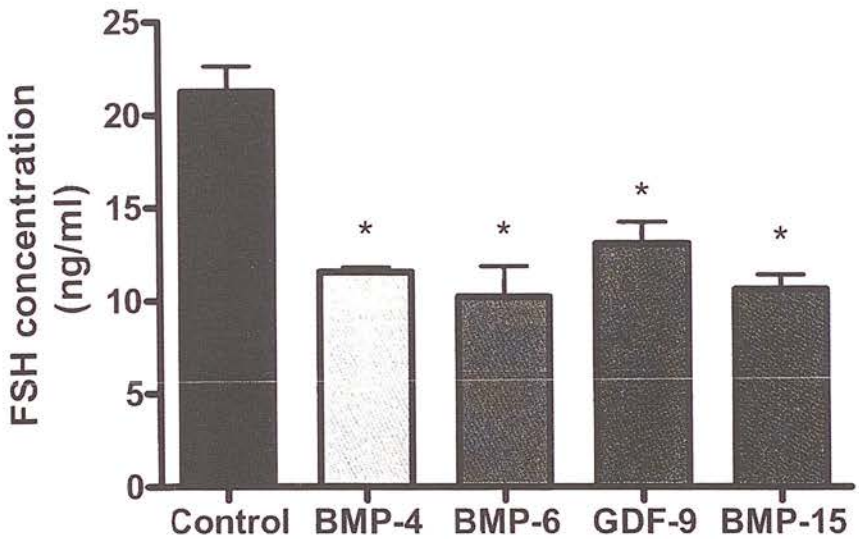


Fig23. **Effect of BMP-4, BMP-6, GDF-9 and BMP-15 on FSH release from ewe pituitary cells.** Cells were cultured in serum-free medium for 48h with 10^{-9} M BMP-4, 2.10^{-9} M BMP-6, 10^{-9} M GDF-9 (kindly given by L. Persani (Milano University)) or 6.10^{-9} M BMP-15 (generously provided by A. Hsueh (Standford University)). Media were collected and FSH concentration was determined by ELISA. Values are the mean ± S.E.M. from one representative experiment with triplicate determination. Three experiments were performed with similar results. a vs b, $P<0.01$.

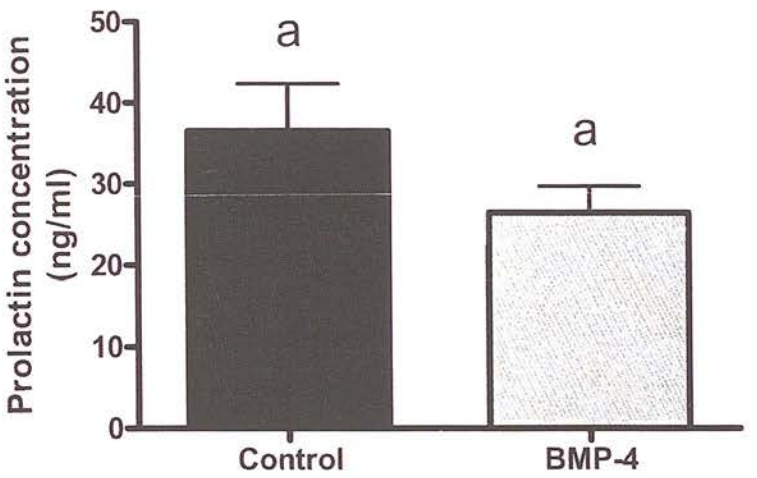


Fig.24. **Effect of BMP-4 on prolactin release in ewe pituitary cells.** Cells were cultured in serum-free medium alone (Control) or in presence of 10^{-9} M BMP-4 (50ng/ml) for 48h. Media were collected and prolactin concentrations were determined by radioimmunological assay as previously described (Kann 1971). Values are the mean ± S.E.M. from five experiments.

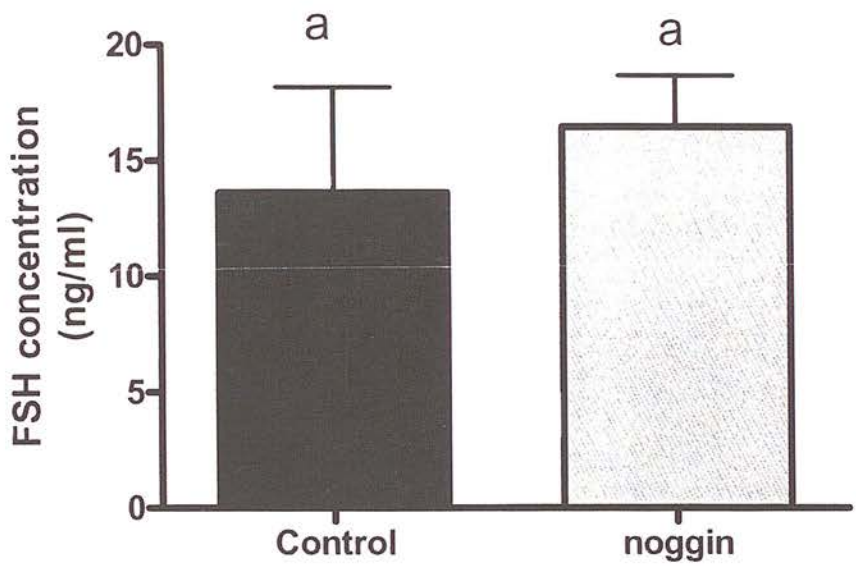


Fig. 25. **Effect of noggin on FSH release from ewe pituitary cells.** Cells were cultured in serum-free medium for 48h with 4.10^{-9} M noggin (100ng/ml) a gift from Regeneron pharmaceuticals, Inc (Tarryton, NY, USA). Media were collected and FSH concentration was determined by ELISA. Values are the mean ± S.E.M. from three experiments with triplicate determination.

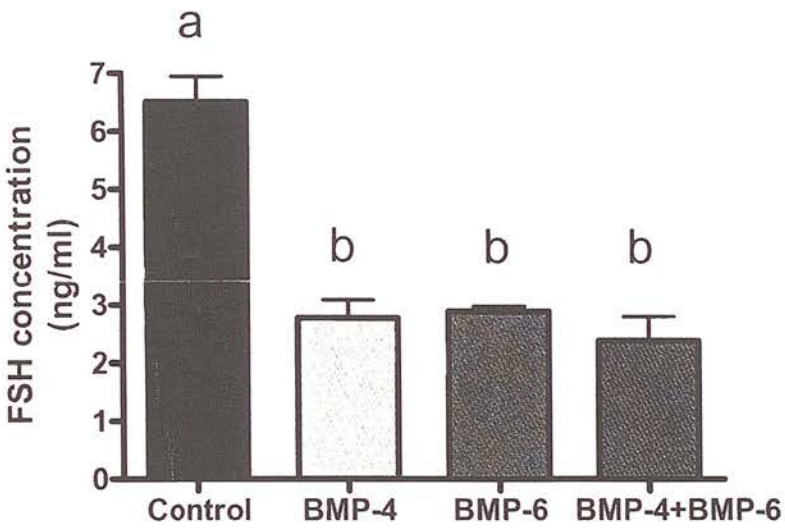


Fig.26. **Interaction between BMP-4 and BMP-6 on FSH release from ewe pituitary cells.** Cells were cultured in serum-free medium for 48h in presence of 10^{-9} M BMP-4 (50ng/ml) and/or 2.10^{-9} M BMP-6 (100ng/ml). Media were collected and FSH concentrations were determined by ELISA. Values are the mean ± S.E.M. from one representative experiment with triplicate determination. Three experiments were performed with similar results. a vs b, $P<0.001$.

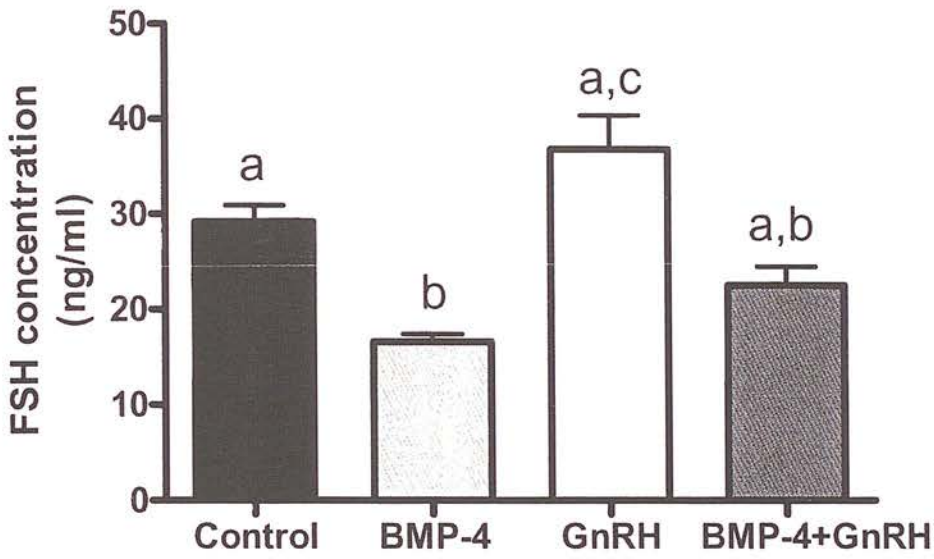
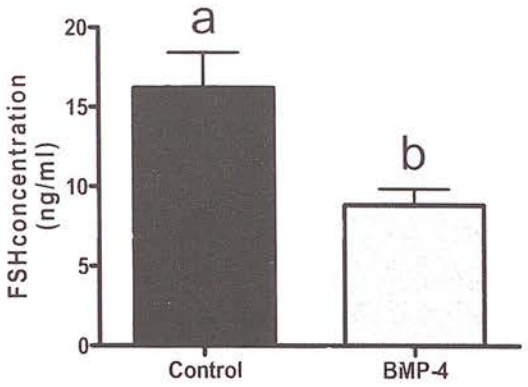


Fig.27. **Interaction between BMP-4 and GnRH on FSH release from ewe pituitary cells.** Cells were cultured in serum-free medium for 48h with or without 10^{-9} M BMP-4 (35ng/ml). GnRH (10^{-8}M) from Ferring (Gentilly, France) was added three hours before the end of 48h treatment. Media were collected at 48h and FSH concentrations were determined by ELISA. Values are the mean ± S.E.M. from one representative experiment with triplicate determination. Two experiments were performed with similar results.

A



B

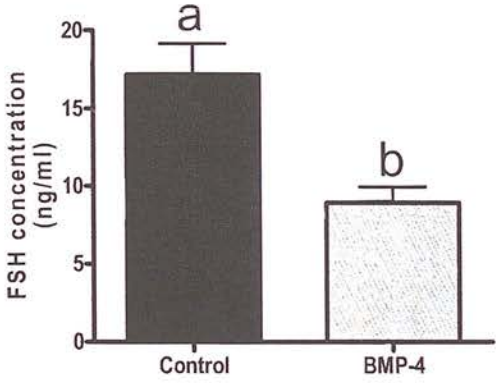


Fig. 28. **Effect of BMP-4 on FSH release from wild type (WT) and FecB/FecB genotypes ewe pituitary cells**. Cells were cultured in serum-free medium alone (Control) or in presence of 10^{-9} M BMP-4 (50ng/ml) for 48h. Media were collected and FSH concentrations were determined by ELISA. Values are the mean ± S.E.M. from five experiments. a vs b, $P<0.01$.

Chapter II:

Does BMP-4 interact with activin signaling pathway for modulating FSH secretion?

A. Introduction

In the previous chapter, BMP-4 was shown to antagonize the stimulatory effect of activin on FSH synthesis and release in sheep pituitary *in vitro* (Faure *et al.* 2005). Activins, BMP mRNAs and their receptors are present in the pituitary suggesting paracrine actions (Meunier *et al.* 1988, for review Knight 1996, Cameron *et al.* 1998, Shimasaki *et al.* 1999, Fafioffe *et al.* 2004). Activin binds to a homodimeric complex of type II receptors (ActRII or ActRIIB). Then, the complex recruits a homodimer of type I receptors (ALK-2/ActRI, ALK-4/ActRIB) before inducing the signaling pathway (ten Dijke *et al.* 1994a, Wrana *et al.* 1994). BMP signaling occurs through a preformed heteromeric complex with a type BMPR-IA (ALK-3) or BMPR-IB (ALK-6) and type BMPR-II serine/threonine kinase receptors (Kawabata *et al.* 1998, Miyazono *et al.* 2001). In both signaling, once the ligand is associated with the receptor complex, the type II receptor trans-phosphorylates the type I receptor. Consequently, the activated type I receptor phosphorylates cytoplasmic proteins called receptor-activated Smads (R-Smads) which interact with a co-Smad, Smad-4. Activin signaling recruits the specific R-Smads, Smad-2 and Smad-3 while BMP signaling acts through Smad-1, Smad-5 and Smad-8. In ovine pituitary cells, the activation of the Smad-2 signaling pathway by activin was previously shown (Dupont *et al.* 2003). The R-Smad-co-Smad complex translocates into the nucleus to promote specific gene expression (Massagué & Wotton 2000, for review: Zwijsen *et al.* 2003).

To counteract the positive effect of activin, follistatin can combine with activin, producing a complex which is unable to bind the activin receptors (Nakamura *et al.* 1990, de Winter *et al.* 1996). Moreover, follistatin has also been described as a BMP antagonist (Fainsod *et al.* 1997, Iemura *et al.* 1998). Activin and BMPs signaling pathways can be inhibited by Smad-7, an inhibitory smad, which able to bind BMP and TGFβ/activin receptors in aim to decrease R-Smads phosphorylation inhibiting their signaling pathways (Zimmerman *et al.* 1996, Nakao *et al.* 1997a, Topper *et al.* 1997, Casellas & Brivanlou 1998, Souchelnytskyi *et al.* 1998, Ishisaki *et al.* 1999). A specific inhibitory Smad, Smad-6, inhibits BMP signaling by preventing the interaction between the phosphorylated form of Smad1 and Smad-4 (Hata *et al.* 1998).

In ovine pituitary cells, BMP-4 prevents the stimulatory effect of exogenous activin on FSH secretion. Besides, activin produced by the pituitary, is known to activate its own

pathway. No data are available on the interaction of BMP-4 and activin. In this chapter, we investigated whether BMP-4 triggers its own signaling pathway and/or reduces the activin signaling pathway.

B. Material & Methods

Reagents

Rabbit polyclonal antibodies against Smad1 (cat# 06-653, lot 22953) and against Smad-2/3 (cat# 07-408, lot 24340) proteins were from Upstate Biotechnology (Lake Placid, NY, USA). Rabbit polyclonal antibody against Smad-2-phosphospecific (ser 465/467) protein (cat# 566415, lot D20646) was from Calbiochem (Merck Biosciences LTD., Nottingham, UK). Rabbit polyclonal antibody against Smad1/5-phosphospecific was kindly given by Professor Carl Heldin, Luwig Institute, Stockholm, Sweden (Persson *et al.* 1998). Rabbit polyclonal antibodies against human Smad-6 and Smad-7 were from TCS Cellworks (Botolph Claydon, Buckingham, UK).

Smad-6 and -7 adenoviruses were a gift from Dr. Aristidis Moustakas (Ludwig Institute for Cancer Research, Uppsala, Sweden).

Human recombinant follistatin-288 was from R&D systems (Minneapolis, USA).

Sheep pituitary cell cultures

Pituitary cell cultures were performed as previously described (Faure *et al.* 2005). After allowing cells to attach, media were replaced with serum-free DMEM containing 0.1% BSA. One hour later, the medium was changed and DMEM–0.1% BSA alone (control) or supplemented with test substances (see *results*). Media were collected 48h later to assay for FSH and LH (Faure *et al.* 2005).

Western blotting analysis of Smad proteins

To investigate Smad activation by BMP-4 or/and activin, ewe pituitary cells were cultured at 2.10^6 cells/well in 6-well plates for 48h. Media were changed to remove nonattached cells and replaced by fresh media containing serum-free DMEM - 0.1% BSA alone or supplemented with 10^{-9} M (35ng/ml) of BMP-4 or/and activin (50ng/ml) for 30, 60 and 90 min. Cells were then washed once on ice with cold PBS and lysed in lysis buffer (150mM NaCl, 10mM Tris pH 7,4, 1mM EDTA, EGTA 1mM, Triton X-100 1%, Igepal CA. 630 0.5%, 100mM Sodium fluoride, 10mM Sodium pyrophosphate, 10mM Sodium

orthovanadate, proteases inhibitor cocktail). The concentrations of the proteins were determined with BC Assay Protein Quantitation kit (Interchim, Montluçon, France). After 1min at 95°C in the reducing SDS-PAGE buffer containing β-mercaptoethanol, 10µg of proteins were separated in 10% SDS-PAGE gels and electroblotted onto Immobilon-P membranes (Millipore corporation, Bedford, MA). Membranes were blocked for 1 h at room temperature in Tris-buffered saline-0.1% Tween (TBST) containing 5% fat-free dry milk and incubated with the anti-Smad1, anti-Smad-2/3, or anti-Smad-2 phosphospecific antibodies diluted at 1:1000 in TBST 1%BSA 5% milk for 1h at RT. After washing, the membranes were incubated with the secondary antibody, a peroxidase-conjugated anti-IgG (Biorad, Marnes la Coquette, France) diluted 1:15000 for 1 h. Immunoreactive proteins were detected using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Orsay, France). Membranes were exposed to Hyperfilm ECL (Amersham Pharmacia Biotech) for 20sec. The same protocol was followed for anti-Smad1/5 phosphospecific antibody except the blocking buffer was 1% BSA in TBST and the antibody was diluted in 1:5000 in 1% BSA TBST.

Reverse transcription

Total RNA from 6-well plates were extracted using Agilent Total RNA Isolation Mini Kit. RNA concentration was measured using RNA 6000 Nano Assay (Agilent Technologies, Stockport, UK,). Aliquots of total RNA were treated with DNase I using DNA-free (Ambion Ltd, Huntingdon, Cambs, UK) according to manufacturer's protocol. Samples were then reverse transcribed, using oligod(T)16, with Taqman Reverse Transcription Reagents (PE Biosystems) according to the manufacturer's protocol, using a program of 10min at 25°C, 30min at 48°C and 5min at 95°C.

Quantitative RT-PCR for Smad-4, Smad-7on pituitary cell cultures

Smad-4 and Smad-7 primers sequences (Smad-4-forward: TCCTCATgTgATCTATgCCC, Smad-4-reverse: TgCAgTgTTAATCCTgAgAg, Smad-7-forward: ATCTTCATCAAgTCCgCCaC, Smad-7-reverse: CTTgACTTCCgAggAATgCC) were synthesized by MWG Biotech AG (Ebersberg, Germany). GapdH sequences were gAC ATC AAg AAg gTg gTg AAg C (forward) and gTC CAC CAC CCT gTT gCT gTA g (reverse). DNase-treated RNA was added at 25-50ng/µl. For PCR, a reaction mix was prepared consisting of Quantitect SYBRGeen Master Mix (1X), Smad-4 or Smad-7 forward and reverse primers (500nM each). A control reaction was done with GapdH forward and

reverse primers (500nM each). This was aliquoted for each sample in duplicate in separated capillaries, then cDNA was added at 1µl/10µl reaction mix. Capillaries were spun for 10sec at 800g. PCR reaction was run on a Lightcycler machine (Roche Diagnostics Ltd, UK) using standard conditions. Controls included cDNA prepared without Multiscribe reverse transcriptase enzyme to check for efficiency of DNAse treatment. Prior to analysis, a validation assay was performed to demonstrate that amplification of the gene of interest and the reference (GapdH) were approximately equal and the fusion curve showed a single surge corresponding to a single PCR product. After the run, capillaries were spun upside down in eppendorf tubes for 10sec at 800g. The PCR product was run on an agarose gel to confirm amplification of a single product of the correct size.

LacZ, Smad-6 and Smad-7 adenoviruses infection of sheep pituitary cells

Viruses were propagated in HEK 293 cells. Cells were cultured on matrigel (1:29) coated plates in MEM with Earles salts, Glutamax I, 25 mM HEPES and 10% FCS. Just before infection (cells approx. 60% confluent) medium was changed to 5% FCS for 1h (5 ml med. per 10 cm plate), then virus (from a 6th seed stock) added (35µl per plate) and incubation continued until a cytopathic effect was apparent (practically 100% cell detachment). Cells/medium were collected and virus released by 5 cycles of freeze/thaw (dry ice-EtOH/37°C bath). Cell debris collected by spinning at 3K for 10 min. Supernatants were aliquoted into eppendorfs and stored at -70 °C.

Viral titre of the working stock (7th seed) was assessed using Rapid Titre kit (BD Biosciences Clontech,Cowley, Oxford, UK). Integrity of virus preps was assessed using restriction analysis of the DNA prep and by western blotting. The Smad 6/ and 7 proteins are Flag-tagged and were detected using a rabbit anti-Flag polyclonal antibody (Sigma). To infect sheep pituitary cells with Smad-6 or Smad-7 adenoviruses, cells were cultured in DMEM+5% FOS in 6-well plates for 24h to allow them to attach. After a wash, media were replaced with OptiMEM (Gibco (Invitrogen), Paisley, UK) alone or supplemented with the adenovirus diluted at 10pfu/cell, 50pfu/cell or 100pfu/cell for 2h. Then, cells were washed and DMEM+10% FOS were added for 4h. To eliminate any trace of serum in the culture, media were replaced with serum-free DMEM containing 0.1% BSA. One hour later, the medium was changed and DMEM–0.1% BSA alone (control) or supplemented with BMP-4 (35ng/ml) or activin (50ng/ml) was added. Media were collected 48h later to assay for FSH and LH

concentrations and cells were used for quantitative RT-PCR for FSHβ and LHβ (Faure *et al.* 2005).

LacZ staining by β-galactosidase

To check adenovirus infection efficiency, ewe pituitary cells infected with 100 pfu/ cell LacZ were fixed in PBS containing 2% Formaldehyde + 0.2% Glutaraldehyde for 5min. After a PBS wash, cells were stained in PBS containing 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$ and β-galactosidase for 2h at 37°C. After PBS wash, slides were examined using LSN 510 meta-confocal microscope.

Smad-6 and Smad-7 immunohistochemistry

After formaldehyde fixation of pituitary cells, permeabilisation with 0.2% NP40, 1% BSA, 10% NGS in PBS for 20min was performed. After a wash in water, the slides were incubated in a hydrogen peroxyde/methanol block for 30min. After two 5min-washes in TBS, a combined avidin-biotin block was performed according to the manufacturer's instructions (Vector Laboratories, Peterborough, UK). Cells were incubated at 4°C overnight with the monoclonal anti- Smad 6 or Smad-7 diluted 1:200 in TBS containing 20% normal goat serum and 5% BSA (TBS/NGS/BSA). Negative controls were performed by replacing the first antibody with normal goat serum. After two 5min-washes in TBS, slides were incubated with goat anti-rabbit biotinylated antibody (Dako, Cambridge, UK) diluted 1:500 for 30 min. After two 5min-washes in TBS, slides were blocked for 30min with ABC complex/HRP according to the manufacturer's instructions (Dako, Cambridge, UK). The slides were revealed by DAB staining and examined using LSN 510 meta-confocal microscope.

Statistical analysis

Results are reported as mean ± S.E.M. All experiments were performed in triplicates except the adenovirus experiments which were performed in duplicate in at least three different experiments. For each figure, the number of experiments shown is indicated in the legend. Comparison between BMP-4, activin, BMP-4+activin and control groups on Smad-4 and Smad-7 mRNA expression levels were analysed by one-way ANOVA followed by Newman-Keuls post-test. Comparison between increasing doses of follistatin supplemented or not with BMP-4 was analysed with a two-way ANOVA followed by unpaired *t*-test. $P<0.05$ was considered significant. Comparison between increasing doses of adenovirus and between

BMP-4 treatment and control groups on oFSHβ, oLHβ mRNA expression and FSH and LH concentrations were analysed with a two-way ANOVA followed followed by Newman-Keuls post-test. For each dose of follistatin, comparison between BMP-4 treatment and control groups were analysed by unpaired *t*-test. The statistical analysis was performed using the GraphPad Prism version 4.00 program for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

C. Results

I. Is Smad pathway used by BMP-4 for inhibiting FSH secretion?

1. BMP-4 stimulates Smad1 phosphorylation

To determine whether BMP-4 could signal via Smad1 signaling pathway, ewe pituitary cells were treated for 1h with 10^{-9}M BMP-4 (35ng/ml). After protein extraction, the activation of Smad1/5 was followed by western blot analysis using a rabbit polyclonal antibody against the phosphorylated form Smad1/5. Figure 1 shows that BMP-4 induced the appearance of a 66 kDa band. In control samples harvested at the same time points, no phospho Smad-1 immunoreactivity was detected. For non-phosphorylated Smad-1protein, the intensity of the band did not change between control and BMP treatment (Fig. 29).

2. Smad-6, BMP specific signaling inhibitor, does not affect the inhibited FSH secretion by BMP-4

To test whether the phosphorylation of Smad-1 is responsible for the inhibition of FSH secretion by BMP-4, Smad-6, BMP specific signaling inhibitor, was overexpressed by adenoviral infection in ewe pituitary cells. Preliminary experiments showed that 90% and 80% of cells expressed LacZ and Smad-6 respectively when adenoviruses were used at dose 100pfu/cell (Fig. 30A-B). When cells were infected with Smad-6 adenovirus, a slight decrease on FSH secretion (around 30%) was observed similar to those with LacZ adenovirus (Fig. 31). However, the inhibition of oFSHβ mRNA expression and FSH release by BMP-4 treatment were not reversed in presence of Smad-6 adenovirus (Fig. 31).

3. Smad-7, TGFβ signaling inhibitor, blocks the inhibited FSH secretion by BMP-4

Meanwhile, Smad-7, TGFβ signaling inhibitor, was overexpressed in ewe pituitary cells by adenoviral infection. An immunohistochemistry using a monoclonal antibody against

Smad-7 revealed that at 100pfu/cell, 80% of the cells were infected (Fig. 32A). Moreover, the overexpression of Smad-7 dose-dependently reduced the phosphorylated form of Smad-1 level (Fig. 32B). Interestingly, the inhibition of oFSHβ mRNA expression and FSH release by BMP-4 treatment was reversed when Smad-7 was overexpressed from dose 10pfu/cell and 50pfu/cell respectively in ewe pituitary cells (Fig. 33).

However, in basal conditions, the Smad-7 adenovirus infection reduced drastically oFSHβ mRNA expression and medium FSH concentrations in a dose-dependent manner up to 85% (Fig. 33). In contrast, LH release was not affected (Fig. 34). The inhibition observed for FSH was specific to Smad-7 adenovirus since infection of pituitary cells with LacZ adenovirus (100pfu/cells) only decreased control FSHβ mRNA expression to 33% and FSH release to 33% (Fig. 31). This result corroborated the presence of an endogenous FSH specific stimulatory factor which was also blocked by Smad-7 overexpression. It is well established that activin acts as endogenous factor using Smad-2/Smad-3 signaling pathway. Indeed, the phosphorylated form of Smad-2 was detected in control conditions (Fig. 35A). The overexpression of Smad-7 reduced in a dose dependent manner the level of Smad-2 phosphorylated form confirming our hypothesis (Fig. 35A). This inhibition is associated to a lack of stimulatory effect of exogenous activin on FSHβ mRNA expression (Fig. 35B). However, activin-stimulated FSH release was not completely blocked by Smad-7 overexpression (data not shown).

II. Does BMP-4 modulate the activin signaling pathway to inhibit FSH secretion?

The overexpression of Smad-7 affected both BMP and activin signaling pathways. To discern whether the preferential signaling pathway used by BMP-4 to inhibit FSH secretion, the activin signaling pathway was specifically blocked using follistatin.

1. BMP-4 still inhibited the FSH secretion when activin signaling pathway is blocked by follistatin

Previous results in our lab showed that the level of phosphorylated form of Smad-2 was suppressed when ewe pituitary cells were treated with follistatin for 24h (Dupont *et al.* 2003). To determine if BMP-4 inhibited-FSH secretion was dependent on the Smad-2/Smad-3 signaling pathway, ewe pituitary cells were simultaneously treated by BMP-4 and follistatin. Follistatin inhibited the FSH secretion from pituitary cells in a dose-dependent manner (Fig.

36). When cells were incubated with both BMP-4 (10^{-9} M) and follistatin (0.03 to 1.3 10^{-9}M), a stronger inhibition of FSH release was observed compared to either factor alone ($p<0.01$, Fig. 36).

2. BMP-4 does not affect the intensity of the phosphorylated form of Smad-2

To determine whether BMP-4 could affect Smad-2 phosphorylation, ewe pituitary cells were treated for 1h with fresh medium supplemented with 10^{-9}M BMP-4, 2. 10^{-9}M activin or both factors. In presence of both treatment, no decrease in the intensity of the 60-65 kDa band corresponding to the phosphorylated form of Smad-2 was noticeable compared to either factor alone (Fig. 37A).

When cells were pretreated for 24h with 1.3 10^{-9}M follistatin (100ng/ml) in order to reduce the endogenous activation of Smad-2, exogenous activin induced the appearance of Smad-2 phosphorylated band. BMP-4 did not modify the intensity of the band compared to control. When cells were treated with both BMP-4 and activin, the intensity of the phosphorylated form of Smad-2 was not different compared to activin alone (Fig. 37B).

III. Does BMP-4 activate downstream common factors of both BMP and activin signaling pathways?

1. BMP-4 does not affect Smad-4 mRNA expression, a cofactor required for both BMPs and activin signaling pathways

To test whether BMP-4 could modify Smad-4 mRNA expression, ewe pituitary cells were treated with 10^{-9} M BMP-4, 2.10^{-9} M activin or both factors. There was no significant difference between the control and each factor alone or simultaneously added into the medium (Fig. 38).

2. BMP-4 stimulates Smad-7 mRNA expression, the TGFβ signaling pathway inhibitor

To determine whether BMP signaling could interfere with the activin pathway modulating the expression level of inhibitors, Smad-7 mRNA expression level was analysed from cells treated with 10^{-9} M BMP-4, 2.10^{-9} M activin or both factors. When cells were treated with activin alone, no significant effect was detected compared to the control. When

cells were incubated with BMP-4 alone or simultaneously added with activin, a 2-fold stimulation was observed ($p<0.05$ vs control, Fig. 39).

D. Discussion

Gonadal and pituitary factors like activins and inhibins are known for differentially modulating the gonadotropin secretion. Furthermore, BMP-4 and BMP-6 were recently shown to inhibit FSH production in sheep (Faure *et al.* 2005). In this study, we investigated the signaling pathway triggered by BMP-4 in ewe pituitary cells.

In ewe pituitary cell cultures, the treatment with exogenous BMP-4 led to the phosphorylation of Smad1/5. To determine whether the activation of Smad1/5 by BMP-4 was required for the oFSHβ mRNA expression and FSH release inhibition, inhibitory Smads were overexpressed to block the signaling pathway. Preliminary experiment showed that ewe pituitary cells were infected successfully with adenoviruses as demonstrated by the expression of the Smad-6 and Smad-7 in more than 80% of cells. In our conditions, the Smad-6 overexpression did not block the inhibitory effect of BMP-4 on FSH secretion. A possible explanation is that Smad-1 signaling pathway is not responsible for the inhibitory effect of BMP-4. However, the absence of interaction between Smad-1 phosphorylated form and Smad-4 has to be checked in order to determine whether Smad-6 is functional in our system.

The overexpression of the TGFβ signaling inhibitor, Smad-7, decreased dose dependently the level of the Smad-1 phosphorylated form. Moreover, the Smad-7 but not LacZ (not shown) overexpression blocked the inhibitory effect of BMP-4 on FSH secretion suggesting the involvement of Smads in BMP-4 action. However, in these conditions, the basal FSH concentrations were decreased by the Smad-7 overerexpression suggesting that Smad-7 has also blocked an endogenous stimulatory signaling pathway. Smad-7 is known to block the Smad-2/Smad3 signaling pathway. Indeed, the Smad-2 phosphorylated form level was decreased by the Smad-7 overexpression. As endogenous activin signaling pathway act through Smad-2/Smad-3 signaling pathway (Dupont et al. 2003, Suszko *et al.* 2003, 2005, Bernard *et al.* 2004), we can hypothesize that Smad-7 has blocked both BMP-4 and activin signaling pathways.

To determine whether BMP-4 effect on FSH secretion interacts with activin signaling pathway, we blocked specifically the activin action by treating the ewe pituitary cells with

follistatin. Follistatin blocked the endogenous phosphorylation of Smad-2 and decreased in a dose-dependent manner the FSH production corroborating the results of Ying *et al.* (1987). Moreover, the co-treatment with BMP-4 showed a further decrease in FSH release suggesting that activin and BMP-4 act independently on FSH secretion. In addition, the phosphorylation of Smad-2 triggered by activin was not altered by BMP-4 treatment. Downstream common factors of BMP and activin signaling pathways, Smad-4 and Smad-7, mRNA expression were also examined after BMP-4 treatment. Smad-7, but not Smad-4 mRNA expression was stimulated by BMP-4 treatment in the ewe pituitary cells. Neither BMP-4 nor activin alone or combined modified Smad-4 mRNA expression level. Surprisingly, BMP-4 but not activin was able to stimulate Smad-7 mRNA expression in the ewe pituitary cells. This result was contradictory to a previous report (Bilezikjian *et al.* 2001) which showed that activin stimulated Smad-7 expression and that follistatin blocked this induction in rat pituitary cells. In our conditions, it is possible that endogenous activin contributed to the elevated levels of Smad-7 mRNA in control preventing further stimulation by exogenous activin. Alternatively, this difference could be due to the species. Altogether, our results suggest that BMP-4 can regulate by feedback loop both activin and BMP signaling pathways keeping the balance between the two effects in the ewe pituitary cells.

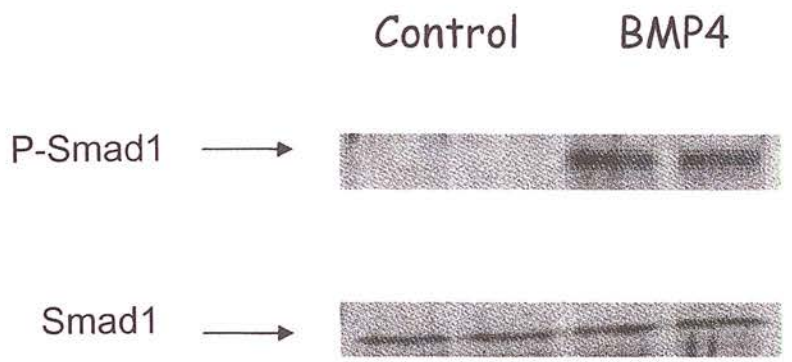


Fig. 29. **BMP-4 effect on Smad1 phosphorylation**. Pituitary cells were cultured in serum-free medium in presence or absence (control) of 10^{-9} M BMP-4 (50ng/ml) for 1h. Total proteins (10µg) were separated on 10% SDS-PAGE and transferred on PVDF membrane. Blots were probed with phospho-Smad1 (upper) and Smad1 (lower) antibodies. Bands were revealed by ECL plus.

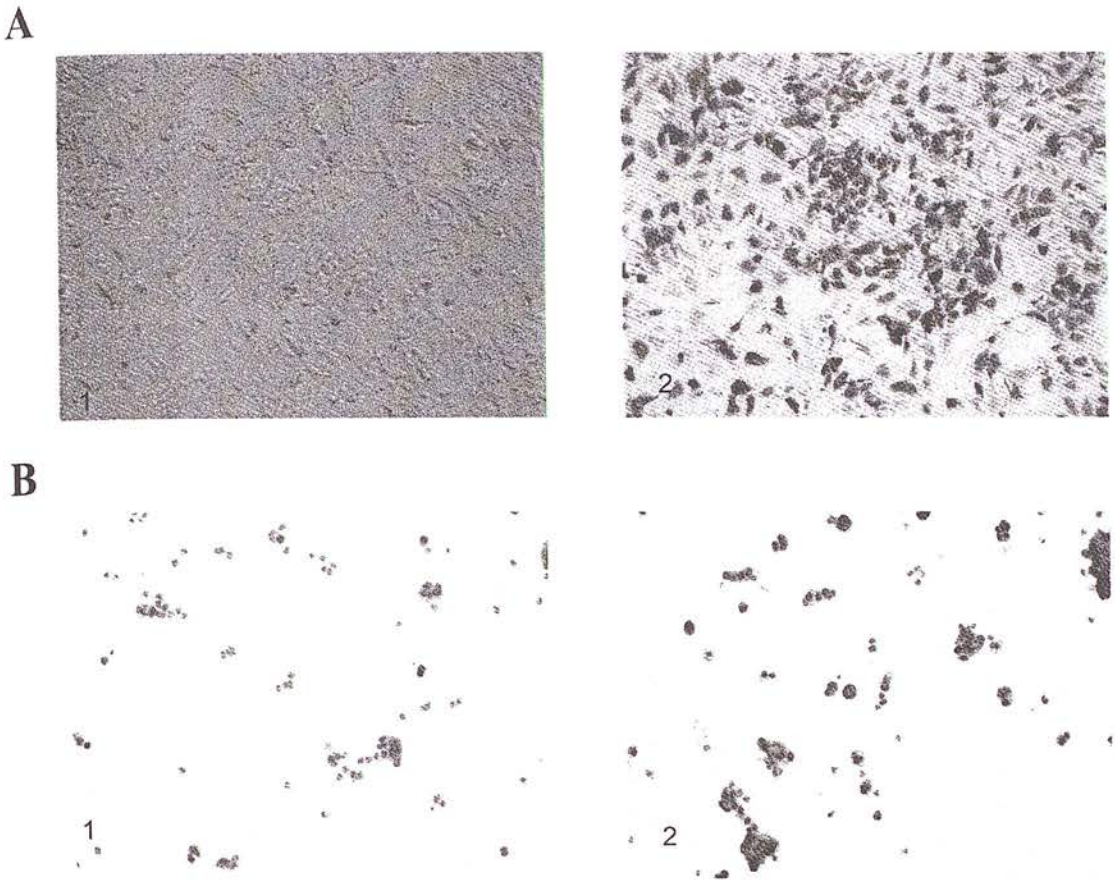


Fig. 30. **Infection of ewe pituitary cells with adenoviruses**. Cells were infected for 2h with LacZ (A) or Smad-6 (B) adenovirus then medium was replaced with 10% FCS medium for 4h. After washing, fresh serum-free medium was added to the cells for 48h. Cells were then fixed with PBS containing 2% Formaldehyde + 0.2% Glutaraldehyde and then incubated with X-gal in PBS containing 5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6, 2 mM MgCl2 (A) or with monoclonal antibody against human Smad-6 and revealed by DAB staining (B).

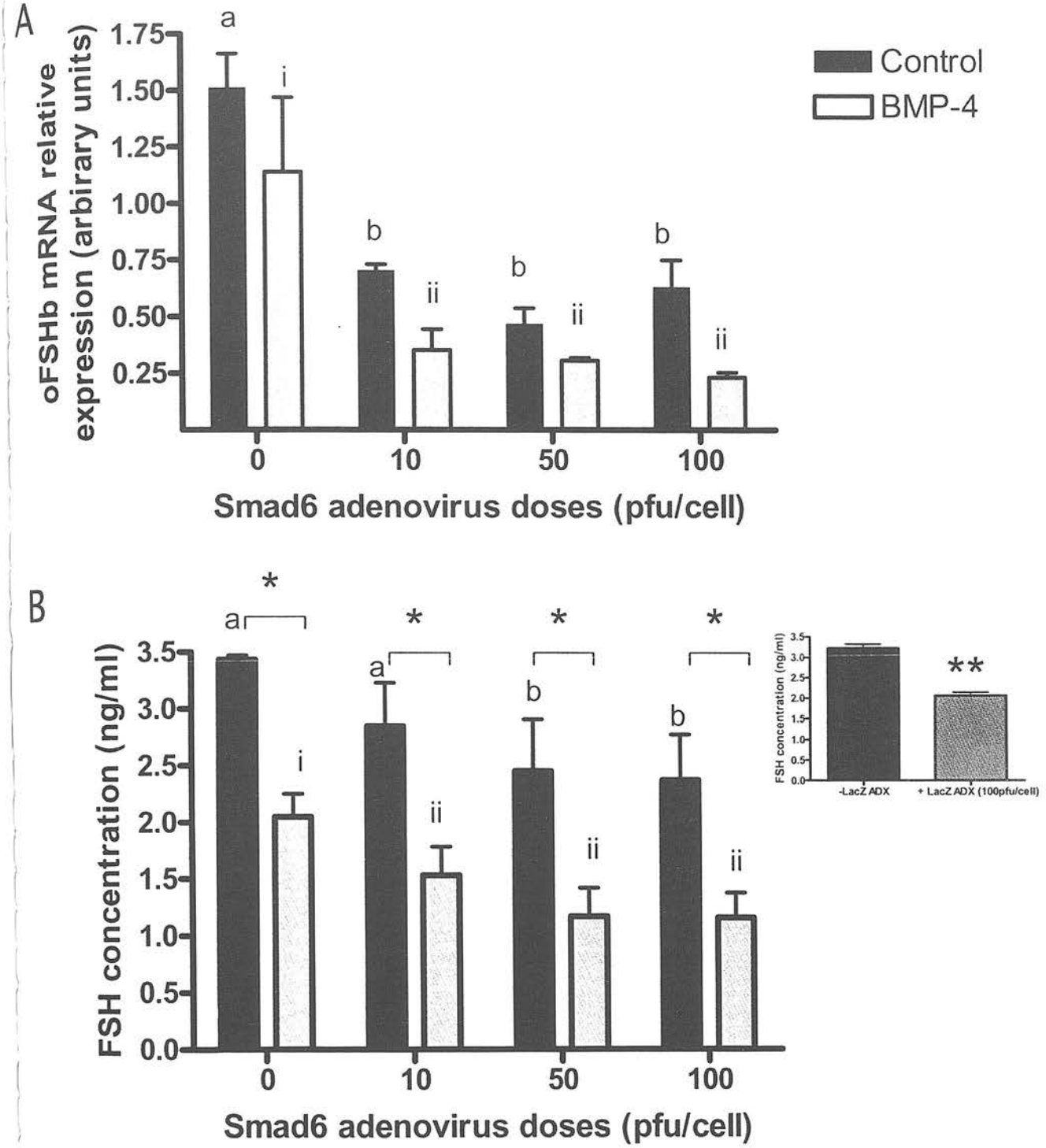


Fig. 31. **Effect of Smad6 overexpression on BMP-4 FSH-inhibited release on ewe pituitary cells.** Cells were infected for 2h with Smad6 adenovirus at indicated doses then medium was replaced with 0% FCS medium for 4h. After washing, fresh serum-free medium alone (Control) or in presence to 10^{-9} M BMP-4 (35ng/ml) was added to the cells for 48h. (A) A real time PCR was performed to detect the relative level of oFSHβ mRNA expression comparing to the 18S mRNA. Values are the mean ± S.E.M. from one representative experiment. a vs b $P<0.05$, i vs ii $P<0.05$. Three experiments were performed with similar results. (B) FSH concentrations were determined by ELISA. Values are the mean ± S.E.M. from four experiments, a vs b $P<0.05$, i vs ii $P<0.05$ * $P>0.05$. ** $P<0.001$

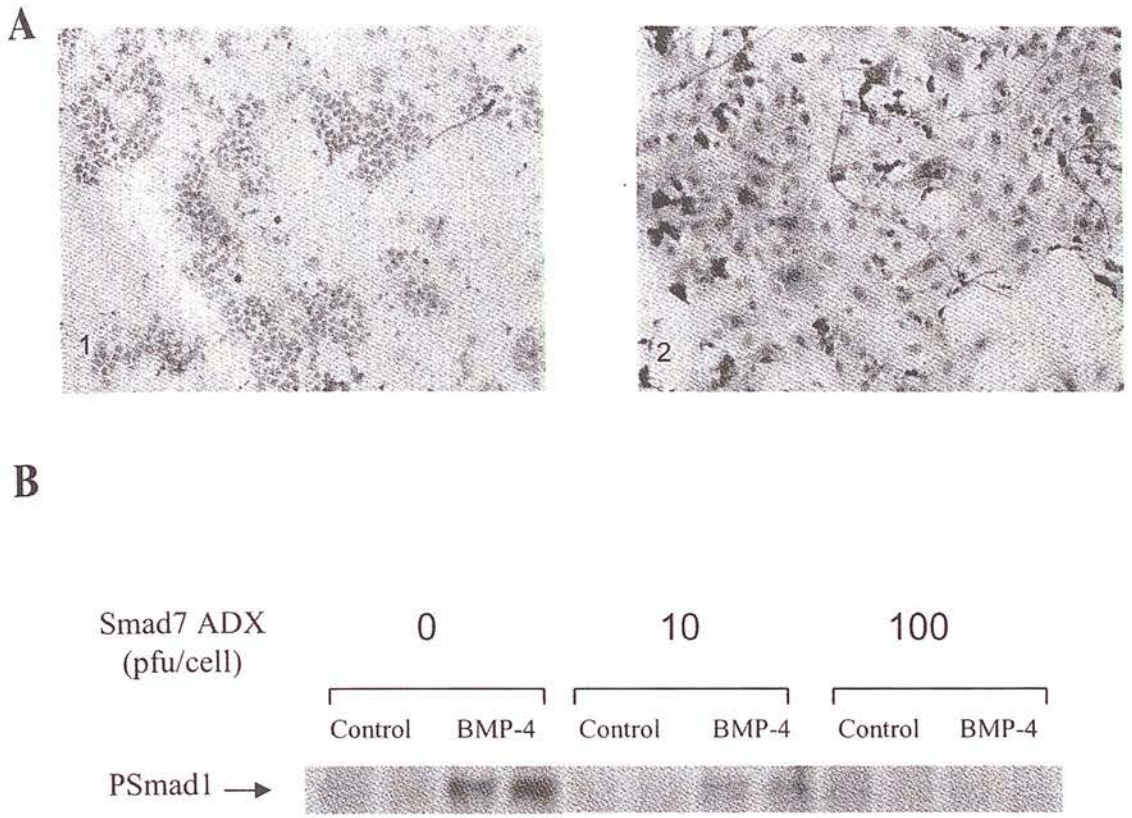


Fig. 32. **Infection of ewe pituitary cells with Smad7**. Cells were infected for 2h with Smad7 adenovirus then medium was replaced with 10% FCS medium for 4h. (A) After washing, fresh serum-free medium was added to the cells for 48h. Cells were then fixed and incubated with monoclonal antibody against human Smad7 (1:200) and revealed by DAB staining. (B) After washing, fresh serum-free medium was added to the cells for 1h. Total proteins (10µg) were separated on 10% SDS-PAGE and transferred on PVDF membrane. Blots were probed with phospho-Smad1 antibody. Bands were revealed by ECL plus.

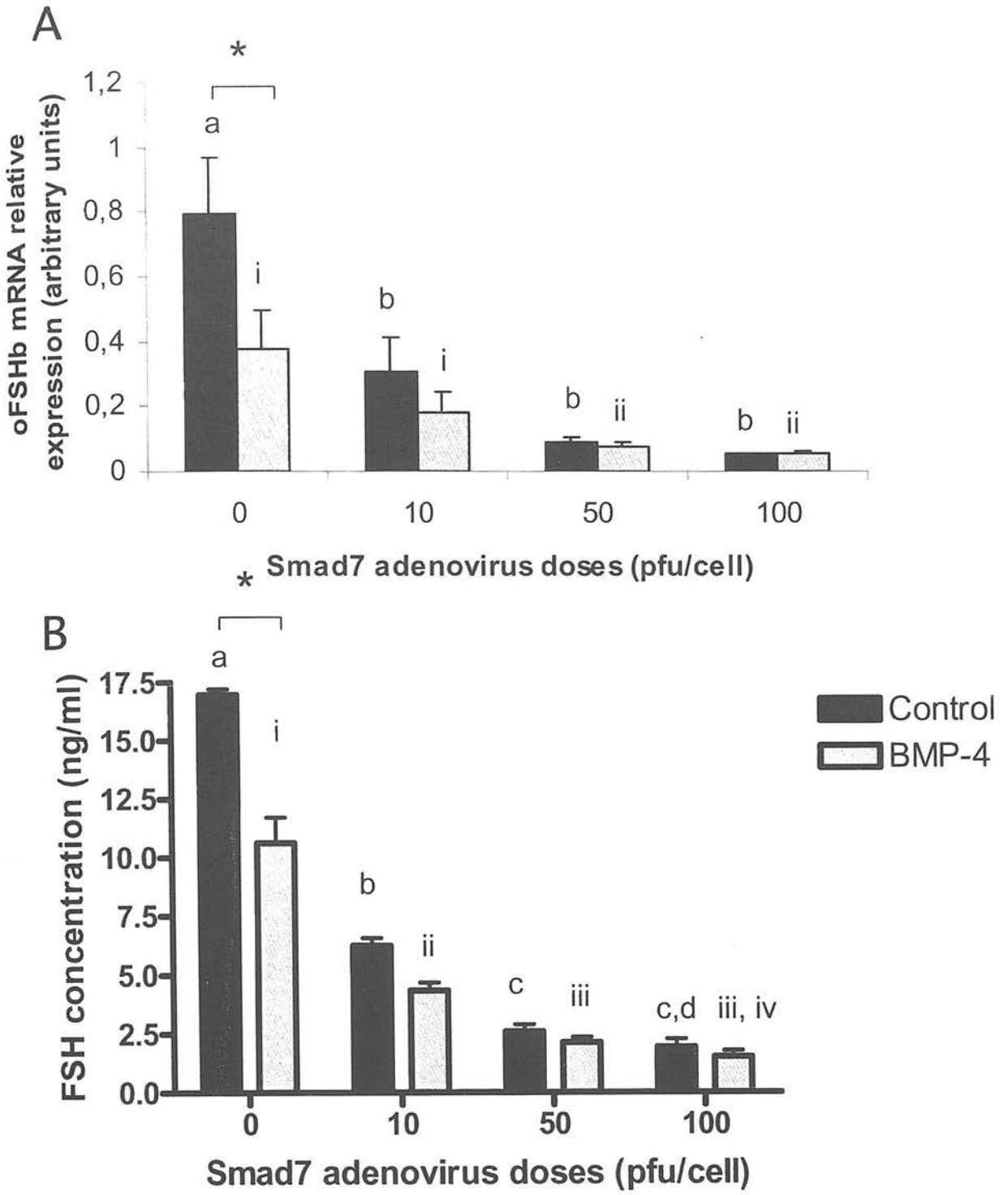


Fig. 33. **Effect of Smad7 overexpression on BMP-4 FSH-inhibited release on ewe pituitary cells.** Cells were infected for 2h with Smad7 adenovirus at indicated doses then medium was replaced with 10% FCS medium. After washing, fresh serum-free medium alone (Control) or in presence to 10^{-9} M BMP-4 (35ng/ml) was added to the cells for 48h. (A). A real time PCR was performed to detect the relative level of oFSHβ mRNA expression comparing to the 18S mRNA. Values are the mean ± S.E.M. from one representative experiment, a vs b $P<0.05$, i vs ii $P<0.05$. Three experiments were performed with the same result. (B) Media were collected and FSH concentrations were determined by ELISA. Values are the mean ± S.E.M. from three experiments. a vs b,c,d $P<0.05$, i vs ii, iii, iv $P<0.05$.

C

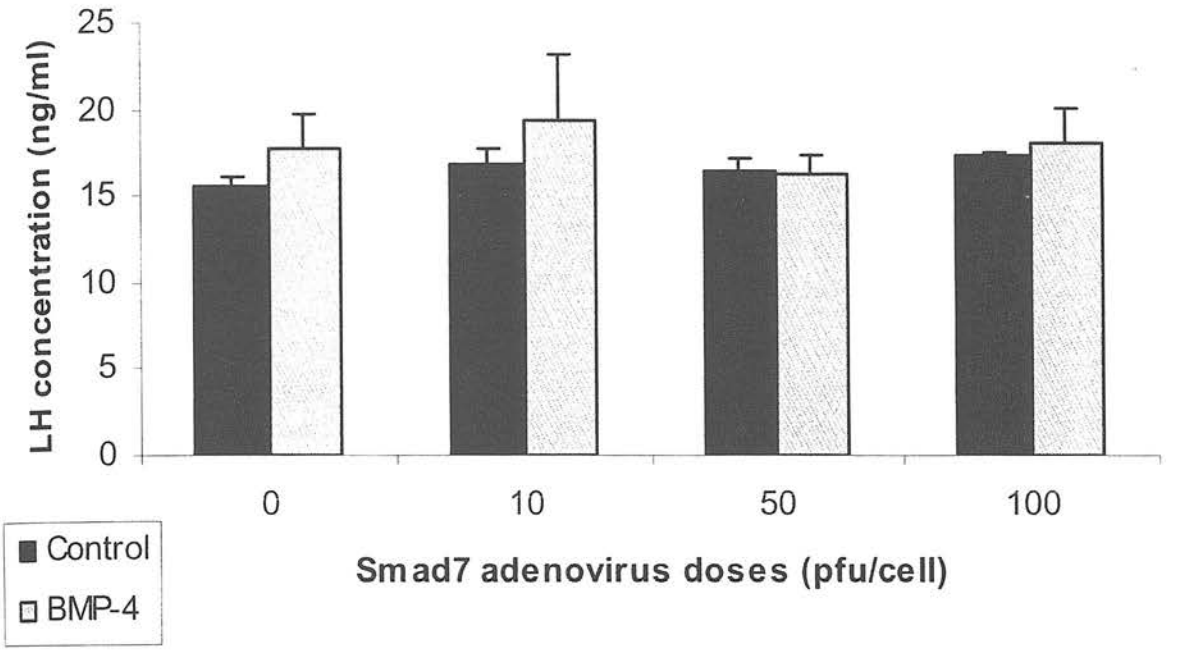


Fig. 34. **Effect of Smad7 overexpression on LH release on ewe pituitary cells**. Cells were infected for 2h with Smad7 adenovirus at indicated doses then medium was replaced with 10% FCS medium for 4h. After washing, fresh serum-free medium alone (Control) or in presence to 10^{-9} M BMP-4 (35ng/ml) was added to the cells for 48h. Media were collected and LH concentrations were determined by ELISA. Values are the mean ± S.E.M. from three experiments.

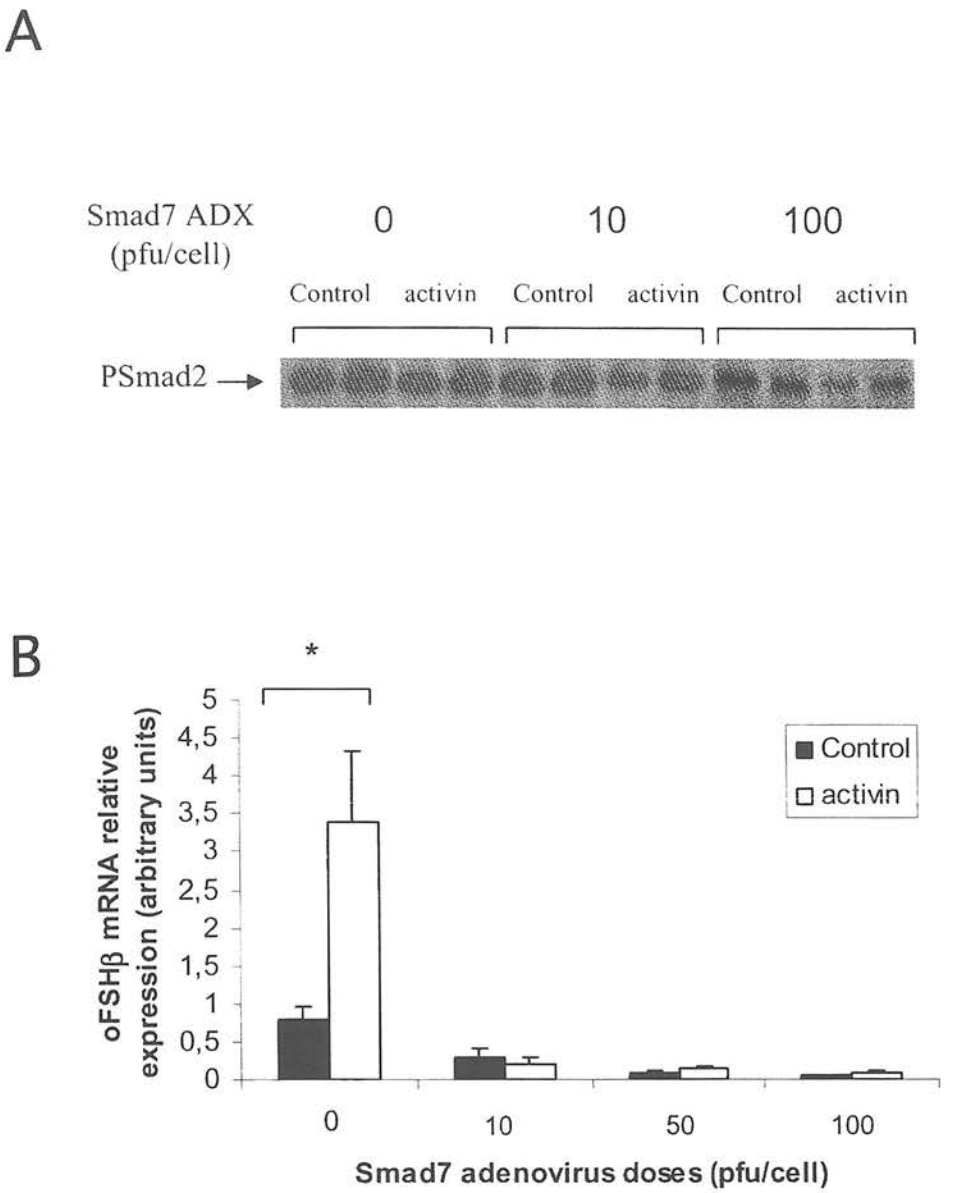


Fig. 35. **Effect of Smad7 overexpression on activin FSH-stimulated release on ewe pituitary cells.** Cells were infected for 2h with Smad7 adenovirus at indicated doses then medium was replaced with 10% FCS medium for 4h. (A) After washing, fresh serum-free medium alone (Control) or in presence to 2.10^{-9} M activin (50ng/ml) was added to the cells for 1h. Total proteins (10µg) were separated on 10% SDS-PAGE and transferred on PVDF membrane. Blots were probed with phospho-Smad2 antibody. Bands were revealed by ECL plus. (B) After washing, fresh serum-free medium alone (Control) or in presence to 2.10^{-9} M activin (50ng/ml) was added to the cells for 48h. Cells were harvested and total mRNAs were extracted. A real time PCR was performed to detect the relative level of oFSHβ mRNA expression comparing to the 18S mRNA (A). Values are the mean ± S.E.M. from one representative experiment, * $P<0.05$. Three experiments were performed with the same result.

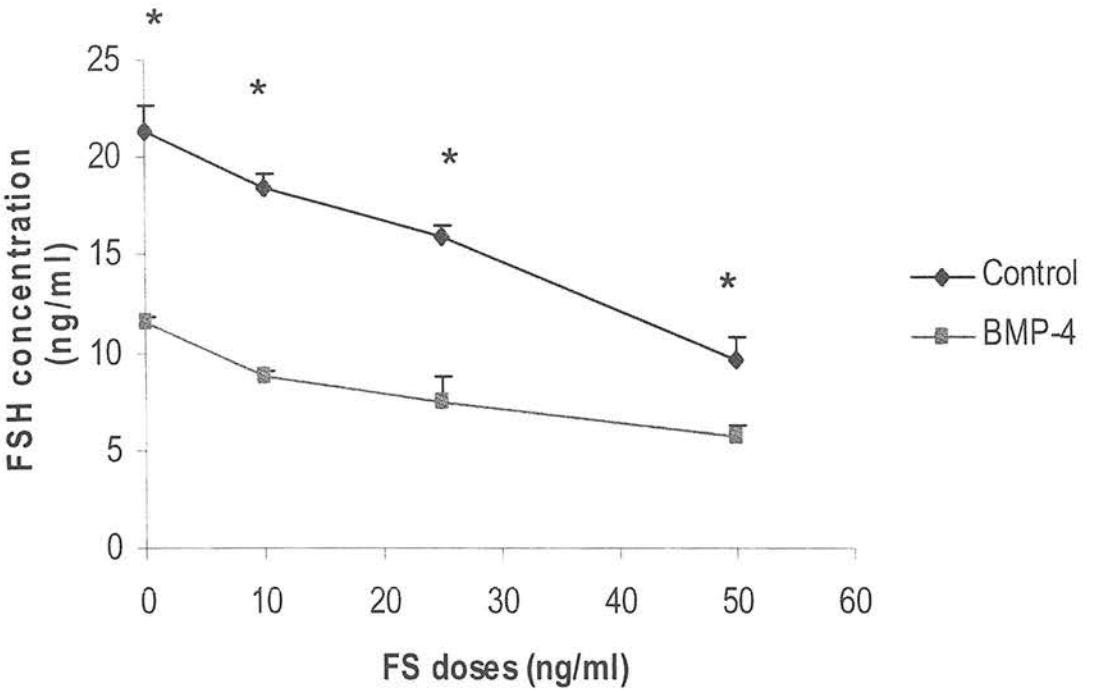


Fig. 36. **Effect of BMP-4 in presence of follistatin on FSH release in ewe pituitary cells.** Cells were cultured in serum-free medium for 48h in the presence of indicated concentrations of follistatin with or without 10^{-9}M BMP-4. Media were collected and the concentrations of FSH were determined by ELISA. Values are the mean+S.E.M. from one representative experiment with triplicate determinations and three experiments were performed with similar results. * $P<0.05$ follistatin alone vs follistatin with BMP-4.

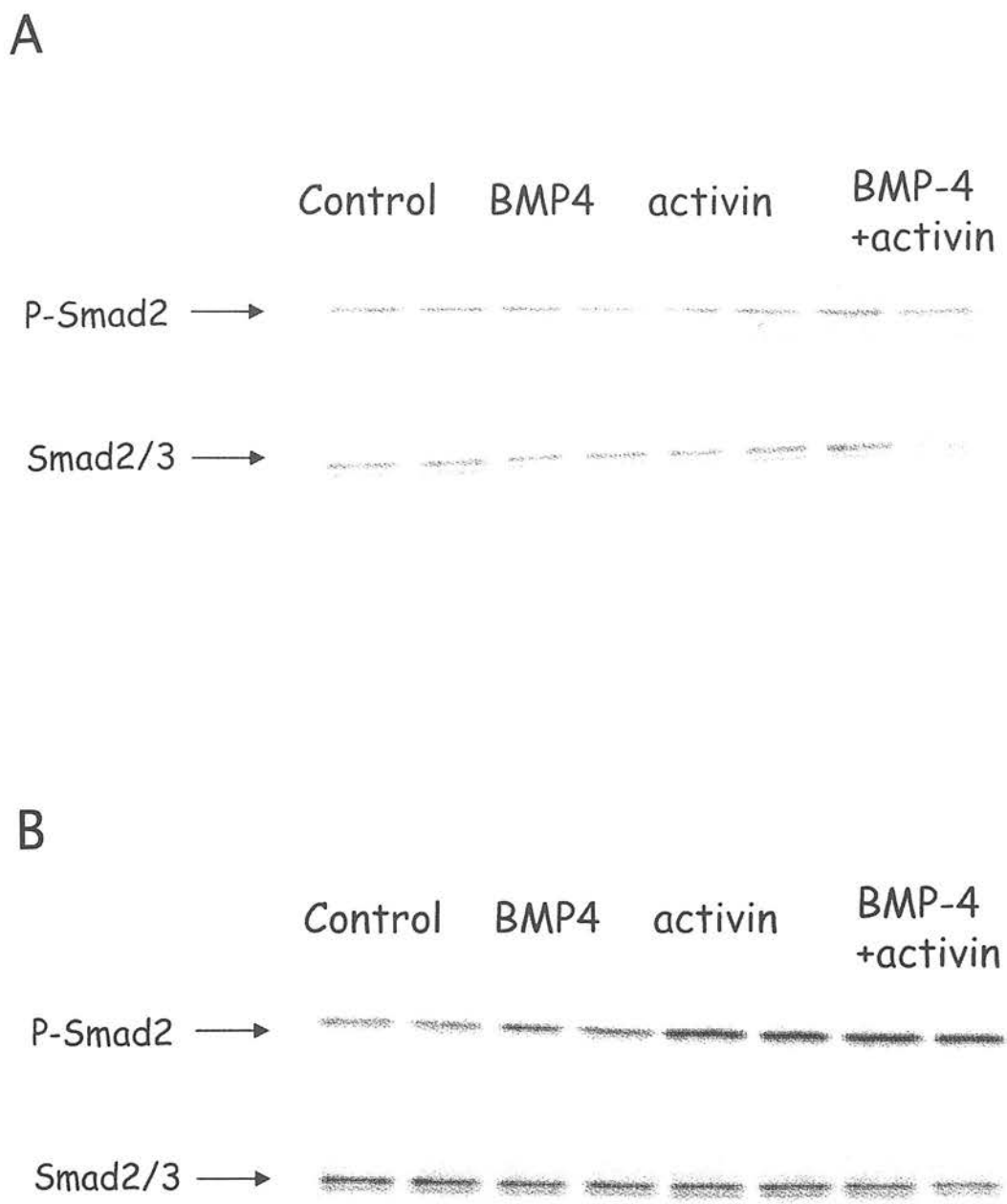


Fig. 37. **Effect of BMP-4 on Smad2 phosphorylation in presence of activin.** (A) Cells were cultured in serum-free medium for 1h with 10^{-9}M BMP-4, 2.10^{-9}M activin, or both factors. Total proteins (10µg) were separated on 10% SDS-PAGE and transferred on PVDF membrane. (B) Similar protocol was performed after 24h follistatin pretreatment at $1.3\ 10^{-9}$M. Blots were probed with phospho-Smad2 (upper) and Smad2/3 (lower) antibodies. Bands were revealed by ECL plus.

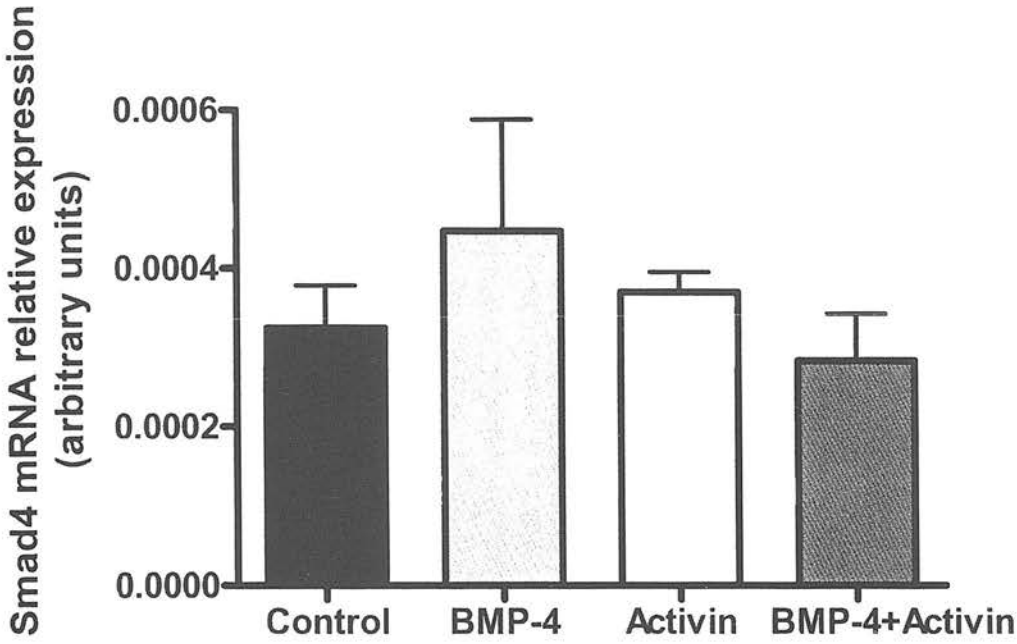


Fig. 38. **Effect of BMP-4 and activin on Smad4 mRNA expression.** Cells were treated with 10^{-9}M BMP-4, 2.10^{-9}M activin, or both factors for 48h. Cells were harvested and total mRNAs were extracted. A real time PCR was performed to detect the relative level of Smad4 mRNA expression comparing to the 18S mRNA. Values are the mean+S.E.M. from one representative experiment with triplicate determinations and three experiments were performed with similar results.

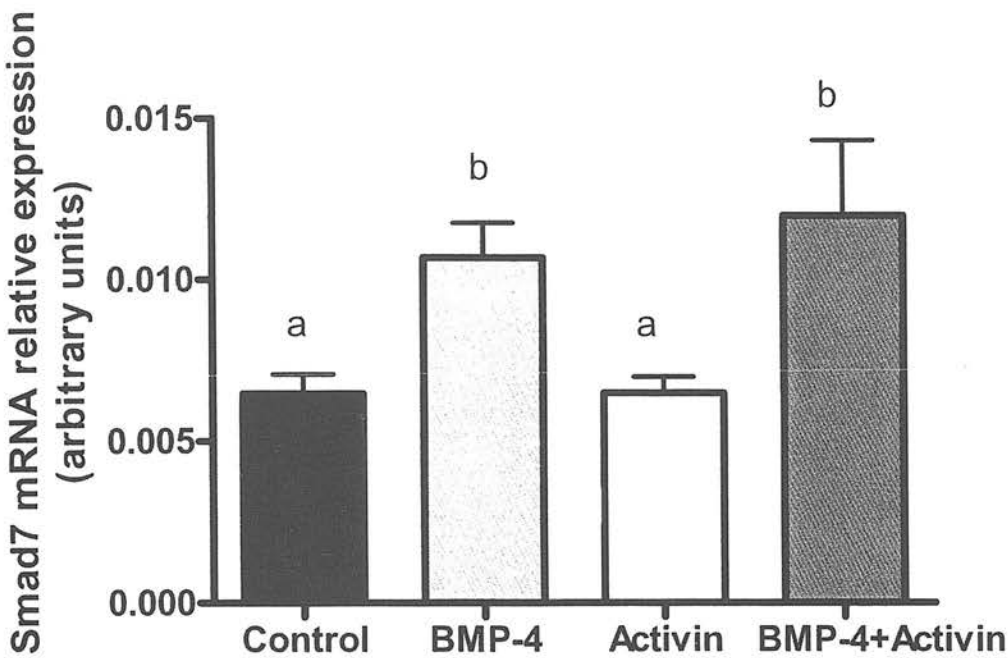


Fig. 39. **Effect of BMP-4 and activin on Smad7 mRNA expression.** Cells were treated with 10^{-9}M BMP-4, 2.10^{-9}M activin, or both factors for 48h. Cells were harvested and total mRNAs were extracted. A real time PCR was performed to detect the relative level of Smad7 mRNA expression comparing to the 18S mRNA. Values are the mean+S.E.M. from one representative experiment with triplicate determinations and three experiments were performed with similar results. b vs a, $P<0.05$.

Chapter III:

BMP-4 differentially modulates gonadotropin secretion by inhibiting GnRH receptor transcription in the mouse gonadodotrope cell line, $L\beta T_2$

A. Introduction

Mellon and co-workers, using targeted oncogenesis in transgenic mice have generated an immortal gonadotrope cell line, $L\beta T_2$ (Mellon *et al.* 1991). These gonadotrope cells can express α, LHβ, FSHβ mRNA and secrete LH and FSH hormones (Turgeon *et al.* 1996, Graham *et al.* 1999). Like primary pituitary cell cultures, gonadotropin secretion is differentially modulated by GnRH, steroids and peptide factors in $L\beta T_2$ gonadotrope cells providing a good reproducible model (Turgeon *et al.* 1996, Nicol *et al.* 2002, Spady *et al.* 2004). GnRH is able to stimulate LH synthesis, translation and secretion (Liu *et al.* 2002, Nguyen *et al.* 2004). As already described in primary cell culture, GnRH pulse frequency also affects differentially LH and FSH production in this murine gonadotrope cell line (Kanasaki *et al.* 2005). Recently, studies of the mouse FSHβ promoter showed the presence of response elements responsible for GnRH-mediated FSH production (Coss *et al.* 2004). Adding to GnRH and steroid effects, activins and inhibins play an important role preferentially on FSH secretion. As with primary ovine pituitary cells, the murine $L\beta T_2$ gonadotrope cell line expresses the activin receptors (types IA, IB, IIA, and IIB) and the Smad proteins (Smad-2, -3, -4, and -7) and can trigger the Smad2/3 signaling pathway required for FSH production (Dupont *et al.* 2003, Bernard 2004). Moreover, studies of the FSHβ promoter showed the presence of response elements responsible for activin-mediated FSH secretion (Suszko *et al.* 2003, Bailey *et al.* 2004). In addition, GnRH and activin can act in synergy since activin can stimulate GnRH-R mRNA expression, and the GnRH induction of FSHβ mRNA expression is reduced by follistatin, an activin antagonist (Graham *et al.* 1999, Pernasetti *et al.* 2001, Nicol *et al.* 2004, Gregory *et al.* 2005).

Recently, other members of the TGFβ superfamily like activins and inhibins were shown to modulate preferentially FSH secretion. TGFβ-1 and -2 inhibit GnRH-R promoter activity assessed by luciferase reporter gene expression at low doses and can also interfere with inhibin-induced inhibition by binding betaglycan allowing activin at higher doses to stimulate FSH secretion in $L\beta T_2$ cell line (Ethier *et al.* 2002). Moreover, Bone Morphogenetic proteins (BMPs) are able to modulate FSH synthesis and release from the pituitary but in an apparently species-specific manner. In contrast to the fact that BMP-4 and BMP-6 were shown to inhibit specifically FSHβ mRNA expression and FSH release in ovine pituitary cells (Faure *et al.* 2005), BMP-6, -7 and -15 were able to stimulate FSH production and the oFSHβ

but not GnRH-R promoters in the murine gonadotrope cell line ($L\beta T_2$) and rat pituitary cells (Huang *et al.* 2001b, Otsuka & Shimasaki 2002).

Whether BMP-4 is able to inhibit gonadotropin secretion in the mouse $L\beta T_2$ cell line was studied. Our results showed that contrary to the previous reports using BMP-6, BMP-7 and BMP-15 (Huang *et al.* 2001b, Otsuka & Shimasaki 2002), BMP-4 was not able to modify either FSH or LH secretion in the gonadotrope cell line on its own. However, we found that BMP-4 stimulated FSH secretion and abolished LH secretion in the combined presence of activin and GnRH whereas GnRH and activin stimulated both FSH and LH. As BMP-4 suppressed GnRH-R mRNA expression in the combined presence of activin and GnRH, our results suggest that the effect of BMP-4 on GnRH-stimulated FSH secretion is due to the inhibition of GnRH-R mRNA expression potentially resulting in a signaling situation similar to a reduced pulse frequency of GnRH which favours FSHβ mRNA expression.

B. Material & Methods

Reagents

Human recombinant activin and BMP-4 were from R&D systems (Abingdon, Oxon, UK) and GnRH was from Bachem (St. Helens, Merseyside, UK).

Rabbit polyclonal antibodies against Smad1 (cat# 06-653, lot 22953) and against Smad2/3 (cat# 07-408, lot 24340) proteins were from Upstate biotechnology (Lake Placid, NY, USA). -Rabbit polyclonal antibody against Smad2-phosphospecific (ser 465/467) protein (cat# 566415, lot D20646) was from Calbiochem (Merck Biosciences LTD., Nottingham, UK). Rabbit polyclonal antibody against Smad1/5-phosphospecific was kindly given by Carl Heldin (Persson *et al.* 1998).

$L\beta T_2$ cell cultures

$L\beta T_2$ cells were kindly provided by Dr. P. Mellon (University of California, San Diego, CA, USA). They were grown and cell cultures were performed as previously described (Nicol *et al.* 2004). Per well, 2.75×10^6 cells were plated in 6-well plates coated with Matrigel (1:4) in 2ml of medium. The following day (day 0), the medium was removed to be assayed for FSH and LH. Phenol red-free DMEM from Sigma (Poole, Dorset, UK) was replaced alone (C) or containing 2.10^{-9} M (50ng/ml) activin (A); 2.10^{-9} M (50ng/ml) activin and 10^{-9}M GnRH (AG); 10^{-9} M (50ng/ml) BMP-4 (B); 2.10^{-9} M (50ng/ml) activin and 10^{-9} M (50ng/ml)

BMP-4 (AB); 2.10^{-9} M (50ng/ml) activin and 10^{-9}M GnRH and 10^{-9} M (50ng/ml) BMP-4 (ABG) for 1h. Cells were then washed with medium to remove residual GnRH. Fresh medium (1.5ml) was added containing the same treatment except that no GnRH was added, and left overnight. This protocol was repeated twice (day 1 and day 2). On day 3, media were collected for assay without adding the GnRH and cells were harvested for RNA extraction.

Mouse FSH and LH assay by RIA

RIA assay on mouse FSH and LH was previously described (McNeilly *et al.* 1996) using reagents supplied by Dr AF Parlow (National Hormone & Peptide Program, Harbor-UCLA, CA, USA). The sensitivity of the FSH and LH RIAs were 0.1 ng/ml and the intra- and inter-assay coefficients of variation were less than 10%.

Reverse transcription-PCR on whole tissues

Total RNA from $L\beta T_2$ cells and mouse ovaries obtained from adult female mice aged 14 weeks were extracted using TRI Reagent (Sigma). Complementary DNA was synthesized from 1 µg RNA in a volume of 20 µl containing 150 ng oligo dT (Promega, Charbonnières, France), 1mM dNTPs, 20 U of RNasin, 1X RT PCR buffer [from 5X assay buffer B (250mM Tris-HCl (pH 8.3), 375mM KCl, 15mM MgCl2, 50mM DTT)], 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 10min and the reverse transcription at 37°C for 1h. PCR was carried out in a volume of 25 µl containing 2.5 µl RT reaction mixture, 1X PCR buffer [from 10X PCR buffer without MgCl2 (500mM KCl, 100mM Tris HCl (pH 9) and 1,0% Triton® X-100), 50 pmol of each primers, indicated concentrations of MgCl2, 0.2mM dNTPs and 10U *Taq* polymerase (Promega). PCR reactions were performed for 30 cycles of 30s at 94°C, 2 min at indicated annealing temperature, and 3 min at 72°C. For primer sequences and details, see chapter I, (Faure *et al.* 2005) and Nicol *et al.* (2002, 2004).

Quantitative RT-PCR

Mouse FSHβ and LHβ primers and probe were designed using Primer Express software (PE Biosystems, Warrington, Cheshire, UK) and synthesized by PE Biosystems. Sequences were given in Nicol *et al.* (2004). Ribosomal 18S primers and probe were from a Taqman RNA Control Reagents kit (VIC labeled probe; PE Biosystem).

Total RNA from 6-well plates was extracted using Tri Reagent (Sigma, Poole, Dorset, UK). Aliquots of total RNA were treated with DNase I using DNA-free (Ambion Ltd, Huntingdon, Cambs, UK) according to manufacturer's protocol. Samples were then reverse transcribed, using oligod(T)16, with Taqman Reverse Transcription Reagents (PE Biosystems) according to the manufacturer's protocol, using a program of 10min at 25°C, 30min at 48°C and 5min at 95°C. DNase-treated RNA was added at 25-50ng/µl. For PCR, a reaction mix was prepared consisting of Taqman Universal PCR Master Mix (1X), FSHβ or LHβ forward and reverse primers (300nM each), FSHβ or LHβ probe (200nM), ribosomal 18S forward and reverse primers (50nM each) and ribosomal 18S probe (200nM). This was aliquoted for each sample in separate tubes, then cDNA was added at 1µl/25µl reaction mix (equivalent to 25-50ng total RNA/25µl). Twenty-five µl aliquots were dispensed into a 96-well PCR plate, with each sample added in duplicate, optical caps fixed onto the plates and the PCR reaction run on an ABI Prism 7900 PCR machine (PE Biosystems) using standard conditions. Controls included cDNA prepared without Multiscribe reverse transcriptase enzyme were performed to check for efficiency of DNAse treatment. Prior to analysis, a validation assay was performed to demonstrate that amplification of FSHβ or LHβ genes comparing to the reference (18S) were approximately equal (Nicol *et al.* 2002). Quantification of FSHβ and LHβ mRNA was performed by the ΔΔCt method and the standard curve method respectively (Bulletin #2; PE Biosystems). A standard PCR reaction using the FSHβ and the LHβ primers was run on an agarose gel to confirm amplification of a single product of the correct size.

Western blotting analysis of Smad proteins

To investigate Smad activation by BMP-4 and/or activin, $L\beta T_2$ cells were cultured at $2.75x10^6$ cells/well in 6-well plates for three days as with exactly the same treatments as described previously (see $L\beta T_2$ cell cultures above). On day 3, GnRH was added with all treatments for 1h after which cells were washed once on ice with cold PBS and lysed in lysis buffer (150mM NaCl, 10mM Tris pH 7,4, 1mM EDTA, EGTA 1mM, Triton X-100 1%, Igepal CA. 630 0.5%, 100mM Sodium fluoride, 10mM Sodium pyrophosphate, 10mM Sodium orthovanadate, proteases inhibitor cocktail). The protein concentration was determined with BC Assay Protein Quantitation kit (Interchim, Montluçon, France). After 1min at 95°C in the reducing SDS-PAGE buffer containing β-mercaptoethanol, 10µg of proteins were separated in 10% SDS-PAGE gels and electroblotted onto Immobilon-P

membranes (Millipore corporation, Bedford, MA). Membranes were blocked for 1 h at room temperature in Tris-buffered saline-0.1% Tween (TBST) containing 5% fat-free dry milk for and incubated with the anti-Smad1, anti-Smad2/3, anti-phosphoSmad2 antibodies diluted at 1:2000, 1:1000 and 1:2000 respectively in TBST 1%BSA 5%milk for 1h at RT. After washing, the membranes were incubated with the secondary antibody, a peroxidase-conjugated anti-IgG (Biorad, Marnes la Coquette, France) diluted 1:15000 for 1 h. Immunoreactive proteins were detected using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Orsay, France). Membranes were exposed to Hyperfilm ECL (Amersham Pharmacia Biotech) for 20sec. The same protocol was followed for anti-phospho Smad1/5 except that the blocking buffer was 1% BSA in TBST and the antibody was diluted in 1:5000 in 1% BSA TBST.

Statistical analysis

Results are reported as mean ± S.E.M from one representative experiment. All experiments were performed in triplicates in at least two different experiments. Comparison between activin (A), GnRH (G), BMP-4 (B), activin+BMP-4 (A+B), activin+BMP-4+GnRH (A+B+G) and control (C) groups on FSH, LH concentrations and FSHβ and LHβ mRNA expression were analysed by one-way ANOVA followed by Newman-Keuls post-test. $P<0.05$ was considered significant. The statistical analysis was performed using the GraphPad Prism version 4.00 program for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

C. Results

1. BMPs and their receptors are expressed in the mouse pituitary and the gonadotrope cell line, $L\beta T_2$

To test whether $L\beta T_2$ cells express BMPs and their receptors, RT-PCR was performed using primers described in Table IA (Chapter I, Faure *et al.* 2005). In $L\beta T_2$ cells, DNA fragments were detected at expected sizes for BMP-6, BMP-7 and the two receptors BMP-RIA (ALK-3) and BMP-RII after 30 cycles (Fig. 40A, B). No PCR product was detected for BMP-2, BMP-4, BMP-15 or BMP-RIB (ALK-6). Positive controls were performed using mouse ovaries and DNA fragments were observed for BMP-2, BMP-4 and BMP-15 (Fig. 40C, D). When negative controls were performed by omitting reverse transcriptase in the RT reaction, no PCR products were detected (not shown).

2. BMP-4 amplifies the stimulatory effect of GnRH on activin-stimulated FSH secretion and inhibits the stimulatory effect of GnRH on activin-stimulated LH secretion

To determine whether BMP-4 was capable of modifying FSH or LH secretion in the $L\beta T_2$ gonadotrope cell line, cells were incubated with BMP-4 (10^{-9}M) for three days following the protocol previously described (Nicol *et al.* 2002). On the last day, the concentration of FSH and LH in the medium was assayed and cells were harvested to extract total RNAs. BMP-4 did not modify FSH or LH concentrations on its own or in presence of activin. However for FSH release, the GnRH stimulatory effect on activin-stimulated FSH release was amplified by the co-treatment with BMP-4 (Fig.41A, b vs d, $P<0.001$) and this was paralleled by changes in FSHβ mRNA expression (Fig.41B, c vs d, $P<0.05$). In contrast, the GnRH stimulatory effect on activin-stimulated LH release was reduced by the co-treatment with BMP-4 (Fig.42A, b vs c, $P<0.001$). The BMP-4 inhibitory effect on GnRH effect was stronger on LHβ mRNA expression as the level of LHβ on activin+GnRH+BMP-4 group was not significantly different from the control group (Fig.42B, b vs a, $P<0.01$).

3. GnRH does not affect the independent BMP and activin signaling pathways

To determine whether BMP-4 and Activin signaling pathways could interact together, proteins were extracted after 60 min treatment with 10^{-9} M BMP-4, 2.10^{-9} M activin or both. These conditions were also carried out with the addition of a 1h pulse of GnRH to amplify the activin effect. Figure 43 shows that BMP-4, but not activin, induced the appearance of a 66 kDa band corresponding to the phosphorylated form of Smad-1 and Smad-5 in the presence or absence of GnRH. When cells were incubated with both BMP-4 and activin, phosphorylated Smad-1 was also detected. Comparing the intensity of this 66kDa band between BMP-4 and BMP-4+activin treatments revealed no significant difference. For Smad-1 protein, the intensity of the band did not change between control and BMP treatment (Fig.43, upper). The activation of Smad2 was assessed with an antibody recognising the dual serine 465/467 with an apparent molecular weight of 58 kDa. Activin induced the appearance of a 58 kDa band whereas no band was detected in the control or after treatment with BMP-4 alone. When cells were treated with both BMP-4 and activin, the band corresponding to the phosphorylated form of Smad-2 was detected and its intensity was not significantly different to the band seen after activin treatment, in the absence or presence of GnRH (Fig.43, lower).

4. BMP-4 inhibits GnRH receptor mRNA expression

Since BMP-4 was able to differentially modulate gonadotropin secretion in the presence of GnRH in the $L\beta T_2$ gonadotrope cell line, cells were incubated with BMP-4 (10^{-9}M) for three days following the same protocol as previously described above (Nicol *et al.* 2002). On the last day, cells were harvested to extract total RNAs. Although BMP-4 did not modify GnRH-R mRNA expression on its own, the GnRH stimulatory effect on GnRH-R mRNA expression was reduced by the co-treatment with BMP-4 (Fig.44, c vs a,b, $P<0.001$).

D. Discussion

While previous studies using the mouse gonadotrope $L\beta T_2$ cell line reported that BMPs could stimulate ovine FSH promoter activity, or mouse FSH mRNA and secretion (Huang *et al.* 2001b, Otsuka & Shimasaki 2002), in our primary sheep pituitary cultures, BMPs consistently inhibited FSH mRNA and secretion (Chapter II, Faure *et al.* 2005). Thus it was important to confirm this apparent species difference in the effect of BMP-4 on FSH production in the ovine pituitary cells and the mouse $L\beta T_2$ cell line. Our results showed that contrary to the previous reports, BMP-4 on its own was not able to modify either FSH or LH secretion in the mouse gonadotrope cell line. However, we found that BMP-4 stimulated FSH secretion and abolished LH secretion in presence of activin and GnRH whereas GnRH and activin stimulated both FSH and LH. BMP-4 also suppressed GnRH-R mRNA expression in presence of activin and GnRH.

In LβT2 gonadotropes, we detected mRNAs for BMP-6 and BMP-7, and the receptors, BMPR-IA (ALK-3) and BMPR-II in $L\beta T_2$ cells corroborating a previous report (Otsuka & Shimasaki 2002). Furthermore we did not detect BMP-2, BMP-4 and BMP-15 DNA fragments after 30 cycles although they are clearly observed in the mouse ovary (Figure 1). This contrasts in part with the previous report in which BMP15 was observed, although only after 35 cycles (Otsuka & Shimasaki 2002).

Although BMP-4 was able to inhibit oFSHβ mRNA expression and FSH release without affecting LH production in ewe pituitary cells, BMP-4 had no effect on either FSH or LH secretion in the murine $L\beta T_2$ gonadotrope cell line. Similar results were obtained with BMP-6 and BMP-7 at 200ng/ml (Otsuka & Shimasaki 2002) although very high dose (1µg/ml) stimulated FSH secretion (Huang *et al.* 2001b). BMP-15 had also stimulatory effect at 100ng/ml (Otsuka & Shimasaki 2002). This difference of effect between BMPs is

surprising as we found that BMP-4, BMP-6, BMP-15 and GDF-9 had the same inhibitory effect in the ewe pituitary cells (Chapter I, data not published). Moreover, several BMPs were found to have similar effects in various cell types (Solloway *et al.* 1998). Nevertheless, $L\beta T_2$ cells express very low level of FSHβ mRNA and release very little FSH without activin or GnRH stimulation suggesting that a potential inhibitory effect of BMP-4 may not be noticeable in these conditions.

Since we showed that BMP-4 effect could antagonize the effect of activin on FSH secretion in the ewe pituitary cells, we determined whether BMP-4 and activin could interact in the murine $L\beta T_2$ gonadotrope cell line. In our culture conditions, activin stimulated FSH production while BMP-4 alone had no effect. However, it is known that the effect of activin on FSH secretion is amplified by co-treatment with GnRH (Graham *et al.* 1999, Nicol *et al.* 2004). When we treated the $L\beta T_2$ cells with both activin and GnRH we found that BMP-4 amplified the stimulatory effect of both activin and GnRH on FSHβ mRNA expression and on FSH release. The transcription factors Smad-2, Smad-3 and Smad-4 mediate activin-stimulated activity of the rat FSHβ promoter and FSH secretion in $L\beta T_2$ cells (Dupont *et al.* 2003, Suszko *et al.* 2003, Bailey *et al.* 2004, Bernard 2004). Since BMP-stimulated phopho-Smad-1 also interacts with Smad-4 we can hypothesize that BMP-4 may act to stimulate FSHβ promoter activity via Smad-4 although promoter activation via different response elements such as AP-1 cannot be ruled out (Coss *et al.* 2004). Further investigations are required to determine the precise mechanisms involved. Interestingly, BMP-4 acts not only on activin and GnRH-stimulated FSH but also on activin and GnRH-stimulated LH production. This surprising result differs from our results in ovine pituitary cells and previous results in $L\beta T_2$ gonadotropes (Otsuka & Shimasaki 2002). We used a daily one hour treatment with GnRH, a GnRH pulse, in our experiments in $L\beta T_2$ cells as described previously (Nicol *et al* 2002, 2004). We found that BMP-4 inhibited the stimulatory effect of both activin and GnRH on both LH release and LHβ mRNA expression. Moreover, BMP-4 was able to inhibit GnRH-R mRNA expression in presence of both activin and GnRH. A recent study showed that using a perifusion system on $L\beta T_2$ gonadotrope cells allowing GnRH pulses, LHβ gene promoter activity was stimulated at higher GnRH pulse frequencies whereas the FSHβ gene promoter was preferentially stimulated at lower GnRH pulse frequencies. Moreover, GnRH-R number was increased at higher frequencies of pulsatile GnRH (Bédécarrats & Kaiser 2003). Therefore, our results suggest that BMP-4, by inhibiting GnRH-R expression, could

potentially mimic a decrease in GnRH pulse frequency by decreasing the GnRH receptor numbers thus promoting activation of the FSHβ gene promoter and inhibiting the LHβ gene promoter. Further studies would determine whether BMP-4 is able to decrease GnRH-R number, although previous studies have indicated a close relationship between GnRH receptor binding sites and levels of GnRH mRNA, at least in sheep (Brooks *et al.* 1992). Moreover, as BMP and activin signaling pathways are not modified in presence of GnRH, it would be interesting to look at P38 and ERK phosphorylation, recently described as varying dependently to GnRH pulses (Kanasaki *et al.* 2005).

In conclusion, the effects of BMPs on pituitary gonadotrophin expression and secretion appear to be opposite in the mouse gonadotropes compared with the sheep pituitary gonadotrope cells. These data may reflect different roles of BMPs in mono- vs pluri-ovulatory species. BMP-4 alone was not able to modify either FSH or LH production in the murine gonadotrope cell line, $L\beta T_2$. However, BMP-4 acted differentially on GnRH and activin-stimulated gonadotropin secretion, by stimulating FSHβ mRNA expression and FSH release and by inhibiting LH production. Moreover, BMP-4 was able to inhibit GnRH-R mRNA expression suggesting a link between the decrease in GnRH-R expression and the inhibition of LH secretion. Thus, in the mouse pituitary BMP-4 may play an important role in gonadotropin secretion by modulating GnRH receptor mRNA expression.

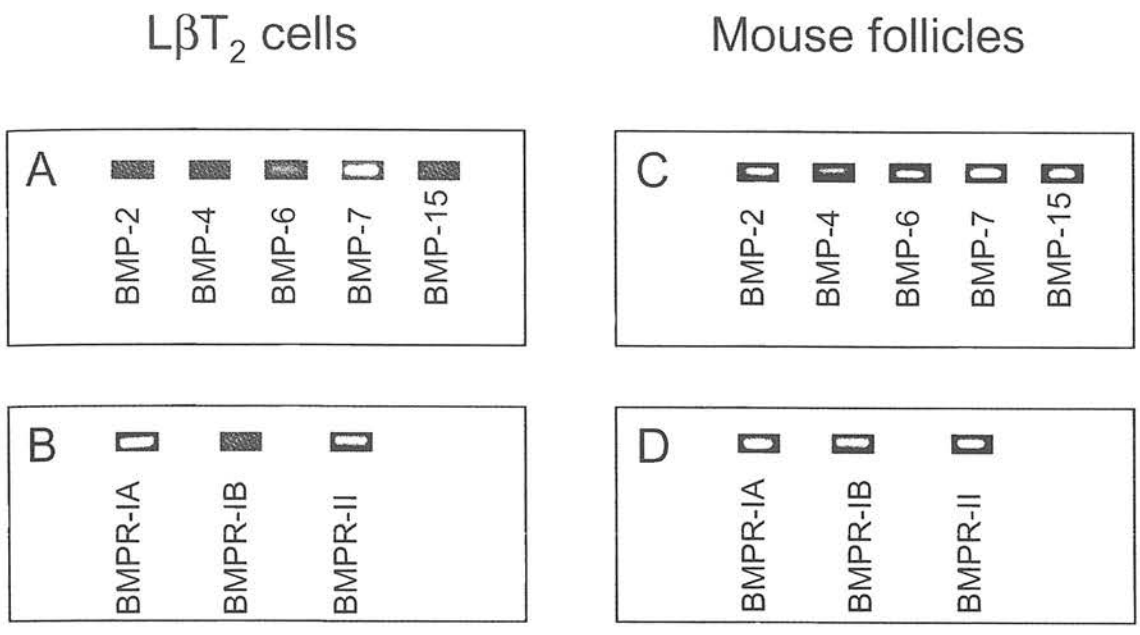
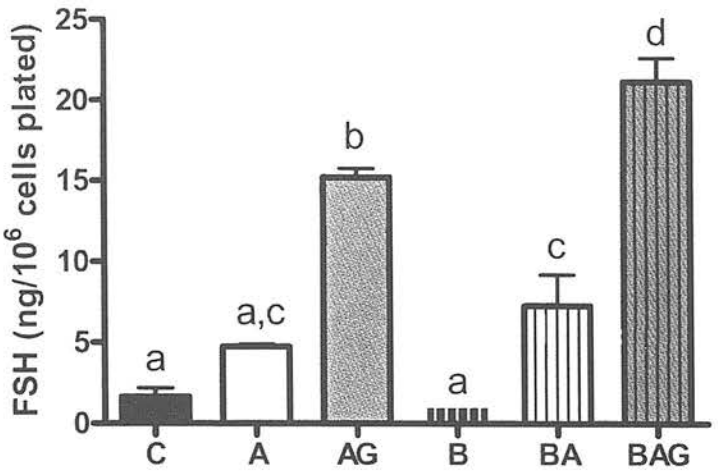


Fig. 40. **Expression of BMP ligands and receptors in $L\beta T_2$ cells and mouse ovaries.** Total RNA was extracted from $L\beta T_2$ cells (A,B) and mouse ovaries (C,D). The mRNAs encoding BMP ligands (A,C) and receptors (B,D) were analysed by RT-PCR at 30 cycles. DNA fragments were electrophoresed in 1% agarose gel and visualized in ethidium bromide staining.

A



B

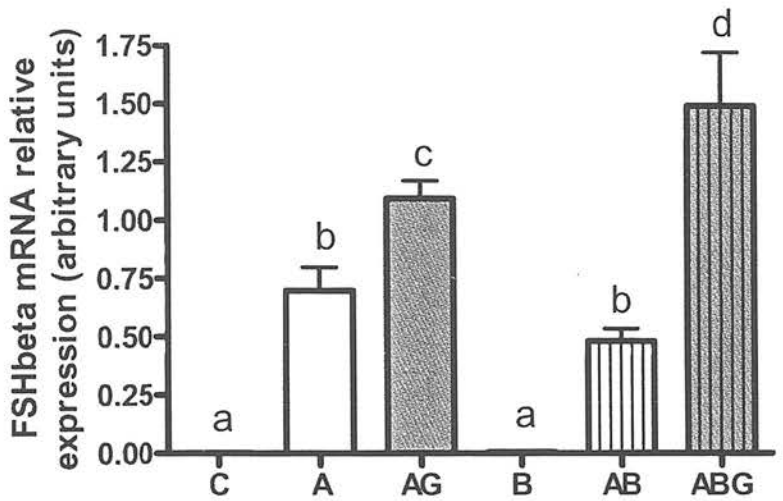
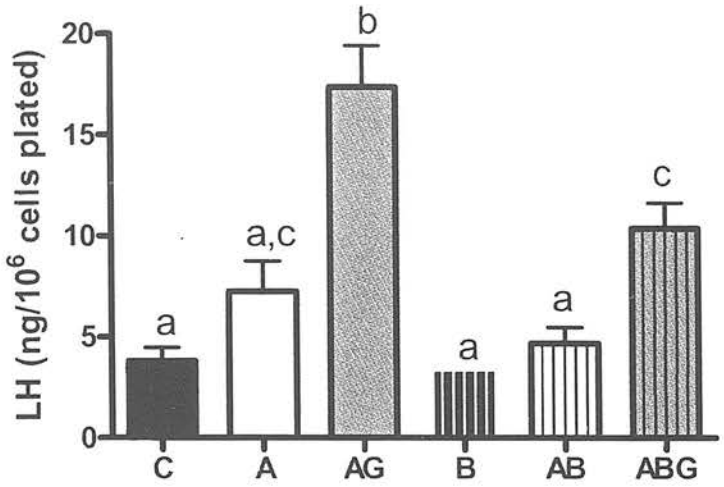


Fig. 41. **FSH release (A) and FSHβ mRNA expression (B)** on treatment day 3 from $L\beta T_2$ cells cultured either in the absence of 2.10^{-9}M activin, 10^{-9}M BMP-4 or 2.10^{-9}M activin+10^{-9}M BMP-4 and given a one hour pulse of 10^{-9}M GnRH per day for 3 days. Values represent mean±S.E.M. from one representative experiment in triplicate. $P<0.05$ was considered as significant and different letters indicate a significant difference.

A



B

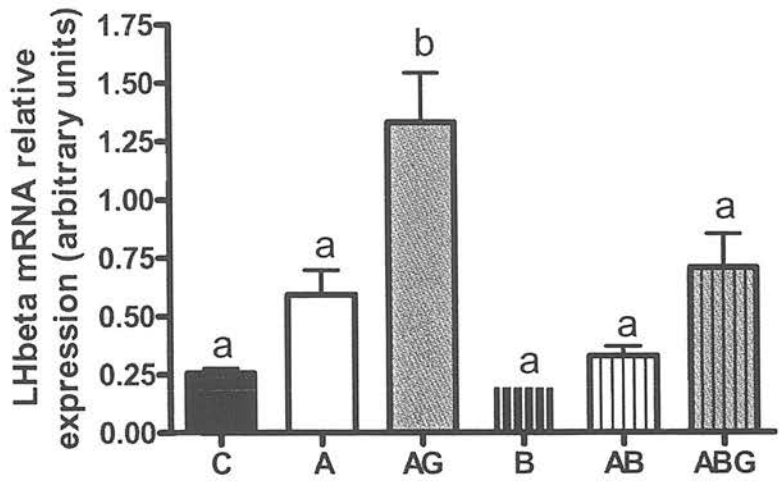


Fig. 42. **LH release (A) and LHβ mRNA expression (B)** on treatment day 3 from $L\beta T_2$ cells cultured either in the absence of 2.10^{-9}M activin, 10^{-9}M BMP-4 or 2.10^{-9}M activin+10^{-9}M BMP-4 and given a one hour pulse of 10^{-9}M GnRH per day for 3 days. Values represent mean±S.E.M. from one representative experiment in triplicate. $P<0.05$ was considered as significant and different letters indicate a significant difference.

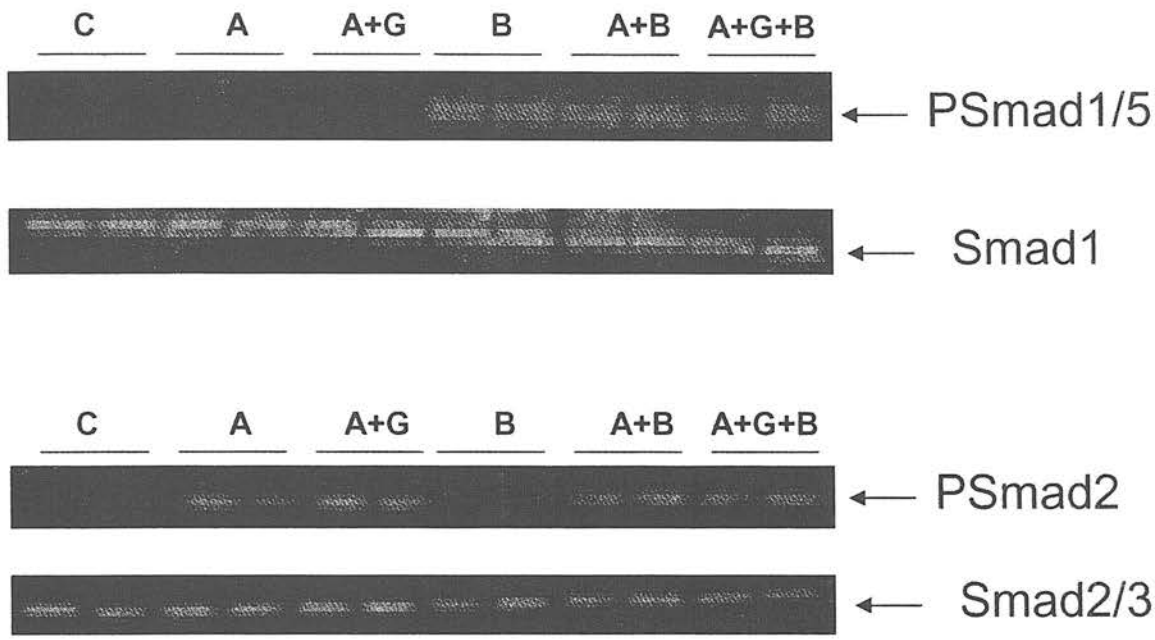


Fig. 43. **Activation of Smad1 and Smad2** on treatment day 4 from $L\beta T_2$ cells cultured either in the absence of 2.10^{-9}M activin, 10^{-9}M BMP-4 or 2.10^{-9}M activin+10^{-9}M BMP-4 and given a one hour pulse of 10^{-9}M GnRH per day for 3 days. Total proteins (10µg) were extracted from cells 60 min after the addition of GnRH on day 4 and separated on 10% SDS-PAGE and transferred on PVDF membrane. Blots were probed with phospho-Smad1 (PSmad1/5) and Smad1 (Smad1) antibodies (upper) and with phospho-Smad2 (PSmad2) and Smad2/3 (Smad2/3) antibodies (lower). Bands were revealed by ECL plus.

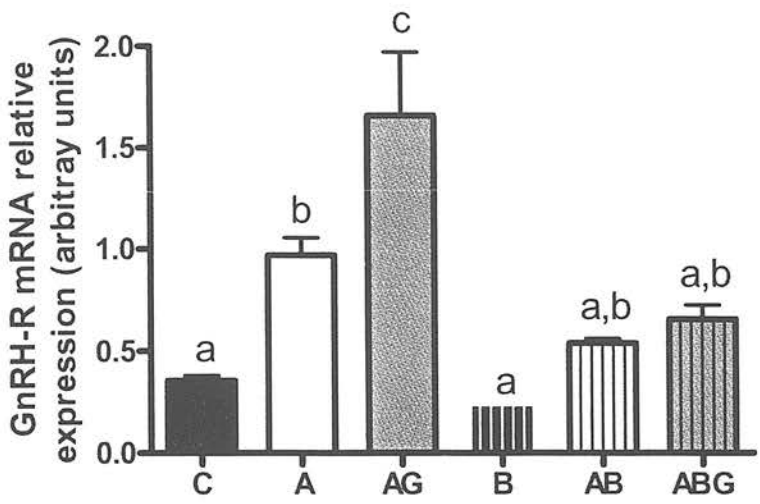


Fig. 44. **GnRH-R mRNA expression** on treatment day 3 from $L\beta T_2$ cells cultured either in the absence of 2.10^{-9}M activin, 10^{-9}M BMP-4 or 2.10^{-9}M activin+10^{-9}M BMP-4 and given a one hour pulse of 10^{-9}M GnRH per day for 3 days. Values represent mean±S.E.M. from one representative experiment in triplicate. $P<0.05$ was considered as significant and different letters indicate a significant difference.

General discussion

The studies in this thesis have shown the presence of several BMPs and their receptors in the ewe pituitary and in the $L\beta T_2$ mouse gonadotrope cell line. BMP-4 and other BMPs were able to regulate differentially gonadotropin secretion in both species. However, the effect of BMP-4 was different among species as BMP-4 inhibited specifically basal and activin-stimulated FSH secretion without altering LH production in sheep, whereas BMP-4 amplified the activin and GnRH stimulatory effect on FSH secretion and inhibited the LH production stimulated by activin and GnRH in mouse.

In the following discussion, three major aspects are examined:

1- What role do BMPs play in the regulation of gonadotropin synthesis and secretion?

2- How do BMPs interact with other gonadotropin modulators?

3- Is there a physiological role for BMPs in the differential regulation of gonadotropin secretion?

I. - What role do BMPs play in the regulation of gonadotropin synthesis and secretion?

A. Do BMPs act as paracrine factors?

BMP-2, BMP-4 and BMP-7 mRNAs were detected in the ewe pituitary whereas BMP-6 and BMP-7 mRNA were detected in the mouse gonadotrope cell line, $L\beta T_2$. These results corroborate Souza and co-workers (2003) for the sheep and Otsuka & Shimasaki (2002) for the $L\beta T_2$ mouse gonadotrope cell line. Surprisingly, we also found GDF-9 mRNA in the ewe pituitary which is known as to be specifically expressed in oocyte (Yan *et al.* 2001). Hence, the pattern of BMP expression appears to be different between the sheep and the mouse.

BMP receptors are expressed in ewe pituitary and in the $L\beta T_2$ cell line. Both BMPR-IA and BMP-RII, not BMPR-IB, were detected by immunohistochemistry in the gonadotrope cells in the ewe pituitary. We then hypothesize that BMPs act through BMPR-IA and BMPR-II. All gonadotrope cells expressed these two receptors but we also found both in other cell types. Further investigations should determine the other pituitary cell types expressing BMPR-IA, BMPR-IB and BMPR-II. As previously described, Booroola Merino ewes showed hyper-prolificacy linked to a mutation in the BMPR-IB receptor. Thus, it would be interesting to find which cell type expresses BMPR-IB to determine which hormone release could be affected by this mutation. The present result that the BMPR-IB is not localised in the

gonadotrope clearly supports previous studies that there is no difference in LH or FSH secretion and that the effects of the BMPR-IB mutation resides within the ovary. Moreover, the two receptors mRNAs (BMPR-IA and BMPR-II) were found in $L\beta T_2$ cell line corroborating the work of Otsuka & Shimasaki (2002).

Expression of both BMPs and BMP receptors in the pituitary suggest a paracrine role for BMPs. To test this hypothesis, ewe pituitary cells were treated with Noggin, BMP-2/BMP-4 antagonist. No significant effect was seen. Since several BMPs are able to inhibit specifically FSH secretion, we suggest that other BMPs can compensate the lack of BMP-2 and BMP-4. To complete these results, it will be interesting to determine which BMPs are produced in the different pituitary cell types using double immunohistochemistry. The incubation of cultured ewe pituitary cells with BMP antibody specific for one selected BMP could help to discern the effect of endogenous BMP on FSH production. Moreover, it would be interesting to assay BMPs in the pituitary and in the blood but no assays are currently available. Nevertheless, the effects of antibody against BMP-6 or against BMP-7 on FSH release in the gonadotrope cell line strongly suggest a potential paracrine role for BMPs (Huang *et al.* 2001). Moreover, the common signaling pathway for BMPs was not activated in our control conditions although activated in presence of BMP-4 suggesting that BMPs act rather as endocrine factors. However, further experiments should investigate whether other signaling pathways can be triggered by BMPs such as MAPK, JAK/STAT or CDK (for review: von Bubnoff & Cho 2001, Miyazono *et al.* 2001, Nohe *at al.* 2004). Another suggestion would be to colocalise elements of the signaling pathway triggered by BMPs within gonadotrope cells. However, TGFβ superfamily signaling pathways are known to interact between each other so it could be difficult to distinguish whether BMPs or another member of the family triggered a selected pathway (Candia *et al.* 1997).

To summarize, BMPs do not seem to act as paracrine factors on gonadotropin production in the sheep in contrast to the mouse gonadotrope $L\beta T_2$ cell line. However, BMPs can be produced and act in the pituitary only in specific physiological conditions.

B. BMPs differentially affect the gonadotropin production

Having demonstrated that the BMP receptors and the BMPs were expressed in the ewe pituitary, further studies were carried out with primary sheep pituitary cell cultures to examine the potential effects of BMPs on gonadotropin secretion. A dose response study showed that

treatment with BMP-4 for 48h inhibited FSH secretion from pituitary cells in a dose-dependant manner. The maximal inhibition of 40% was obtained with doses from $5x10^{-10}$M (25ng/ml) of BMP-4. A time course experiment showed that a significant effect with 10^{-9}M BMP-4 (50ng/ml) was observed from 24h of incubation. The same inhibition of FSH secretion was found with $2x10^{-9}$ M BMP-6 (100 ng/ml), 10^{-9} M GDF-9 (50 ng/ml) or $6x10^{-9}$ M BMP-15 (200ng/ml) for 48h. Although all tested BMPs inhibited FSH production, each BMP was effective at different concentrations (from $5x10^{-10}$M to $6x10^{-9}$ M) maybe reflecting their affinity for their receptors. Moreover, the co-treatment with BMP-4 and BMP-6 showed no additive effect. We also found 40% of inhibition on FSHβ mRNA expression when cells were treated with 10^{-9}M BMP-4 (50ng/ml) or $2x10^{-9}$ M BMP-6 (100ng/ml) alone for 48h. This inhibitory effect on FSH secretion due to BMPs treatment on ewe pituitary cells was not found with LH secretion. Moreover, no significant effect was observed in presence of 10^{-9}M BMP-4 for 48h on prolactin secretion. These results show that several BMPs act preferentially on FSH secretion in the ewe pituitary cell culture by inhibiting FSHβ mRNA expression and its release. Our results were surprising as other studies showed that BMPs were able to stimulate FSH secretion. However, studies were undertaken in rodents. Similar doses of BMP-6 and BMP-7 had no effect on FSH synthesis and release in mouse pituitary cells or the $L\beta T_2$ gonadotrope cell line, whereas BMP-15 stimulated FSH secretion in rodents (Otsuka & Shimasaki 2002). The same stimulatory effect was found with BMP-6 and BMP-7 but only with a very high dose (1μg/ml, i.e. $2x10^{-8}$M) in mouse pituitary cells or $L\beta T_2$ cell line (Huang *et al.* 2001). As we found that several BMPs had the same effect on FSH secretion in the sheep pituitary primary cultures, we tested whether BMP-4 in our hands had a different effect on gonadotropin secretion in rodents.

A treatment with 10^{-9}M BMP-4 (50ng/ml) of the mouse $L\beta T_2$ cells, showed that BMP-4 was not able to modulate either FSH or LH production using the protocol previously described by Nicol and co-workers (2004). This result was similar to these found by Otsuka & Shimasaki using BMP-6 and BMP-7 (2002). However, they found a stimulatory effect of BMP-15. They also showed BMP-15 bound BMPR-IB in granulosa cells and activated Smad-1 phosphorylation (Moore *et al.* 2003). BMP-15 can also act via activin signaling pathway as it is able to bind weakly ALK-2 (ActRI) and ActRII and to stimulate weakly the phosphorylation of Smad-2 (Moore *et al.* 2003). In our experiments, BMP-4 was able to activate the phosphorylation of Smad1/5, not Smad-2. Moreover, we found an effect of BMP-4 on activin and GnRH-stimulated gonadotropin secretion. These very surprising and

interesting results showed BMP-4 was able to amplify the activin and GnRH-stimulatory effect on FSH production and to inhibit the activin and GnRH-stimulatory effect on LH secretion in the $L\beta T_2$ cell line. This result shows a differential effect of BMP-4 on gonadotropin production modulating the effects of activin and GnRH.

C. Which pathway(s) are triggered by BMPs for modulating the gonadotropin secretion?

The BMP common signaling pathway is via Smad1/5/8. The treatment with BMP-4 activated the phosphorylation of Smad1/5 in both ewe pituitary cells and mouse gonadotrope cells. However, whether the activation of Smad1/5 was responsible of the inhibition of FSH secretion due to BMP-4 treatment in the ewe pituitary cells is not known. Moreover, even though Smad1/5 was activated in $L\beta T_2$ cells, no effect of BMP-4 alone on FSH production was noticeable. This suggests a fundamental difference in the effect BMP among species. Adenoviral vector induced overexpression of Smad-6 which is a specific BMP signaling pathway inhibitor would allow to answer the question. Results showed that overexpression of Smad-6 did not prevent the BMP-4 inhibition on FSH synthesis and release. However, whether Smad-6 overexpression is able to block the signaling via Smad-1 has to be checked, particularly, if the interaction between the phosphorylated form of Smad-1 and Smad-4 is prevented. Adenoviral vector inducing overexpression of the TGFβ signaling pathway inhibitor Smad-7 reversed BMP-4 inhibition on FSH secretion in ewe pituitary cells. However, Smad-7 is also an activin signaling pathway inhibitor (Ishisaki *et al.* 1999), we cannot conclude whether BMP-4 effect while initially activating the phospho Smad-1, acts only through Smad1/5/8 or interacts with the Smad2/3 signaling pathways as both were inhibited. The signaling pathway suppression was confirmed by the reduction of the phosphorylated form of Smad1/5 and Smad-2. Then, to specifically block the activin signaling pathway, we used follistatin which binds activin. The co-treatment with both follistatin and BMP-4 showed a further decrease suggesting the independence of activin and BMP-4 signaling pathways in our system. In addition, BMP-4 was not able to modify Smad-2 phosphorylation. Nevertheless, BMP-4 may inhibit FSH production using other pathways as mitogen-activated protein kinase (MAPK), Ca2+/calmoduline, CDK or JAK/STAT signaling pathways (for review: von Bubnoff & Cho 2001, Miyazono *et al.* 2001, Nohe *et al.* 2004). Further experiments should determine which pathway is required for BMP-4 inhibition of FSH secretion in ewe pituitary cells and stimulation in the mouse gonadotrope cell line, $L\beta T_2$.

D. Which level(s) of gonadotropin regulation are affected by BMP effect?

BMP-4 is able to specifically inhibit FSH secretion in ewe pituitary cells and to differentially affect gonadotropin secretion in $L\beta T_2$ cells. Whatever the signaling pathway triggered by BMP-4, it is interesting to determine at what level FSH regulation could be modulated by BMP-4. This effect could act at gene level by modulating gene expression, at mRNA level by affecting stability like activin, at protein level through affecting translation or at the level of secretion. Moreover, to underline the complexity, the effect of BMP-4 could act directly on FSH promoter or via modulation of the expression of other proteins which would then affect the FSHβ promoter.

After Smad signaling pathway activation, the phosphorylated form of R-Smad proteins associates with a common partner, Smad-4 and translocates into the nucleus. The R-Smad-Smad4 complex (es) are then capable of binding DNA on a Smad binding element (SBE). The regulation of target gene requires additional interaction with transcription factors, either coactivators or corepressors (for review, Massague and Wotton 2000) and both SBEs and transcription factors may be cell type specific. For instance, a SBE was recently identified in the rat FSHβ subunit gene that is required for full activin responsiveness (Suszko *et al.* 2003). Interestingly, whereas this site is conserved in the mouse gene, it is not present in the ovine, bovine, porcine or human genes suggesting that important species-specific differences exist in activin regulation of FSHβ gene expression. Similar differences probably exist in BMP regulation of target genes. Given that BMP-15 was found to stimulate the activity of the ovine FSHβ promoter transfected into mouse $L\beta T_2$ gonadotrope cells, the presence of specific repressors rather than particular SBEs in ovine gonadotropes could be favored. Moreover, BMP-4 has a different effect depending on the species reinforcing the presence of species-specific cofactors. Alternatively, because we did not determine whether BMPs act directly on the FSHβ promoter, we cannot exclude the possibility that BMPs affect FSH production via another target gene in gonadotropes or other cell type.

In $L\beta T_2$ cell line, BMP-4 inhibited the LH production stimulated by both activin and GnRH. Activin A is known to increase both LHβ mRNA expression and LH secretion and to act synergistically with GnRH through the activation of transfected LHβ promoter (Nicol *et al.* 2004, Yamada *et al* 2004). The activation of Smad-binding and homeobox elements on LHβ promoter by activin was shown (Coss *et al.* 2005). Similarly to activin, response

elements specific to BMP signaling could be present on LHβ promoter and participate with GnRH and activin response elements to modulate LH production.

BMP-4 was able to inhibit oFSHβ mRNA expression by 40% in the ewe pituitary cells. Cycloheximide had a drastic inhibitory effect on its own on oFSHβ mRNA expression but not LHβ mRNA. This showed the importance of one or several proteins for maintaining the oFSHβ mRNA level or for synthesizing new transcripts and emphasizing the differences in t/2 of the mRNAs (see Brown *et al* 2001). Following our hypothesis that the FSH secretory pathway is more constitutive than LH secretory pathway, FSH new transcripts are translated and the new protein is secreted without being stored in electron dense secretory granules (McNeilly *et al* 2003). This hypothesis has been reinforced by studies on $L\beta T_2$ cells showing that LH, but not FSH, released in response to GnRH was co-released with SgII via a regulated, granin-dependent pathway, (Nicol *et al* 2002; 2004). Nevertheless, further experiments on kinetics should help to determine whether BMP-4 could affect transcription and mRNA expression of other proteins. Because BMP-4 inhibited FSH transcription, we should also test its impact on FSHβ mRNA stability by an actinomycin-D treatment especially since activin may act via Smad2/3-Smad 4 to stabilize FSHβ mRNA.

Although our experiments in sheep suggest that BMP-4 affects the synthesis of FSHβ mRNA rather than the translation into protein, we can not exclude an effect on the protein synthesis and the secretory process because of $L\beta T_2$ experiments showing also an effect on LH secretion. Protein synthesis could be tested by following the FSH incorporation of labelled amino acids in presence of BMP-4 treatment. Both FSH and LH are produced in the same gonadotrope cells but chromogranin A (CgA) was associated with secretory granules containing FSH and LH hormones whereas secretogranin II (SgII) was only associated with LH in the gonadotrope cells in rodents (Crawford *et al* 2002, Watanabe *et al.* 1991). We could suggest that, because in the mouse $L\beta T_2$ gonadotropes BMP-4 enhanced activin plus GnRH–stimulated FSH secretion and inhibited activin plus GnRH –stimulated LH secretion, BMP-4 could affect the secretory pathway of FSH and LH by modifying the storage of FSH and LH into specific secretory granules. Further experiments could determine the specific secretory granules selected by BMP effect by following SgII and CgA with FSH and LH or by electronic microscopy after BMP-4 treatment.

In conclusion, BMPs play a role in the differential regulation of gonadotropin secretion depending on the species. In sheep, BMPs inhibit specifically FSH synthesis and release without affecting LH production whereas in mouse $L\beta T_2$ gonadotrope cell line, BMP-4 has no effect on its own but amplified activin and GnRH-stimulated FSH secretion and inhibits activin and GnRH-stimulated LH production. Even if the Smad1/5/8 signaling pathway is activated, our experiments suggest that the different effects of BMPs in sheep and mouse gonadotropes may be related to the activation of different signaling pathways in these gonadotropes.

II. How BMPs interact with other gonadotropin modulators?

Gonadotropin synthesis and release are regulated by hypothalamic, gonadal and pituitary factors. The hypothalamic GnRH and the gonadal steroids particularly progesterone and oestradiol can act differentially on gonadotropin synthesis and release. In addition to GnRH and steroids, some members of the Transforming Growth Factor-Beta (TGF-β) superfamily, particularly activins and inhibins, are involved in specific regulation of FSH production, stimulating and inhibiting respectively FSH synthesis and release without affecting LH production.

A. The GnRH stimulatory effect on gonadotropin secretion is differentially modulated by BMP-4 depending on species

In ewe pituitary cells, we showed that the stimulatory effect of GnRH on FSH secretion was counterbalanced by BMP-4 treatment. However, our protocol using GnRH have to be modified to ensure the stimulatory effect of GnRH on FSH secretion. As previously shown (Nicol *et al.* 2002, 2004) mouse $L\beta T_2$ cells treated with both activin and GnRH secreted more FSH than with GnRH alone. A similar protocol should be used in sheep cell culture. In these conditions, and in complete contrast to the sheep, BMP-4 was able to amplify the stimulatory effect of both treatments on FSH secretion in the $L\beta T_2$ cell line. Surprisingly, BMP-4 also inhibited the stimulatory effect of activin and GnRH both on LH release and LHβ mRNA expression. More interestingly, BMP-4 also inhibited GnRH-R mRNA expression similar to the changes in LHβ mRNA expression. A recent study showed that using a perifusion system with $L\beta T_2$ gonadotrope cells allowing GnRH to be given in a series of pulses, LHβ gene promoter activity was stimulated at higher GnRH pulse frequencies whereas the FSHβ gene promoter was preferentially stimulated at lower GnRH pulse frequencies.

Moreover, GnRH-R number was increased at higher frequencies of pulsatile GnRH (Bédécarrats & Kaiser 2003). Therefore, our results showed with a single daily pulse of GnRH (which is a low frequency) that BMP-4, by inhibiting GnRH-R expression, could decrease the GnRH receptor numbers. As the density of GnRH receptor on gonadotropes determines their ability to respond to GnRH (Wise *et al.* 1984), we could suggest that a lower expression of GnRH receptors due to BMP-4 treatment could activate the FSHβ gene promoter and inhibits the LHβ gene promoter. These findings are reinforced by the different effects between sheep and rodents of another member of the TGFβ superfamily, activin on GnRH receptor expression and numbers in both species. In sheep, the number of GnRH receptors was was decreased by activin A (Gregg *et al.* 1991). In contrast, activin stimulated GnRH receptors number in rodents (Braden & Conn 1992). These results underline the difference among species. Therefore, BMP-4 may play a role in differential gonadotropin regulation by modulating the number of GnRH receptors.

B. The negative effect of oestradiol on FSH secretion is enhanced by BMP-4 in sheep

In additon to pituitary factors, another main regulator of FSH is estradiol. Estrogen receptors are expressed in gonadotropes in sheep (Sheng *et al.* 1998, Tobin *et al.* 2001), injections of 17-β estradiol in ewes reduced FSHβ mRNA concentrations and FSH release directly at the pituitary level (Mercer *et al.* 1993, Phillips *et al.* 1998, Molter-Gérard *et al.* 2000) while immunoneutralisation of estradiol increased plasma concentrations of FSH (Mann *et al.* 1990). Moreover, the treatment of ovine pituitary cells with 17-β estradiol showed a direct negative effect on the ovine FSH promoter (Miller & Miller 1996). In our study, estradiol also exerted a strong inhibition on FSHβ mRNA expression and FSH release. Moreover, BMP-4 amplified this effect of estradiol. In other tissues, it was shown that estrogens and BMPs could interact through overlapping intracellular signaling mechanisms (Yamamoto *et al.* 2002). In rodent and human pituitary prolactinomas where BMP-4 as well as 17-β estradiol induce cell proliferation, an additive effect of both BMP-4 and 17-β estradiol was observed (Paez-Pereda *et al.* 2003). In that model, Smad-1 and Smad-4 physically interacted with the estrogen receptor under BMP-4 stimulation (Paez-Pereda *et al.* 2003, Wu *et al.* 2003). To determine whether a similar mechanism can occur in ewe gonadotrope cells, co-immunoprecipitation studies could demonstrate that under BMP-4 stimulation some element of BMP signaling pathway could interact with the estrogen receptor. Moreover, the

analysis of estrogen receptor promoter may identify response elements related to the BMP effect on FSH secretion.

C. The local specific FSH stimulatory factor, activin, is antagonized by BMP-4 in sheep and not affected in the mouse gonadotrope cell line

Local factors like activins and inhibins preferentially act on FSH secretion by stimulating and inhibiting respectively FSHβ gene transcription, translation and FSH production (De Kretser & Robertson 1989, Vale *et al.* 1990, Ling *et al.* 1986, Vale *et al.* 1986). Inhibin and activin subunits were detected in the rat by immunolocalization in the ovary (especially granulosa cells, theca cells) and anterior pituitary (Meunier *et al.* 1988). Receptors for activin were also identified in granulosa and theca cells, oocyte and the pituitary (Cameron *et al.* 1998, Fafioffe *et al.* 2004) indicating an autocrine/paracrine role of activin (review Findlay *et al.* 2001, Pangas *et al.* 2002). The incubation of cultured rat pituitary cells with a mouse monoclonal antibody specific for the activin B reduced the FSHβ mRNA expression and FSH concentration without affecting LH production (Corrigan *et al.* 1991). As activins are a main regulator of FSH production and had the opposite effect of BMPs, we treated the ewe pituitary cells with both 10^{-9}M BMP-4 and $2x10^{-9}$M activin-A for 48h. Results showed that BMP-4 antagonized the stimulatory effect of activin. In contrast, no effect was noticeable on either FSH or LH production in $L\beta T_2$ cells suggesting a different mode of regulation in rodents. Because of the interaction between BMP-4 and activin in the ewe pituitary, we tried to determine at which level they could interact. At an intracellular level, in both cell types, the BMP-4 induced phosphorylation of Smad-1 was not affected by activin and the activin-induced phosphorylation of Smad-2 was not affected by BMP-4. Thus, the activin and BMP Smad pathways do not appear to interact at this level, and the results of our last experiments using the adenoviral Smad-6 and -7 vectors suggest that BMP could act via other pathways. Nevertheless, further experiments should determine whether BMP pathway could interact with activin pathway. However, other levels of interaction need to be examined, particularly at the level of ligands, receptors and at promoter regulation. Co-immunoprecipitation would help to discern if BMP could interact with activin itself and with which kind of receptor. However, results on follistatin experiments showed that BMP-4 decreased FSH production even more than follistatin alone suggesting independent pathways. As studies on gene promoters showed that activin regulated the rat FSHβ promoter by consensus Smad-binding element(s) in the mouse $L\beta T_2$ gonadotrope cell line (Suszko *et al.*

2003), we can suggest competition between BMP and activin signaling pathways transcription factors. Moreover, GnRH activated the FSHβ promoter, both GnRH and activin A had synergistic role on FSHβ promoter stimulation, and this effect was abolished by mutation of the Smad-binding element (Gregory *et al.* 2005). In our $L\beta T_2$ cell experiments, because we found an interaction in presence of GnRH, we can suggest that the transcription factor(s) induced by BMP-4 could potentially bind DNA on this important Smad-binding element required for the synergistic effect of GnRH and activin. The same experiment could be done on the LHβ promoter as Yamada and others (2004) found that activin A was able to increase both LHβ mRNA expression and LH secretion and to increase synergistically with GnRH a transfected LHβ promoter-reporter construct in $L\beta T_2$ cells.

In conclusion, BMP-4 effect interacts with the other gonadotropin modulators GnRH, oestradiol and activins. In sheep, BMP-4 inhibits the stimulatory effect of GnRH and activin-A and is additive to the inhibitory effect of oestradiol on both oFSHβ mRNA expression and FSH release without affecting LH secretion. In sheep, the effect of BMPs may interact with activin by modulating directly its signaling pathways thus affecting the FSHβ promoter, or indirectly by modulating intermediary, as yet unidentified, factors like inhibitors or cofactors. In contrast, in the mouse $L\beta T_2$ gonadotrope cell line, the main role of BMPs is to modulate GnRH effect, acting to affect both FSH and LH production. BMP effect could influence GnRH effect by modulating GnRH receptor expression and then, GnRH receptor numbers. This interaction with other modulators underlines the important role of BMPs on differential gonadotropin secretion depending on species.

III. Is there a physiological role for BMPs in the differential regulation of gonadotropin secretion?

BMPs affect the differential regulation of gonadotropin secretion. However, we would like to determine whether BMPs effect is reproducible in physiological conditions, during the oestrous cycle and in *in vivo* experiments.

A. Do other factors modulate BMPs expression?

To determine whether BMP-4 mRNA expression is modulated by FSH regulators, ewe pituitary cells were treated with 10^{-9}M BMP-4, $2x10^{-9}$M activin and 10^{-13}M 17β- oestradiol

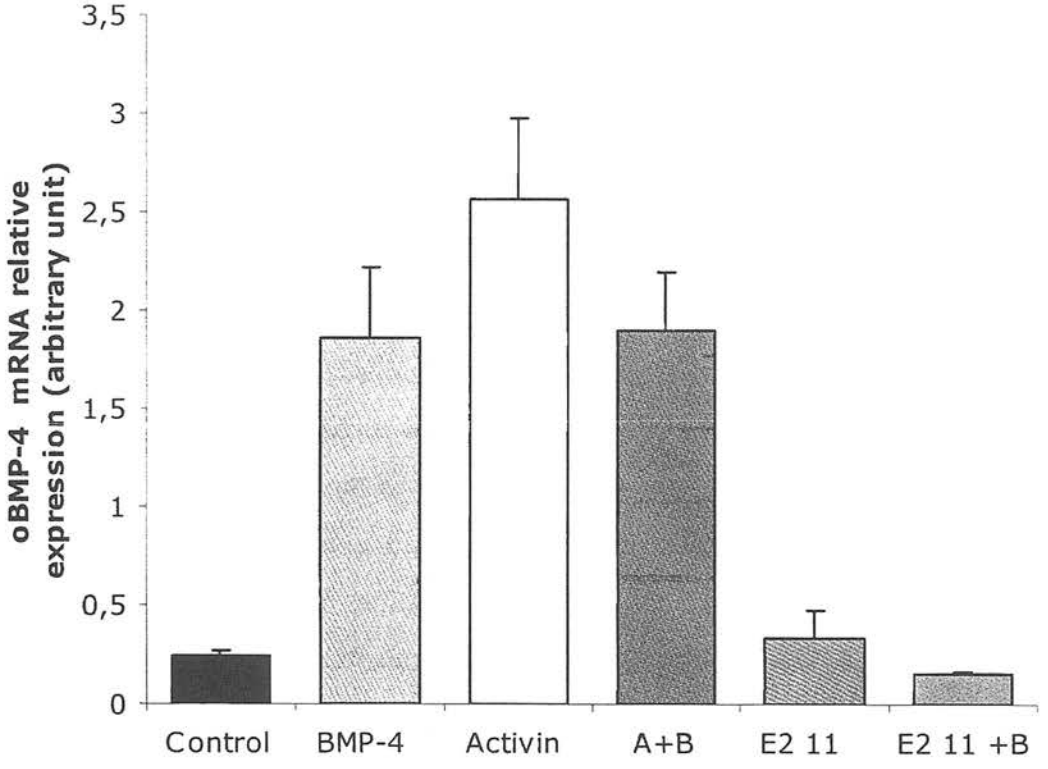


Fig. 45. **Effect of activin and oestradiol treatment on BMP-4 mRNA expression relative level**. Ewe pituitary cells were treated with 10^{-9}M BMP-4, 2.10^{-9}M activin, both, 10^{-11}M E2, 10^{-11}M E2 + 10^{-9}M BMP-4 for 48h. Total mRNA were extracted and real time PCR was performed.

for 48h. Preliminary data showed that activin was able to stimulate by 2-fold oBMP-4 mRNA expression whereas oestradiol had no effect (Fig.45). This result reinforced the possibility that endogenous BMP synthesis is modulated by local factors.

B. Does the effect of BMPs depend on the stage of the oestrous cycle?

The effects of BMP-4 treatment was examined in cell cultures from non-cyclic ewe at different times throughout the year and no difference was noticeable between experiments. Nevertheless, it would be interesting to look at the effect of BMPs on FSH secretion at different stages of the oestrous cycle. Maybe, BMPs are present all the time but during the FSH second surge, they could be blocked by antagonists or gonadotrope cells are less responsive to the effects of BMPs. To test these two hypotheses, we should determine the pattern of expression of BMP antagonists, during the oestrous cycle, and test, by *in vitro* studies, the effect of BMPs on FSH secretion in cell cultures of pituitaries collected at different stages of the sheep oestrous cycle. When the BMP assays become available, it would be also interesting to follow their concentrations during the oestrous cycle.

C. Are BMPs able to modulate gonadotropin secretion *in vivo*?

Our results on BMP effect were obtained on ewe pituitary cells which were allowed to attach for two days before being treated. During this time, the cells are obviously not exposed to the *in vivo* modulators such as circulating steroids or other growth factors. To complete our study, *in vivo* experiments would help to determine the physiological effect of BMPs. BMPs can be directly delivered in the pituitary by stereotaxical approach. However, the quantity of BMPs delivered would be still important. Alternatively, adenoviral vectors expressing BMP-4 or signaling pathway inhibitor can be delivered into the ovine pituitary gland by stereotaxy This original approach was successfully developed by Davis and co-workers (2001). Our results showed a very high efficiency of adenovirus infection in ewe pituitary cells in culture, confirming the efficacy of this method. After determining the signaling pathway(s) required for BMPs to inhibit FSH secretion, we could transfect cells with a specific inhibitor mediated by adenoviral vectors.

Meanwhile, natural mutations have been described and affected folliculogenesis in sheep. Booroola ewes are hyper-prolific due to a mutation in the intracellular serine/threonine kinase domain of BMPR-IB. Two groups found higher FSH concentrations in the presence of the mutation attributed to a deficiency in ovarian feedback (Bindon *et al.* 1984, McNatty *et al.*

1987) while one group found no difference in gonadotropin secretion between the two genotypes (Souza *et al.* 1997). A study of gonadotropin subunits mRNA expression in pituitaries by Northern blots showed no significant difference between the FecB carrier and the non-carrier genotypes (Fleming *et al.* 1995). In our experiments, we found the same 40% inhibition on FSH secretion in both genotypes. Moreover, we showed that the BMPR-IB was not expressed in gonadotrope cells by immunohistochemistry in the sheep pituitary. We can not exclude a very low expression of this receptor in gonadotropes, but on-going experiments will determine which pituitary cell type expresses this receptor. Therefore, we would like to measure changes in the hormone corresponding to the cell type expressing the BMPR-IB and determine whether the mutation of BMPR-IB could affect its secretion. Another sheep breed is known for hyper-prolificacy, the Inverdale ewes have a mutation in the *bmp-15* gene. In homozygous $FecX^{I}/FecX^{I}$ ewes, gonadotropin plasma concentrations are high whereas no significant difference is noticeable for heterozygous ewes compared to wild type. With our results showing the inhibition of FSH by BMP-15, we cannot exclude a role at the pituitary level. It would be interesting to test whether BMP-15 treatment on pituitary cell culture from homozygous $FecX^{I}/FecX^{I}$ ewes would reverse the high level of FSH concentration.

In conclusion, for the first time, our data provide evidence for a role of BMPs in the differential regulation of gonadotropin synthesis and release in ewe pituitary. Furthermore, we demonstrate their ability to interplay with main FSH modulators.

References

A

Abraham IM, Han SK, Todman MG, Korach KS & Herbison AE 2003 Estrogen receptor beta mediates rapid estrogen actions on gonadotropin-releasing hormone neurons in vivo. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **23**: 5771-5777.

Amstalden M, Zieba DA, Garcia MR, Stanko RL, Welsh TH Jr, Hansel WH & Williams GL 2004 Evidence that lamprey GnRH-III does not release FSH selectively in cattle. *Reproduction* **127**: 35-43.

Andres JL, Stanley K, Cheifetz S & Massague J 1989 Membrane-anchored and soluble forms of betaglycan, a polymorphic proteoglycan that binds transforming growth factor-beta. *The Journal of cell biology* **109**: 3137-3145.

Anouar Y & Duval J 1991 Differential expression of secretogranin II and chromogranin A genes in the female rat pituitary through sexual maturation and estrous cycle. *Endocrinology* **128**: 1374-1380.

Arai K, Taya WT & Sasamoto S 1996 Roles of inhibin and estradiol in the regulation of follicle-stimulating hormone and luteinizing hormone secretion during the estrous cycle of the rat. *Biology of Reproduction* **55**: 127-133.

Attisano L &Wrana JL 2000 Smads as transcriptional co-modulators. *Current opinion in cell biology* **12**: 235-243. Review

Attisano L & Wrana JL 2002 Signal transduction by the TGF-β superfamily. Science **296**: 1646-1647.

Aubin J, Davy A & Soriano P 2004 In vivo convergence of BMP and MAPK signaling pathways: impact of differential Smad1 phosphorylation on development and homeostasis. *Genes & Development* **18**: 1482-1494.

B

Baenziger JU & Green ED 1988 Pituitary glycoprotein hormone oligosaccharides: structure, synthesis and function of the asparagine-linked oligosaccharides on lutropin, follitropin and thyrotropin. *Biochimica et biophysica acta* **947**: 287-306. Review

Bai RY, Koester C, Ouyang T, Hahn SA, Hammerschmidt M, Peschel C & Duyster 2002 SMIF, a Smad4-interacting protein that functions as a co-activator in TGFbeta signalling. *Nature cell biology* **4**: 181-190.

Bai S, Shi X, Yang X & Cao X 2000 Smad6 as a transcriptional corepressor. *Journal of Biological Chemistry* **275**: 8267-8270.

Bailey JS, Rave-Harel N, McGillivray SM, Coss D & Mellon PL 2004 Activin regulation of the follicle-stimulating hormone beta-subunit gene involves Smads and the TALE homeodomain proteins Pbx1 and Prep1. *Molecular Endocrinolology* **18**: 1158-1170.

Balemans W & Van Hul W 2002 Extracellular regulation of BMP signaling in vertebrates: a cocktail of modulators. *Developmental Biology* **250**: 231-250. Review.

Baratta M, West LA, Turzillo AM & Nett TM 2001 Activin modulates differential effects of estradiol on synthesis and secretion of follicle-stimulating hormone in ovine pituitary cells. *Biology of reproduction* **64**: 714-719.

Bartlewski PM, Beard AP, Cook SJ, Chandolia RK, Honaramooz A & Rawlings NC 1999 Ovarian antral follicular dynamics and their relationships with endocrine variables throughout the oestrous cycle in breeds of sheep differing in prolificacy. *Journal of reproduction and fertility* **115**: 111-124.

Batra SK & Miller WL 1985 Progesterone decreases the responsiveness of ovine pituitary cultures to luteinizing hormone-releasing hormone. *Endocrinology* **117**: 1436-1440.

Batra SK & Miller WL 1985 Progesterone inhibits basal production of follicle-stimulating hormone in ovine pituitary cell culture. *Endocrinology* **117**: 2443-2448.

Beato M & Klug J 2000 Steroid hormone receptors: an update. *Human reproduction update* **6**: 225-236. Review.

Bédécarrats GY, Linher KD & Kaiser UB 2003 Two common naturally occurring mutations in the human gonadotropin-releasing hormone (GnRH) receptor have differential effects on gonadotropin gene expression and on GnRH-mediated signal transduction. *The Journal of clinical endocrinology and metabolism* **88**: 834-843.

Bédécarrats GY & Kaiser UB 2003 Differential regulation of gonadotropin subunit gene promoter activity by pulsatile gonadotropin-releasing hormone (GnRH) in perifused L beta T2 cells: role of GnRH receptor concentration. *Endocrinology* **144**: 1802-1811.

Benus GF, Wierenga AT, de Gorter DJ, Schuringa JJ, van Bennekum AM, Drenth-Diephuis L, Vellenga E & Eggen BJ 2005 Inhibition of the transforming growth factor beta (TGFbeta) pathway by interleukin-1beta is mediated through TGFbeta-activated kinase 1 phosphorylation of SMAD3. *Molecular biology of the cell* **16**: 3501-3510.

Bernard DJ 2004 Both SMAD2 and SMAD3 mediate activin-stimulated expression of the follicle-stimulating hormone beta subunit in mouse gonadotrope cells. *Molecular Endocrinology* **18**: 606-623.

Besecke LM, Guendner MJ, Sluss PA, Polak AG, Woodruff TK, Jameson JL, Bauer-Dantoin AC & Weiss J 1997 Pituitary follistatin regulates activin-mediated production of follicle-stimulating hormone during the rat estrous cycle. *Endocrinology* **138**: 2841-2848.

Bilezikjian LM, Corrigan AZ, Blount AL, Chen Y & Vale WW 2001 Regulation and actions of Smad7 in the modulation of activin, inhibin, and transforming growth factor-beta signaling in anterior pituitary cells. *Endocrinology* **142**: 1065-1072.

Bilezikjian LM, Blount AL, Leal AM, Donaldson CJ, Fisher WH & Vale WW 2004 Autocrine/paracrine regulation of pituitary function by activin, inhibin and follistatin. *Molecular and Cellular Endocrinology* **225**: 29-36. Review.

Bindon BM 1984 Reproductive biology of the Booroola Merino sheep. *Australian journal of biological sciences* **37**: 163-189. Review.

Blomenrohr M, Heding A, Sellar R, Leurs R, Bogerd J, Eidne KA & Willars GB. 1999 Pivotal role for the cytoplasmic carboxyl-terminal tail of a nonmammalian gonadotropin-releasing hormone receptor in cell surface expression, ligand binding, and receptor phosphorylation and internalization. *Molecular pharmacology* **56**: 1229-1237. Erratum in: Mol Pharmacol 2000 Jan;57(1):211.

Bouamoud N, Lerrant Y, Ribot G & Counis R 1992 Differential stability of mRNAs coding for alpha and gonadotropin beta subunits in cultured rat pituitary cells. *Molecular and Cellular Endocrinology* **88**: 143-151.

Bousfield GR, Perry WM & Darrell NW 1994 Gonadotropins. Chemistry and biosynthesis. *In*: Knobil E, Neill J, eds. The physiology of reproduction. New York: Raven Press. 1749-1788.

Braden TD & Conn PM 1992 Activin-A stimulates the synthesis of gonadotropin-releasing hormone receptors. *Endocrinology* **130**: 2101-2105.

Braden TD & Conn PM 1992 Activin-A stimulates the synthesis of gonadotropin-releasing hormone receptors. *Endocrinology* **130**: 2101-2105.

Braw-Tal R, McNatty KP, Smith P, Heath DA, Hudson NL, Phillips DJ, McLeod BJ & Davis GH 1993 Ovaries of ewes homozygous for the X-linked Inverdale gene (FecXI) are devoid of secondary and tertiary follicles but contain many abnormal structures. *Biology of Reproduction* **49**: 895-907.

Brooks J, Crow WJ, McNeilly JR & McNeilly AS 1992 Relationship between gonadotrophin subunit gene expression, gonadotrophin-releasing hormone receptor content and pituitary and plasma gonadotrophin concentrations during the rebound release of FSH

after treatment of ewes with bovine follicular fluid during the luteal phase of the cycle. *Journal of molecular endocrinology* **8**: 109-118.

Brooks J, Taylor PL, Saunders PT, Eidne KA, Struthers WJ & McNeilly AS 1993 Cloning and sequencing of the sheep pituitary gonadotropin-releasing hormone receptor and changes in expression of its mRNA during the estrous cycle. *Molecular and cellular endocrinology* **94**: R23-R7.

Brooks J & McNeilly AS 1994 Regulation of gonadotrophin-releasing hormone receptor mRNA expression in the sheep. *Journal of Endocrinology* **143**: 175-182.

Brown P, McNeilly JR, Evans JG, Crawford GM, Walker M, Christian HC & McNeilly AS 2001 Manipulating the in vivo mRNA expression profile of FSH beta to resemble that of LH beta does not promote a concomitant increase in intracellular storage of follicle-stimulating hormone. *Journal of Neuroendocrinology* **13**: 50-62.

von Bubnoff & Cho KWY 2001 Intracellular BMP signaling regulation in vertebrates: pathway or network ? *Developmental Biology* **239**: 1-14.

Burger LL, Dalkin AC, Aylor KW, Workman LJ, Haisenleder DJ & Marshall JC 2001 Regulation of gonadotropin subunit transcription after ovariectomy in the rat: measurement of subunit primary transcripts reveals differential roles of GnRH and inhibin. *Endocrinology* **142**: 3435-3442.

Burger LL, Dalkin AC, Aylor KW, Haisenleder J & Marshall JC 2002 GnRH pulse frequency modulation of gonadotropins subunit gene transcription in normal gonadotropes-Assessment by primary transcript assay provides evidence for roles of GnRH and follistatin. *Endocrinology* **143**: 3243-3249.

Burger LL, Haisenleder DJ, Dalkin AC & Marshall JC 2004 Regulation of gonadotropin subunit gene transcription. *Journal of molecular endocrinology* **33**: 559-584. Review.

Burgon PG, Stanton PG & Robertson DM 1996 In vivo bioactivities and clearance patterns of highly purified human luteinizing hormone isoformes. *Endocrinology* **137**: 4827-4836.

C

de Caestecker MP, Yahata T, Wang D, Parks WT, Huang S, Hill CS, Shioda T, Roberts AB & Lechleider RJ 2000 The Smad4 activation domain (SAD) is a proline-rich, p300-dependent transcriptional activation domain. *The Journal of biological chemistry* **275**: 2115-2122.

Cameron VA, Nishimura E, Mathews LS, Lewis KA, Sawchenko PE & Vale WW 1994 Hybridization histochemical localization of activin receptor subtypes in rat brain, pituitary, ovary, and testis. *Endocrinology* **134**: 799-808.

Candia AF, Watabe T, Hawley SH, Onichtchouk D, Zhang Y, Derynck R, Niehrs C & Cho KW 1997 Cellular interpretation of multiple TGF-beta signals: intracellular antagonism between activin/BVg1 and BMP-2/4 signaling mediated by Smads. *Development* **124**: 4467-4480.

Caraty A, Martin GB & Montgomery G 1984 A new method for studying pituitary responsiveness in vivo using pulses of LH-RH analogue in ewes passively immunized against native LH-RH. *Reproduction, nutrition, development* **24**: 439-448.

Carroll RS, Corrigan AZ, Gharib SD, Vale W & Chin WW 1989 Inhibin, activin, and follistatin: regulation of follicle-stimulating hormone messenger ribonucleic acid levels. *Molecular Endocrinology* **3**: 1969-1976.

Carroll RS, Corrigan AZ, Vale W & Chin WW 1991 Activin stabilizes follicle-stimulating hormone-beta messenger ribonucleic acid levels. *Endocrinology* **129**: 1721-1726.

Casellas R & Brivanlou AH 1998 Xenopus Smad7 inhibits both the activin and BMP pathways and acts as a neural inducer. *Developmental biology* **198**: 1-12.

Centrella M, McCarthy TL, Canalis E 1987 Transforming growth factor beta is a bifunctional regulator of replication and collagen synthesis in osteoblast-enriched cell cultures from fetal rat bone. *The Journal of biological chemistry* **262**: 2869-2874.

Chapman SC & Woodruff TK 2003 Betaglycan localization in the female rat pituitary: implications for the regulation of FSH by inhibin. *Endocrinology* **144**: 5640-5649.

Chen YG, Liu F & Massague J 1997 Mechanism of TGFbeta receptor inhibition by FKBP12. *The EMBO Journal* **16**: 3866-3876.

Chen YG, Hata A, Lo RS, Wotton D, Shi Y, Pavletich N & Massague J 1998 Determinants of specificity in TGF-beta signal transduction. *Genes & development* **12**: 2144-2152.

Chen J, Hersmus N, Van Duppen V, Caesens P, Denef C & Vankelecom H 2005 The adult pituitary contains a cell population displaying stem/progenitor cell and early embryonic characteristics. *Endocrinology* **146**: 3985-3998.

Cherrington BD, Farmerie TA, Lents CA, Cantlon JD, Roberson MS & Clay CM 2005 Activin responsiveness of the murine gonadotropin-releasing hormone receptor gene is mediated by a composite enhancer containing spatially distinct regulatory elements. *Molecular Endocrinolology* **19**: 898-912.

Childs GV, Ellison DG & Garner LL 1980 An immunocytochemist's view of gonadotropin storage in the adult male rat: cytochemical and morphological heterogeneity in serially sectioned gonadotropes. *The American journal of anatomy* **158**: 397-409.

Childs GV 1985 Shifts in gonadotropin storage in cultured gonadotropes following GnRH stimulation, in vitro. *Peptides* **6**: 103-107.

Childs GV, Unabia G, Tibolt R & Lloyd JM 1987 Cytological factors that support nonparallel secretion of luteinizing hormone and follicle-stimulating hormone during the estrous cycle. *Endocrinology* **121**: 1801-1813. Erratum in: *Endocrinology* 1988 **122**:1436.

Childs GV, Unabia G & Komak S 2001 Differential expression of estradiol receptors alpha and beta by gonadotropes during the estrous cycle. *The Journal of Histochemistry & Cytochemistry* **49**: 665-666.

Clark DE, Tisdall DJ, Fidler AE & McNatty KP 1996 Localization of mRNA encoding c-kit during the initiation of folliculogenesis in ovine fetal ovaries. *Journal of reproduction and fertility* **106**: 329-335.

Clarke IJ, Cummins JT & de Kretser DM 1983 Pituitary gland function after disconnection from direct hypothalamic influences in the sheep. *Neuroendocrinology* **36**: 376-384.

Clarke IJ & Cummins JT 1984 Direct pituitary effects of estrogen and progesterone on gonadotropin secretion in the ovariectomized ewe. *Neuroendocrinology* **39**: 267-274.

Clarke IJ, Cummins JT, Crowder ME & Nett TM 1987 Pituitary receptors for gonadotropin-releasing hormone in relation to changes in pituitary and plasma luteinizing hormone in ovariectomized-hypothalamo pituitary disconnected ewes. I. Effect of changing frequency of gonadotropin-releasing hormone pulses. *Biology of Reproduction* **37**: 749-754.

Clarke IJ, Cummins JT, Crowder ME & Nett TM 1988 Pituitary receptors for gonadotropin-releasing hormone in relation to changes in pituitary and plasma gonadotropins in ovariectomized hypothalamo/pituitary-disconnected ewes. II. A marked rise in receptor number during the acute feedback effects of estradiol. *Biology of Reproduction* **39**: 349-354.

Clarke IJ, Cummins JT, Jenkin M & Phillips DJ 1989 The oestrogen-induced surge of LH requires a 'signal' pattern of gonadotrophin-releasing hormone input to the pituitary gland in the ewe. *The Journal of endocrinology* **122**: 127-134.

Clarke IJ, Rao A, Fallest PC & Shupnik MA 1993 Transcription rate of the follicle stimulating hormone (FSH) beta subunit gene is reduced by inhibin in sheep but this

does not fully explain the decrease in mRNA. *Molecular and cellular endocrinology* **91**: 211-216.

Clarke IJ 2002 Multifarious effects of estrogen on the pituitary gonadotrope with special emphasis in the ovine species. *Archives of Physiology and Biochemistry* **110**: 62-73. Review.

Clarke IJ & Pompolo S 2005 Synthesis and secretion of GnRH. *Animal reproduction science* **88**: 29-55.

Condon TP, Sawyer CH, Heber D, Stewart JM & Whitmoyer DI 1985 Postcastration rise in plasma gonadotropins is blocked by a luteinizing hormone-releasing hormone antagonist. *Biology of reproduction* **33**: 715-721.

Corbani M, Counis R, Starzec A & Jutisz M 1984 Effect of gonadectomy on pituitary levels of mRNA encoding gonadotropin subunits and secretion of luteinizing hormone. *Molecular and cellular endocrinology* **35**: 83-87.

Corrigan AZ, Bilezikjian LM, Caroll RS, Bald LN, Schmelzer CH, Fendly BM, Mason AJ, Chin WW, Schwall RH & Vale W 1991 Evidence for an autocrine role of activin B within rat anterior pituitary cultures. *Endocrinology* **128**: 1682-1684.

Coss D, Jacobs SB, Bender CE & Mellon PL 2004 A novel AP-1 site is critical for maximal induction of the follicle-stimulating hormone beta gene by gonadotropin-releasing hormone. *The Journal of biological chemistry* **279**: 152-162.

Coss D, Thackray VG, Deng CX & Mellon PL 2005 Activin Regulates Luteinizing Hormone {beta}-Subunit Gene Expression through Smad-Binding and Homeobox Elements. *Molecular endocrinology* **19**: 2610-2633.

Counis R, Laverriere JN, Garrel G, Bleux C, Cohen-Tannoudji J, Lerrant Y, Kottler ML & Magre S 2005 Gonadotropin-releasing hormone and the control of gonadotrope function. *Reproduction, nutrition, development* **45**: 43-254.

Cowley MA, Rao A, Wright PJ, Illing N, Millar RP & Clarke IJ 1998 Evidence for differential regulation of multiple transcripts of the gonadotropin releasing hormone receptor in the ovine pituitary gland; effect of estrogen. *Molecular and cellular endocrinology* **146**: 141-149.

Crawford JL, Currie RJ & McNeilly AS 2000 Replenishment of LH stores of gonadotrophs in relation to gene expression, synthesis and secretion of LH after the preovulatory phase of the sheep oestrous cycle. *Journal of Endocrinology* **167**: 453-463.

Crawford JL, McNeilly JR, Nicol L & McNeilly AS 2002 Promotion of intragranular co-aggregation with LH by enhancement of secretogranin II storage resulted in increased

intracellular granule storage in gonadotrophs of GnRH-deprived male mice. *Reproduction* **124**: 267-277.

Crawford JL & McNeilly AS 2002 Co-localisation of gonadotrophins and granins in gonadotrophs at different stages of the oestrous cycle in sheep. *Journal of Endocrinology* **174**: 179-194.

Culler MD & Negro-Vilar A 1986 Evidence that pulsatile follicle-stimulating hormone secretion is independent of endogenous luteinizing hormone-releasing hormone. *Endocrinology* **118**: 609-612.

Cupp AS, Kojima FN, Roberson MS, Stumpf TT, Wolfe MW, Werth LA, Kittok RJ, Grotjan HE & Kinder JE 1995 Increasing concentrations of 17b-estradiol has differential effects on secretion of luteinizing hormone and follicle-stimulating hormone and amounts of mRNA for gonadotropin subunits during the follicular phase of the bovine estrous cycle. *Biology of Reproduction* **52**: 288-296.

Currie RJ & McNeilly AS 1995 Mobilization of LH secretory granules in gonadotrophs in relation to gene expression, synthesis and secretion of LH during the preovulatory phase of the sheep oestrous cycle. *Journal of Endocrinology* **147**: 259-270.

D

Dacheux F 1978 Ultrastructural localization of gonadotrophic hormones in the porcine pituitary using the immunoperoxidase technique. *Cell and tissue research* **191**: 219-232.

Dalkin AC, Haisenleder DJ, Ortolano GA, Ellis TR & Marshall JC 1989 The frequency of gonadotropin-releasing-hormone stimulation differentially regulates gonadotropin subunit messenger ribonucleic acid expression. *Endocrinology* **125**: 917-924.

Dalkin AC, Haisenleder DJ, Ortolano GA, Suhr A & Marshall JC 1990 Gonadal regulation of gonadotropin subunit gene expression: evidence for regulation of follicle-stimulating hormone-beta messenger ribonucleic acid by nonsteroidal hormones in female rats. *Endocrinology* **127**: 798-806.

Dalkin AC, Knight CD, Shupnik MA, Haisenleder DJ, Aloi J, Kirk SE, Yasin M & Marshall JC 1993 Ovariectomy and inhibin immunoneutralization acutely increase follicle-stimulating hormone-beta messenger ribonucleic acid concentrations: evidence for a nontranscriptional mechanism. *Endocrinology* **132**: 1297-1304. Erratum in: *Endocrinology* 1997 **138** :5496.

Dalkin AC, Haisenleder DJ, Gilrain JT, Aylor K, Yasin M & Marshall JC 1999 Gonadotropin-releasing hormone regulation of gonadotropin subunit gene expression in

female rats: actions on follicle-stimulating hormone beta messenger ribonucleic acid (mRNA) involve differential expression of pituitary activin (beta-B) and follistatin mRNAs. *Endocrinology* **140**: 903-908.

Dasen JS, O'Connell SM, Flynn SE, Treier M, Gleiberman AS, Szeto DP, Hooshmand F, Aggarwal AK & Rosenfeld MG 1999 Reciprocal interactions of Pit1 and GATA2 mediate signaling gradient-induced determination of pituitary cell types. *Cell* **97**: 587-598.

Datto M & Wang XF 2005 Ubiquitin-mediated degradation a mechanism for fine-tuning TGF-beta signaling. *Cell* **121**: 2-4.

Davidson JS, Wakefield IK & Millar RP 1994 Absence of rapid desensitization of the mouse gonadotropin-releasing hormone receptor. *The Biochemical journal* **300**: 299-302.

Davis GH, McEwan JC, Fennessy PF, Dodds KG & Farquhar PA 1991 Evidence for the presence of a major gene influencing ovulation rate on the X chromosome of sheep. *Biology of Reproduction* **44**: 620-624.

Davis JR, McVerry J, Lincoln GA, Windeatt S, Lowenstein PR, Castro MG & McNeilly AS 2001 Cell type-specific adenoviral transgene expression in the intact ovine pituitary gland after stereotaxic delivery: an in vivo system for long-term multiple parameter evaluation of human pituitary gene therapy. *Endocrinology* **142**: 795-801.

Davis GH, Balakrishnan L, Ross IK, Wilson T, Galloway SM, Lumsden BM, Hanrahan JP, Mullen M, Mao XZ, Wang GL, Zhao ZS, Zeng YQ, Robinson JJ, Mavrogenis AP, Papachristoforou C, Peter C, Baumung R, Cardyn P, Boujenane I, Cockett NE, Eythorsdottir E, Arranz JJ & Notter DR 2005 Investigation of the Booroola (FecB) and Inverdale (FecX(I)) mutations in 21 prolific breeds and strains of sheep sampled in 13 countries. *Animal reproduction science* Jun 24; in press, available at http://www.elsevier.com

ten Dijke P, Yamashita H, Ichijo H, Franzen P, Laiho M, Miyazono K & Heldin CH 1994 Characterization of type I receptors for transforming growth factor-beta and activin. *Science* **264**: 101-104.

ten Dijke P, Yamashita H, Sampath TK, Reddi AH, Estevez M, Riddle DL, Ichijo H, Heldin CH & Miyazono K 1994 Identification of type I receptors for osteogenic protein-1 and bone morphogenetic protein-4. *Journal of Biological Chemistry* **269**: 16985-16988.

Dong J, Albertini DF, Nishimori K, Kumar TR, Lu N & Matzuk M 1996 Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature* **383**: 531-535.

Donaldson CJ, Vaughan JM, Corrigan AZ, Fischer WH &Vale WW 1999 Activin and inhibin binding to the soluble extracellular domain of activin receptor II. *Endocrinology* **140**: 1760-1766.

Driancourt MA, Gauld IK, Terqui M & Webb R 1986 Variations in patterns of follicle development in prolific breeds of sheep. *Journal of reproduction and fertility* **78**: 565-575.

Dudley AT, Lyons KM & Robertson EJ 1995 A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye. *Genes & Development* **9**: 2795-2807.

Dupont J, McNeilly J, Vaiman A, Canepa S, Combarnous Y & Taragnat C 2003 Activin signaling pathways in ovine pituitary and LbetaT2 gonadotrope cells. *Biology of Reproduction* **68**: 1877-1887.

Durlinger AL, Visser JA, Themmen AP 2002 Regulation of ovarian function: the role of anti-Mullerian hormone. *Reproduction* **124**: 601-609. Review.

Duval DL, Ellsworth BS & Clay CM 1999 Is gonadotrope expression of the gonadotropin releasing hormone receptor gene mediated by autocrine/paracrine stimulation of an activin response element? *Endocrinology* **140**: 1949-1952.

E

Ebisawa T, Fukuchi M, Murakami G, Chiba T, Tanaka K, Imamura T & Miyazono K 2001 Smurf1 interacts with transforming growth factor-beta type I receptor through Smad7 and induces receptor degradation. *Journal of Biological Chemistry* **276**: 12477-12480.

Eickelberg O, Centrella M, Reiss M, Kashgarian M & Wells RG 2002 Betaglycan inhibits TGF-beta signaling by preventing type I-type II receptor complex formation. Glycosaminoglycan modifications alter betaglycan function. *Journal of Biological Chemistry* **277**: 823-829.

Ericson J, Norlin S, Jessell TM & Edlund T 1998 Integrated FGF and BMP signaling controls the progression of progenitor cell differentiation and the emergence of pattern in the embryonic anterior pituitary. *Development* **125**: 1005-1015.

Ethier JF, Farnworth PG, Findlay JK & Ooi GT 2002 Transforming growth factor-beta modulates inhibin A bioactivity in the LbetaT2 gonadotrope cell line by competing for binding to betaglycan. *Molecular Endocrinology* **16**: 2754-2763.

Esparza-Lopez J, Montiel JL, Vilchis-Landeros MM, Okadome T, Miyazono K & Lopez-Casillas F 2001 Ligand binding and functional properties of betaglycan, a co-receptor of

the transforming growth factor-beta superfamily. Specialized binding regions for transforming growth factor-beta and inhibin A. *Journal of Biological Chemistry* **276**: 14588-14596.

F

Fabre S, Pierre A, Pisselet C, Mulsant P, Lecerf F, Pohl J, Monget P & Monniaux D 2003 The Booroola mutation in sheep is associated with an alteration of the bone morphogenetic protein receptor-IB functionality. *Journal of Endocrinology* **177**: 435-444.

Fafioffe A, Ethier JF, Fontaine J, JeanPierre E, Taragnat C & Dupont J 2004 Activin and inhibin receptor gene expression in the ewe pituitary throughout the oestrous cycle. *Journal of Endocrinology* **182**: 55-68.

Fainsod A, Deissler K, Yelin R, Marom K, Epstein M, Pillemer G, Steinbeisser H & Blum M. 1997 The dorsalizing and neural inducing gene follistatin is an antagonist of BMP-4. *Mechanisms of development* **63**: 39-50.

Farnworth PG, Thean E, Robertson DM & Schwartz J 1995 Ovine anterior pituitary production of follistatin in vitro. *Endocrinology* **136**: 4397-4406.

Farnworth PG 2000 Gonadotrophin secretion revisited. How many ways can FSH leave a gonadotroph ? *Journal of Endocrinology* **145**: 387-395.

Faure MO, Nicol L, Fabre S, Fontaine J, Mohoric N, McNeilly A & Taragnat C 2005 BMP-4 inhibits follicle-stimulating hormone secretion in ewe pituitary. *Journal of Endocrinology* **186**: 109-121.

Findlay JK, Drummond AE, Dyson M, Baillie AJ, Robertson DM & Ethier JF 2001 Production and actions of inhibin and activin during folliculogenesis in the rat. *Molecular and cellular endocrinology* **180**: 139-144. Review.

Fink G 1988 Gonadotropin secretion and its control. In: Knobil E, Neill J, eds. The physiology of reproduction. New York: Raven Press. 1349-1377.

Fleming JS, Greenwood PJ, Heath DA, Hudson NL, Lun S, Shaw L & McNatty KP 1995 Expression of gonadotrophin subunit genes in sheep that were homozygous carriers and non-carriers of the Booroola fecundity gene FecB. *Journal of Reproduction and Fertility* **103**: 315-321.

Fernandez-Vazquez G, Kaiser UB, Albarracin CT, Chin WW 1996 Transcriptional activation of the gonadotropin-releasing hormone receptor gene by activin A. *Molecular Endocrinology* **10**: 356-366.

Fox KM, Dias JA & Van Roey P 2001 Three-dimensional structure of human follicle-stimulating hormone. *Molecular Endocrinology* **15**: 378-389.

Freeman ME 1988 The ovarian cycle of the rat. In: Knobil E, Neill J, eds. The physiology of reproduction. New York: Raven Press. 1893-1928.

Fry RC, Clarke IJ, Cummins JT, Bindon BM, Piper LR & Cahill LP 1988 Induction of ovulation in chronically hypophysectomized Booroola ewes. *Journal of reproduction and fertility* **82**: 711-715.

Fukuda M, Miyamoto K, Hasegawa Y, Ibuki Y & Igarashi M 1987 Action mechanism of inhibin in vitro--cycloheximide mimics inhibin actions on pituitary cells. *Molecular and cellular endocrinology* **51**: 41-50.

G

Galet C, Lecompte F & Combarnous Y 2004 Association/dissociation of gonadotropin subunits involves disulfide bridge disruption which is influenced by carbohydrate moiety. *Biochemical and biophysical research communications* **324**: 868-873.

Galloway SM, McNatty KP, Cambridge LM, Laitinen MP, Juengel JL, Jokiranta TS, McLaren RJ, Luiro K, Dodds KG, Montgomery GW, Beattie AE, Davis GH & Ritvos O 2000 Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner. *Nature Genetics* **25**: 279-283.

Gharib SD, Wierman ME, Badger TM & Chin WW 1987 Sex steroid hormone regulation of follicle-stimulating hormone subunit messenger ribonucleic acid (mRNA) levels in the rat. *The Journal of clinical investigation* **80**: 294-299.

Gault PM, Morgan K, Pawson AJ, Millar RP & Lincoln GA 2004 Sheep exhibit novel variations in the organization of the mammalian type II gonadotropin-releasing hormone receptor gene. *Endocrinology* **145**: 2362-2374.

Gazzerro E, Gangji V & Canalis E 1998 Bone morphogenetic proteins induce the expression of noggin, which limits their activity in cultured rat osteoblasts. *The Journal of clinical investigation* **102**: 2106-2114.

Ghosh BR, Wu JC, Strahl BD, Childs GV & Miller WL 1996 Inhibin and estradiol alter gonadotropes differentially in ovine pituitary cultures: changing gonadotrope numbers and calcium responses to gonadotropin-releasing hormone. *Endocrinology* **137**: 5144-5154.

Gilboa L, Nohe A, Geissendorfer T, Sebald W, Henis YI & Knaus P 2000 Bone morphogenetic protein receptor complexes on the surface of live cells: a new

oligomerization mode for serine/threonine kinase receptors. *Molecular biology of the cell* **11**:1023-1035.

Glister C, Kemp CF & Knight PG 2004 Bone morphogenetic protein (BMP) ligands and receptors in bovine ovarian follicle cells: actions of BMP-4, -6 and -7 on granulosa cells and differential modulation of Smad-1 phosphorylation by follistatin. *Reproduction* **127**: 239-254.

Goodman RL 1988 Neuroendocrine control of the ovine estrous cycle. In: Knobil E, Neill J, eds. The physiology of reproduction. New York: Raven Press. 1929-1969.

Graham KE, Nusser KD & Low MJ 1999 LbetaT2 gonadotroph cells secrete follicle stimulating hormone (FSH) in response to active A. *Journal of Endocrinology* **162**: R1-R5.

Gray PC, Greenwald J, Blount AL, Kunitake KS, Donaldson CJ, Choe S & Vale W. 2000 Identification of a binding site on the type II activin receptor for activin and inhibin. *The Journal of biological chemistry* **275**: 3206-3212.

Gregg DW, Allen MC & Nett TM 1990 Estradiol-induced increase in number of gonadotropin-releasing hormone receptors in cultured ovine pituitary cells. *Biology of reproduction* **43**: 1032-1036.

Gregg DW, Schwall RH & Nett TM 1991 Regulation of gonadotropin secretion and number of gonadotropin-releasing hormone receptors by inhibin, activin-A, and estradiol. *Biology of Reproduction* **44**: 725-732.

Gregory SJ, Lacza CT, Detz AA, Xu S, Petrillo LA & Kaiser UB 2005 Synergy between activin A and gonadotropin-releasing hormone in transcriptional activation of the rat follicle-stimulating hormone-beta gene. *Molecular Endocrinology* **19**: 237-254.

Gronroos E, Hellman U, Heldin CH, Ericsson J 2002 Control of Smad7 stability by competition between acetylation and ubiquitination. *Molecular Cell* **10**: 483-493.

Grotewold L, Plum M, Dildrop R, Peters T & Ruther U 2001 Bambi is coexpressed with Bmp-4 during mouse embryogenesis. *Mechanisms of Development* **100**: 327-330.

H

Haisenleder DJ, Cox ME, Parsons SJ & Marshall JC 1998 Gonadotropin-releasing hormone pulses are required to maintain activation of mitogen-activated protein kinase: role in stimulation of gonadotrope gene expression. *Endocrinology* **139**: 3104-3111.

Haisenleder DJ, Ferris HA & Shupnik MA 2003 The calcium component of gonadotropin-releasing hormone-stimulated luteinizing hormone subunit gene transcription is

mediated by calcium/calmodulin-dependent protein kinase type II. *Endocrinology* **144**: 2409-2416.

Halvorson LM, Weiss J, Bauer-Dantoin AC & Jameson JL 1994 Dynamic regulation of pituitary follistatin messenger ribonucleic acids during the rat estrous cycle. *Endocrinology* **134**: 1247-1253.

Hanrahan JP, Gregan SM, Mulsant P, Mullen M, Davis GH, Powell R & Galloway SM. 2004 Mutations in the genes for oocyte-derived growth factors GDF9 and BMP15 are associated with both increased ovulation rate and sterility in Cambridge and Belclare sheep (Ovis aries). *Biology of Reproduction* **70**: 900-909.

Hanyu A, Ishidou Y, Ebisawa T, Shimanuki T, Imamura T & Miyazono K 2001 The N domain of Smad7 is essential for specific inhibition of transforming growth factor-beta signaling. *The Journal of cell biology* **155**: 1017-1027.

Harland RM 1994 The transforming growth factor beta family and induction of the vertebrate mesoderm: bone morphogenetic proteins are ventral inducers. *Proceedings of the National Academy of Sciences of the United States of America* **91**: 10243-10246. Review.

Harris D, Bonfil D, Chuderland D, Kraus S, Seger R & Naor Z 2002 Activation of MAPK cascades by GnRH: ERK and Jun N-terminal kinase are involved in basal and GnRH-stimulated activity of the glycoprotein hormone LHbeta-subunit promoter. *Endocrinology* **143**: 1018-1025.

Harrison CA, Gray PC, Fischer WH, Donaldson C, Choe S & Vale W 2004 An activin mutant with disrupted ALK4 binding blocks signaling via type II receptors. *Journal of Biological Chemistry* **279**: 28036-28044.

Hata A, Lagna G, Massagué J & Hemmati-Brivanlou A 1998 Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumor suppressor. *Genes & Development* **12**: 186-197.

Hayes S, Chawla A & Corvera S 2002 TGF beta receptor internalization into EEA1-enriched early endosomes: role in signaling to Smad2. *The Journal of cell biology* **158**: 1239-1249.

Herbison AE 1995 Neurochemical identity of neurones expressing oestrogen and androgen receptors in sheep hypothalamus. *Journal of reproduction and fertility. Supplement* **49**: 271-283. Review.

Hodges JK 1978 Effects of gonadectomy and oestradiol treatment on plasma luteinizing hormone concentrations in the marmoset monkey, Callithrix jacchus. *Journal of Endocrinology* **76**: 271-281.

Hoffmann A, Preobrazhenska O, Wodarczyk C, Medler Y, Winkel A, Shahab S, Huylebroeck D, Gross G & Verschueren K 2005 Transforming growth factor-beta-activated kinase-1 (TAK1), a MAP3K, interacts with Smad proteins and interferes with osteogenesis in murine mesenchymal progenitors. *Journal of Biological Chemistry* **280**: 27271-27283.

Hsu DR, Economides AN, Wang X, Eimon PM & Harland RM 1998 The Xenopus dorsalizing factor Gremlin identifies a novel family of secreted proteins that antagonize BMP activities. *Molecular cell* **1**: 673-683.

Huse M, Chen YG, Massagué J & Kuriyan J 1999 Crystal structure of the cytoplasmic domain of the type I TGF beta receptor in complex with FKBP12. *Cell* **96**: 425-436.

Huang EJ, Manova K, Packer AI, Sanchez S, Bachvarova RF & Besmer P 1993 The murine steel panda mutation affects kit ligand expression and growth of early ovarian follicles. *Developmental biology* **157**: 100-109.

Huang HJ, Sebastian J, Strahl BD, Wu JC & Miller WL 2001 The promoter for the ovine follicle-stimulating hormone-beta gene (FSHbeta) confers FSHbeta-like expression on luciferase in transgenic mice: regulatory studies in vivo and in vitro. *Endocrinology* **142** : 2260-2266.

Huang HJ, Sebastian J, Strahl BD, Wu JC & Miller WL 2001 Transcriptional regulation of the ovine follicle-stimulating hormone-beta gene by activin and gonadotropin-releasing hormone (GnRH): involvement of two proximal activator protein-1 sites for GnRH stimulation. *Endocrinology* **142**: 2267-2274.

Huang HJ, Wu JC, Su P, Zhirnov O & Miller WL 2001 A novel role for bone morphogenetic proteins in the synthesis of follicle-stimulating hormone. *Endocrinology* **142**: 2275-2283.

I

Iemura S, Yamamoto TS, Takagi C, Uchiyama H, Natsume T, Shimasaki S, Sugino H & Ueno N 1998 Direct binding of follistatin to a complex of bone-morphogenetic protein and its receptor inhibits ventral and epidermal cell fates in early Xenopus embryo. *Proceedings of the National Academy of Sciences U S A* **95**: 9337-42.

Ikeda T, Takahashi H, Suzuki A, Ueno N, Yokose S, Yamaguchi A, Yoshiki S 1996 Cloning of rat type I receptor cDNA for bone morphogenetic protein-2 and bone morphogenetic

protein-4, and the localization compared with that of the ligands. *Developmental dynamics : an official publication of the American Association of Anatomists* **206**: 318-329.

Illing N, Jacobs GF, Becker II, Flanagan CA, Davidson JS, Eales A, Zhou W, Sealfon SC & Millar RP 1993 Comparative sequence analysis and functional characterization of the cloned sheep gonadotropin-releasing hormone receptor reveal differences in primary structure and ligand specificity among mammalian receptors. *Biochemical and biophysical research communications* **196**:745-51.

Imakawa K, Day ML, Zalesky DD, Garcia-Winder M, kittok RJ, Kinder JE 1986 Regulation of pulsatile LH secretion by ovarian steroids in the heife. *Journal of Animal Science* **63**: 162-168.

Imamura T, Takase M, Nishihara A, Oeda E, Hanai JI, Kawabata M & Miyazono K 1997 Smad6 inhibits signaling by the TGF-b superfamily. *Nature* **389**: 622-626.

Inoue K & Kurosumi K 1984 Ultrastructural immunocytochemical localization of LH and FSH in the pituitary of the untreated male rat. *Cell Tissue Research* **235**: 77-83.

Ishida W, Hamamoto T, Kusanagi K, Yagi K, Kawabata M, Takehara K, Sampath TK, Kato M & Miyazono K 2000 Smad6 is a Smad1/5-induced smad inhibitor. Characterization of bone morphogenetic protein-responsive element in the mouse Smad6 promoter. *Journal of Biological Chemistry* **275**: 6075-6079.

Ishisaki A, Yamato K, Hashimoto S, Nakao A, Tamaki K, Nonaka K, ten Dijke P, Sugino H & Nishihara T 1999 Differential inhibition of Smad6 and Smad7 on bone morphogenetic protein- and activin-mediated growth arrest and apoptosis in B cells. *Journal of Biological Chemistry* **274**: 13637-13642.

Israel DI, Nove J, Kerns KM, Kaufman RJ, Rosen V, Cox KA & Wozney JM 1996 Heterodimeric bone morphogenetic proteins show enhanced activity in vitro and in vivo. *Growth Factors* **13**: 291-300.

Itoh F, Asao H, Sugamura K, Heldin CH, ten Dijke P & Itoh S 2001 Promoting bone morphogenetic protein signaling through negative regulation of inhibitory Smads. *The EMBO Journal* **20**: 4132-4142.

Itoh S, Thorikay M, Kowanetz M, Moustakas A, Itoh F, Heldin CH & ten Dijke P 2003 Elucidation of Smad requirement in transforming growth factor-beta type I receptor-induced responses. *The Journal of biological chemistry* **278**: 3751-3761.

J

Jameson JL, Becker CB, Lindell CM & Habener JF 1988 Human follicle-stimulating hormone beta-subunit gene encodes multiple messenger ribonucleic acids. *Molecular Endocrinolology* **2**: 806-815.

K

Kaiser UB, Lee BL, Carroll RS, Unabia G, Chin WW & Childs GV 1992 Follistatin gene expression in the pituitary: localization in gonadotropes and folliculostellate cells in diestrous rats. *Endocrinology* **130**: 3048-3056.

Kaiser UB, Sabbagh E, Katzenellenbogen RA, Conn PM & Chin WW 1995 A mechanism for the differential regulation of gonadotropin subunit gene expression by gonadotropin-releasing hormone. *Proceedings of the National Academy of Sciences of the United States of America* **92**: 12280-12284.

Kaiser UB, Jakubowiak A, Steinberger A & Chin W 1997 Differential effects of gonadotropin-releasing hormone (GnRH) pulse frequency on gonadotropin subunit and GnRH receptor messenger ribonucleic acid levels *in vitro*. *Endocrinology* **138**: 1224-1231.

Kaiser UB, Sabbagh E, Saunders BD & Chin WW 1998 Identification of cis-acting deoxyribonucleic acid elements that mediate gonadotropin-releasing hormone stimulation of the rat luteinizing hormone beta-subunit gene. *Endocrinology* **139**: 2443-2451.

Kanasaki H, Bedecarrats GY, Kam KY, Xu S & Kaiser UB 2005 GnRH Pulse Frequency-Dependent Activation of ERK Pathways in Perifused L{beta}T2 Cells. *Endocrinology* 2005 Sep 1; in press, available at http://endo.endojournals.org

Kann G 1971 Dosage radioimmunologique de la prolactine plasmatique chez les ovins. *Comptes Rendus de l'Académie des Sciences* III **272** : 2808-2811.

Karsch FJ, Bowen JM, Caraty A, Evans NP, Moenter SM 1997 Gonadotropin-releasing hormone requirements for ovulation. *Biology of Reproduction* **56**: 303-309. Review.

Kasa-Vubu JZ, Dahl GE, Evans NP, Thrun LA, Moenter SM, Padmanabhan V & Karsch FJ 1992 Progesterone blocks the estradiol-induced gonadotropin discharge in the ewe by inhibiting the surge of gonadotropin-releasing hormone. *Endocrinology* **131**: 208-212.

Kavsak P, Rasmussen RK, Causing CG, Bonni S, Zhu H, Thomsen GH & Wrana JL 2000 Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF beta receptor for degradation. *Molecular Cell* **6**: 1365-1375.

Kawabata M, Imamura T & Miyazono K, 1998 Signal transduction by Bone Morphogenetic Proteins. *Cytokine & Growth Factor Reviews* **9**: 49-61.

Kimura N, Matsuo R, Shibuya H, Nakashima K & Taga T 2000 BMP2-induced apoptosis is mediated by activation of the TAK1-p38 kinase pathway that is negatively regulated by Smad6. *Journal of Biological Chemistry* **275**: 17647-17652.

Kirk SE, Dalkin AC, Yasin M, Haisenleder DJ, Marshall JC. Related Articles, Links Gonadotropin-releasing hormone pulse frequency regulates expression of pituitary follistatin messenger ribonucleic acid: a mechanism for differential gonadotrope function. *Endocrinology* **135**: 876-880.

Klein R, Findlay JK, Clarke IJ, de Kretser DM & Robertson DM 1993 Radioimmunoassay of FSH-suppressing protein in the ewe: concentrations during the oestrous cycle and following ovariectomy. *The Journal of endocrinology* **137**: 433-443.

Klein J, Lobel L, Pollak S, Ferin M, Xiao E, Sauer M & Lustbader JW 2002 Pharmacokinetics and pharmacodynamics of single-chain recombinant human follicle-stimulating hormone containing the human chorionic gonadotropin carboxyterminal peptide in the rhesus monkey. *Fertility and sterility* **77**: 1248-1255.

Knight PG 1996 Roles of inhibins, activins, and follistatin in the female reproductive system. *Frontiers of Neuroendocrinology* **17**: 476-509. Review.

Knight PG & Glister C 2003 Local roles of TGF-β superfamily members in the control of ovarian follicle development. *Animal Reproduction Sciences* **78** 165-183. Review.

Kottler ML, Counis R & Bouchard P 1999 Mutations of the GnRH receptor gene: a new cause of autosomal-recessive hypogonadotropic hypogonadism. *Archives of medical research* **30**: 481-5. Review.

De Kretser DM & Robertson DM 1989 The isolation and physiology of inhibin and related proteins. *Biology of reproduction* **40**: 33-47.

Kretzschmar M, Doody J & Massague J 1997 Opposing BMP and EGF signalling pathways converge on the TGF-beta family mediator Smad1. *Nature* **389**: 618-622.

Krystal G, Lam V, Dragowska W, Takahashi C, Appel J, Gontier A, Jenkins A, Lam H, Quon L, Lansdorp P 1994 Transforming growth factor beta 1 is an inducer of erythroid differentiation. *The Journal of experimental medicine* **180**: 851-860.

Kumar TR, Wang Y, Lu N & Matzuk MM 1997 Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. *Nature genetics* **15**: 201-204.

L

Lalloz MR, Detta A & Clayton RN 1988 Gonadotropin-releasing hormone desensitization preferentially inhibits expression of the luteinizing hormone beta-subunit gene in vivo. *Endocrinology* **122**: 1689-1694.

Lapthorn AJ, Harris DC, Littlejohn A, Lustbader JW, Canfield RE, Machin KJ, Morgan FJ & Isaacs NW 1994 Crystal structure of human chorionic gonadotropin. *Nature* **369**: 455-461.

Lee WS, Otsuka F, Moore RK & Shimasaki S 2001 Effect of bone morphogenetic protein-7 on folliculogenesis and ovulation in the rat. *Biology of reproduction* **65** 994-999.

Lemyre E, Lemieux N, Decarie JC & Lambert M 1998 Del(14)(q22.1q23.2) in a patient with anophthalmia and pituitary hypoplasia. *American journal of medical genetics* **77**: 162-165. Review.

Lewis KA, Gray PC, Blount AL, MacConell LA, Wiater E, Bilekzikjian LM & Vale W 2000 Betaglycan binds inhibin and can mediate functional antagonism of activin signaling. *Nature* **404**: 411-414.

Li X, Ionescu AM, Schwarz EM, Zhang X, Drissi H, Puzas JE, Rosier RN, Zuscik MJ & O'Keefe RJ 2003 Smad6 is induced by BMP-2 and modulates chondrocyte differentiation. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* **21**: 908-913.

Lin SY, Morrison JR, Phillips DJ & de Kretser DM 2003 Regulation of ovarian function by the TGF-β superfamily and follistatin. *Reproduction* **126**: 133-148.

Lin X, Liang M, Feng XH 2000 Smurf2 is a ubiquitin E3 ligase mediating proteasome-dependent degradation of Smad2 in transforming growth factor-beta signaling. *Journal of Biological Chemistry* **275**: 36818-36822.

Lin X, Liang M, Liang YY, Brunicardi FC & Feng XH 2003 SUMO-1/Ubc9 promotes nuclear accumulation and metabolic stability of tumor suppressor Smad4. *Journal of Biological Chemistry* **278**: 31043-31048.

Lin X, Liang YY, Sun B, Liang M, Shi Y, Brunicardi FC, Shi Y & Feng XH 2003 Smad6 recruits transcription corepressor CtBP to repress bone morphogenetic protein-induced transcription. *Molecular and cellular biology* **23**: 9081-9093.

Ling N, Ying SY, Ueno N, Shimasaki S, Esch F, Hotta M & Guillemin R 1986 Pituitary FSH is released by a heterodimer of the beta-subunits from the two forms of inhibin. *Nature* **321**: 779-782.

Liu F, Ventura F, Doody J & Massagué J 1995 Human type II receptor for Bone Morphogenetic Proteins (BMPs): extension of the two-kinase receptor model to the BMPs. *Molecular and cellular biology* **15**: 3479-3486.

Liu F, Pouponnot C & Massague J 1997 Dual role of the Smad4/DPC4 tumor suppressor in TGFbeta-inducible transcriptional complexes. *Genes & Development* **11**: 3157-3167.

Liu F, Austin DA, Mellon PL, Olefsky JM & Webster NJ 2002 GnRH activates ERK1/2 leading to the induction of c-fos and LHbeta protein expression in LbetaT2 cells. *Molecular Endocrinolology* **16**: 419-434.

Lo RS, Chen YG, Shi Y, Pavletich NP & Massague J 1998 The L3 loop: a structural motif determining specific interactions between SMAD proteins and TGF-beta receptors. *The EMBO Journal* **17**: 996-1005.

Lo RS & Massague J 1999 Ubiquitin-dependent degradation of TGF-beta-activated smad2. : *Nature cell biology* **1**:472-478.

Lumpkin MD, Moltz JH, Yu WH, Samson WK & McCann SM 1987 Purification of FSH-releasing factor: its dissimilarity from LHRH of mammalian, avian, and piscian origin. *Brain research bulletin* **18**: 175-178.

Lumpkin MD, McDonald JK, Samson WK & McCann SM 1989 Destruction of the dorsal anterior hypothalamic region suppresses pulsatile release of follicle stimulating hormone but not luteinizing hormone. *Neuroendocrinology* **50**: 229-235.

M

MacConell LA, Leal AM & Vale WW 2002 The distribution of betaglycan protein and mRNA in rat brain, pituitary, and gonads: implications for a role for betaglycan in inhibin-mediated reproductive functions. *Endocrinology* **143**: 1066-1075.

Macias-Silva M, Abdollah S, Hoodless PA, Pirone R, Attisano L & Wrana JL 1996 MADR2 is a substrate of the TGFbeta receptor and its phosphorylation is required for nuclear accumulation and signaling. *Cell* **87**: 1215-1224.

Mann GE, Campbell BK, McNeilly AS & Baird DT 1990 Effects of passively immunizing ewes against inhibin and oestradiol during the follicular phase of the oestrous cycle. *Journal of Endocrinology* **125**: 417-424.

Manning G, Whyte DB, Martinez R, Hunter T & Sudarsanam S 2002 The protein kinase complement of the human genome. *Science* **298**: 1912-1934. Review.

Marek A, Brodzicki J, Liberek A, Korzon M 2002 TGF-beta (transforming growth factor-beta) in chronic inflammatory conditions - a new diagnostic and prognostic marker?

Medical science monitor : international medical journal of experimental and clinical research **8**: RA145-51. Review.

Martin GB, Wallace JM, Taylor PL, Fraser HM, Tsonis CG & McNeilly AS 1986 The roles of inhibin and gonadotrophin-releasing hormone in the control of gonadotrophin secretion in the ewe. *Journal of Endocrinology* **111**: 287-296.

Mason AJ, Hayflick JS, Zoeller RT, Young WS 3rd, Phillips HS, Nikolics K & Seeburg PH 1986 A deletion truncating the gonadotropin-releasing hormone gene is responsible for hypogonadism in the hpg mouse. *Science* **234**: 1366-1371.

Massagué J, Cheifetz S, Endo T, Nadal-Ginard B 1986 Type beta transforming growth factor is an inhibitor of myogenic differentiation. *Proceedings of the National Academy of Sciences of the United States of America* **83**: 8206-8210.

Massagué J & Wotton D 2000 Transcriptional control by the TGF-beta/Smad signaling system. *The EMBO journal* **19**: 1745-1754.

Massagué J & Chen YG 2000 Controlling TGF-β signaling. *Genes & Development* **14**: 627-644.

Massague J 2003 Integration of Smad and MAPK pathways: a link and a linker revisited. *Genes & Development* **17**: 2993-2997.

Mathoux F, Vigneron C., Fontaine J, McNeilly AS, Phillips DJ & Taragnat C 2001 Evidence for activin release by ovine pituitary cells and its regulation by gonadotropin releasing hormone (GnRH). Symposium "Actions and interactions at pituitary", Christchurch, New Zealand, 25-27 august 2001. Abstract.

Matsuura I, Denissova NG, Wang G, He D, Long J & Liu F 2004 Cyclin-dependent kinases regulate the antiproliferative function of Smads. *Nature* **430**: 226-231.

Matzuk MM 2000 Revelations of ovarian follicle biology from gene knockout mice. *Molecular and Cellular Endocrinology* **163**: 61-66. Review.

Mazerbourg S & Hsueh AJ 2003 Growth differentiation factor-9 signaling in the ovary. *Molecular and Cellular Endocrinology* **202**: 31-36. Review.

McIntosh JE & McIntosh RP 1986 Varying the patterns and concentrations of gonadotrophin-releasing hormone stimulation does not alter the ratio of LH and FSH released from perifused sheep pituitary cells. *Journal of Endocrinology* **109**: 155-61.

McNatty KP, Lun S, Heath DA, Ball K, Smith P, Hudson NL, McDiarmid J, Gibb M & Henderson KM 1986 Differences in ovarian activity between booroola X merino ewes which were homozygous, heterozygous and non-carriers of a major gene influencing their ovulation rate. *Journal of Reproduction and Fertility* **77**: 193-205.

McNatty KP, Hudson N, Henderson KM, Gibb M, Morrison L, Ball K & Smith P 1987 Differences in gonadotrophin concentrations and pituitary responsiveness to GnRH between Booroola ewes which were homozygous (FF), heterozygous (F+) and non-carriers (++) of a major gene influencing their ovulation rate. *Journal of Reproduction and Fertility* **80**: 577-588.

McNeilly AS, Crow W, Brooks J & Evans G 1992 Luteinizing hormone pulses, follicle stimulating hormone and the control of follicle selection. *Journal of Reproduction and Fertility supplement* **45**: 5-18.

McNeilly AS, Crawford JL, Taragnat C, Nicol L & McNeilly JR 2003 The differential secretion of FSH and LH: regulation through genes, feedback and packaging. *Reproduction Supplement* **61**: 463-476.

McPherson JC 3rd, Costoff A & Mahesh VB 1975 Influence of estrogen-progesterone combinations on gonadotropin secretion in castrate female rats (1). *Endocrinology* **97**: 771-779.

Meeran D, Urbanski HF, Gregory SJ, Towsend J & Tortones DJ 2003 Developmental changes in the hormonal identity of gonadotroph cells in the rhesus monkey pituitary gland. *The Journal of Clinical & Metabolism* **88**: 2934-2942.

Mellon PL, Windle JJ & Weiner RI 1991 Immortalization of neuroendocrine cells by targeted oncogenesis. *Recent progress in hormone research* **47**: 69-93; discussion 93-6.

Mercer JE, Phillips DJ & Clarke IJ 1993 Short-term regulation of gonadotropin subunit mRNA levels by estrogen: studies in the hypothalamo-pituitary intact and hypothalamo-pituitary disconnected ewe. *Journal of Neuroendocrinology* **5**: 591-596.

Meunier H, Rivier C, Evans RM & Vale W 1988 Gonadal and extragonadal expression of inhibin alpha, beta A, and beta B subunits in various tissues predicts diverse functions. *Proceedings of the National Academy of Sciences of the United States of America* **85**: 247-251.

Micevych P, Sinchak K, Mills RH, Tao L, LaPolt P & Lu JK 2003The luteinizing hormone surge is preceded by an estrogen-induced increase of hypothalamic progesterone in ovariectomized and adrenalectomized rats. *Neuroendocrinology* **78**: 29-35.

Millar RP, Lu ZL, Pawson AJ, Flanagan CA, Morgan K & Maudsley SR 2004 Gonadotropin-releasing hormone receptors. *Endocrine Reviews* **25**: 235-75. Review.

Miller CD & Miller WL 1996 Transcriptional repression of the ovine follicle-stimulating hormone- beta gene by 17 beta-estradiol. *Endocrinology* **137**: 3437-3446.

Miller WL, Shafiee-Kermani F, Strahl BD & Huang HJ 2002 The nature of FSH induction by GnRH. Trends in endocrinology and metabolism: TEM **13**: 257-263. Review.

Miro F, Smyth CD & Hillier SG 1991 Development-related effects of recombinant activin on steroid synthesis in rat granulosa cells. *Endocrinology* **129**: 3388-3394.

Miyazono K, Kusanagi K & Inoue H 2001 Divergence and convergence of TGF-beta/BMP signaling. *Journal of Cellular Physiology* **187**: 265-276. Review.

Miyazono K, Maeda S & Imamura T 2005 BMP receptor signaling: Transcriptional targets, regulation of signals, and signaling cross-talk. *Cytokine Growth Factor Reviews* **16**: 251-263

Mochizuki T, Miyazaki H, Hara T, Furuya T, Imamura T, Watabe T & Miyazono K Roles for the MH2 domain of Smad7 in the specific inhibition of transforming growth factor-beta superfamily signaling. *Journal of Biological Chemistry* **279**: 31568-31574.

Moenter SM, Caraty A & Karsch FJ 1990 The estradiol-induced surge of gonadotropin-releasing hormone in the ewe. *Endocrinology* **127**: 1375-1384.

Molter-Gerard C, Fontaine J, Guerin S & Taragnat C 1999 Differential regulation of the gonadotropin storage pattern by gonadotropin-releasing hormone pulse frequency in the ewe. *Biology of Reproduction* **60**: 1224-1230.

Molter-Gerard C, Caraty A, Guerin S, Fontaine J & Taragnat C 2000 Dynamic changes in the gonadotrope cell subpopulations during an estradiol-induced surge in the ewe. *Biology of Reproduction* **63**: 1084-1091.

Monniaux D & Pisselet C 1992 Control of proliferation and differentiation of ovine granulosa cells by insulin-like growth factor-I and follicle-stimulating hormone in vitro. *Biology of Reproduction* **46**: 109-119.

Monniaux D, Huet C, Besnard N, Clement F, Bosc M, Pisselet C, Monget P & Mariana JC 1997 Follicular growth and ovarian dynamics in mammals. *Journal of reproduction and fertility. Supplement* **51**: 3-23. Review.

Montesano R, Orci L 1988 Transforming growth factor beta stimulates collagen-matrix contraction by fibroblasts: implications for wound healing. *Proceedings of the National Academy of Sciences U S A* **85**: 4894-4897.

Montgomery GW, Galloway SM, Davis GH & McNatty KP 2001 Genes controlling ovulation rate in sheep. *Reproduction* **121**: 843-852. Review

Moore RK, Otsuka F & Shimasaki S 2003 Molecular basis of bone morphogenetic protein-15 signaling in granulosa cells. *Journal of Biological Chemistry* **278**: 304-310.

Moser M, Binder O, Wu Y, Aitsebaomo J, Ren R, Bode C, Bautch VL, Conlon FL & Patterson C 2003 BMPER, a novel endothelial cell precursor-derived protein, antagonizes bone morphogenetic protein signaling and endothelial cell differentiation. *Molecular and cellular biology* **23**: 5664-5679.

Mulsant P, Lecerf F, Fabre S, Schibler L, Monget P, Lanneluc I, Pisselet C, Riquet J, Monniaux D, Callebaut I, Cribiu E, Thimonier J, Teyssier J, Bodin L, Cognie Y, Chitour N & Elsen JM 2001 Mutation in bone morphogenetic protein receptor-IB is associated with increased ovulation rate in Booroola Merino ewes. *Proceedings of the National Academy of Sciences U S A* **98**: 5104-5109.

Murakami G, Watabe T, Takaoka K, Miyazono K & Imamura T 2003 Cooperative inhibition of Bone Morphogenetic Protein signaling by Smurf1 and inhibitory Smads. *Molecular Biology of the Cell* **14**: 2809-2817.

N

Nagarajan RP, Zhang J, Li W & Chen Y 1999 Regulation of Smad7 promoter by direct association with Smad3 and Smad4. *Journal of Biological Chemistry* **274**: 33412-33418.

Nakamura T, Takio K, Eto Y, Shibai H, Titani K & Sugino H 1990 Activin-binding protein from rat ovary is follistatin. *Science* **247**: 836-838.

Nakao A, Afrakhte M, Morén A, Nakayama T, Christian JL, Heuchel R, Itoh S, Kawabata M, Heldin NE, Heldin CH & ten Dijke P 1997 Identification of Smad7, a TGFβ-inducible antagonist of TGF-β signaling. *Nature* **389**: 631-635.

Nakao A, Imamura T, Souchelnytskyi S, Kawabata M, Ishisaki A, Oeda E, Tamaki K, Hanai J, Heldin CH, Miyazono K & ten Dijke P 1997 TGF-beta receptor-mediated signalling through Smad2, Smad3 and Smad4. *The EMBO journal* **16**: 5353-5362.

Nakashima K, Yanagisawa M, Arakawa H, Kimura N, Hisatsune T, Kawabata M, Miyazono K & Taga T 1999 Synergistic signaling in fetal brain by STAT3-Smad1 complex bridged by p300. *Science* **284**: 479-482.

Nakayama T, Berg LK & Christian JL 2001 Dissection of inhibitory Smad proteins: both N- and C-terminal domains are necessary for full activities of Xenopus Smad6 and Smad7. *Mechanisms of Development* **100**: 251-262.

Naor Z, Harris D & Shacham S 1998 Mechanism of GnRH receptor signaling: combinatorial cross-talk of Ca2+ and protein kinase C. *Frontiers in Neuroendocrinology* **19**: 1-19.

Naor Z, Benard O & Seger R 2000 Activation of MAPK cascades by G-protein-coupled receptors: the case of gonadotropin-releasing hormone receptor. *Trends in endocrinology and metabolism: TEM.* **11**: 91-99. Review.

Nett TM, Turzillo AM, Baratta M & Rispoli LA 2002 Pituitary effects of steroid hormones on secretion of follicle-stimulating hormone and luteinizing hormone. *Domestic animal endocrinology* **23**: 33-42. Review.

Nguyen KA, Santos SJ, Kreidel MK, Diaz AL, Rey R & Lawson MA 2004 Acute regulation of translation initiation by gonadotropin-releasing hormone in the gonadotrope cell line .LbetaT2. *Molecular Endocrinology* **18**: 1301-1312.

Nicol L, McNeilly JR, Stridsberg M, Crawford JL & McNeilly AS 2002 Influence of steroids and GnRH on biosynthesis and secretion of secretogranin II and chromogranin A in relation to LH release in LbetaT2 gonadotroph cells. *Journal of Endocrinolology* **174**: 473-483.

Nicol L, McNeilly JR, Stridsberg M & McNeilly AS 2004 Differential secretion of gonadotrophins: investigation of the role of secretogranin II and chromogranin A in the release of LH and FSH in LbetaT2 cells. *Journal of molecular endocrinology* **32**: 467-480.

Nilsson S, Makela S, Treuter E, Tujague, Thomsen J, Anderson G, Enmark E, Pettersson K, Warner M & Gustafsson JA 2001 Mechanisms of estrogen action. *Physiological Reviews* **81**: 1535-1565.

Nilsson EE & Skinner MK 2003 Bone morphogenetic protein-4 acts as an ovarian follicle survival factor and promotes primordial follicle development. *Biology of Reproduction* **69**: 1265-1272.

Niswender GD, Moore RT, Akbar AM, Nett TM & Diekman MA 1975 Flow of blood to the ovaries of ewes throughout the estrous cycle. *Biology of Reproduction* **13**: 381-388.

Nohe A, Hassel S, Ehrlich M, Neubauer F, Sebald W, Henis YI & Knaus P 2002 The mode of bone morphogenetic protein (BMP) receptor oligomerization determines different BMP-2 signaling pathways. *Journal of Biological Chemistry* **277**: 5330-5338.

Nohe A, Keating E, Underhill TM, Knaus P & Petersen NO 2003 Effect of the distribution and clustering of the type I A BMP receptor (ALK3) with the type II BMP receptor on the activation of signalling pathways. *Journal of cell science* **116**: 3277-3284.

Nohe A, Keating E, Knaus P & Peterson NO 2004 Signal transduction of bone morphogenetic protein receptors. *Cellular Signalling* **16**: 291-299.

Norwitz ER, Xu S, Jeong KH, Bédécarrats GY, Winebrenner LD, Chin WW & Kaiser UB 2002 Activin A augments GnRH-mediated transcriptional activation of the mouse GnRH receptor gene. *Endocrinology* **143**: 985-997.

O

Ohashi N, Yamamoto T, Uchida C, Togawa A, Fukasawa H, Fujigaki Y, Suzuki S, Kitagawa K, Hattori T, Oda T, Hayashi H, Hishida A & Kitagawa M 2005 Transcriptional induction of Smurf2 ubiquitin ligase by TGF-beta. *FEBS letters* **579**: 2557-2563.

Okadome T, Oeda E, Saitoh M, Ichijo H, Moses HL, Miyazono K & Kawabata M 1996 Characterization of the interaction of FKBP12 with the transforming growth factor-beta type I receptor in vivo. *Journal of Biological Chemistry* **271**: 21687-21690.

O'Malley BW & Tsai MJ 1992 Molecular pathways of steroid receptor action. *Biology of Reproduction* **46**: 163-167.

Omori Y, Nakamura K, Yamashita S, Matsuda H, Mizutani T, Miyamoto K & Minegishi T 2005 Effect of follicle-stimulating hormone and estrogen on the expression of betaglycan messenger ribonucleic acid levels in cultured rat granulosa cells. *Endocrinology* **146**: 3379-3386.

Onichtchouk D, Chen YG, Dosch R, Gawantka V, Delius H, Massagué J & Niehrs C 1999 Silencing of TGF-beta signalling by the pseudoreceptor BAMBI. *Nature* **401**: 480-485.

Otsuka F, Yao Z, Lee T, Yamamoto S, Erickson GF & Shimasaki S 2000 Bone morphogenetic protein-15. Identification of target cells and biological functions. *The Journal of Biological Chemistry* **275**: 39523-39528.

Otsuka F, Yamamoto S, Erickson GF & Shimasaki S 2001 Bone morphogenetic protein-15 inhibits follicle-stimulating hormone (FSH) action by suppressing FSH receptor expression. *Journal of Biological Chemistry* **276**: 11387-11392.

Otsuka F & Shimasaki S 2002 A novel function of bone morphogenetic protein-15 in the pituitary: selective synthesis and secretion of FSH by gonadotropes. *Endocrinology* **143**: 4938-4941.

P

Padmanabhan V, McFadden K, Mauger DT, Karsch FJ & Midgley AR Jr 1997 Neuroendocrine control of follicle-stimulating hormone (FSH) secretion. I. Direct evidence for separate episodic and basal components of FSH secretion. *Endocrinology* **138**: 424-432.

Padmanabhan V & Sharma TP 2001 Neuroendocrine vs. paracrine control of follicle-stimulating hormone. *Archives of medical research* **32**: 533-543. Review.

Padmanabhan V, Battaglia D, Brown MB, Karsch FJ, Lee JS, Pan W, Phillips DJ & Van Cleeff J 2002 Neuroendocrine control of follicle-stimulating hormone (FSH) secretion: II. Is follistatin-induced suppression of FSH secretion mediated via changes in activin availability and does it involve changes in gonadotropin-releasing hormone secretion? *Biology of Reproduction* **66**: 1395-1402.

Padmanabhan V, Brown MB, Dahl GE, Evans NP, Karsch FJ, Mauger DT, Neill JD & Van Cleeff J 2003 Neuroendocrine control of follicle-stimulating hormone (FSH) secretion: III. Is there a gonadotropin-releasing hormone-independent component of episodic FSH secretion in ovariectomized and luteal phase ewes? *Endocrinology* **144**: 1380-1392.

Paez-Pereda M, Giacomini D, Refojo D, Nagashima AC, Hopfner U, Grubler Y, Chervin A, Goldberg V, Goya R, Hentges ST, Low MJ, Holsboer F, Stalla GK & Arzt E 2003 Involvement of bone morphogenetic protein 4 (BMP-4) in pituitary prolactinoma pathogenesis through a Smad/estrogen receptor crosstalk. *Proceedings of the National Academy of Sciences of the U S A* **100**: 1034-109.

Pangas SA, Rademaker AW, Fishman DA & Woodruff TK 2002 Localization of the activin signal transduction components in normal human ovarian follicles: implications for autocrine and paracrine signaling in the ovary. *The Journal of clinical endocrinology and metabolism* **87**: 2644-2657.

Page RB & Dovey-Hartman BJ 1984 Neurohemal contact in the internal zone of the rabbit median eminence. *The Journal of comparative neurology* **226**: 274-288.

Parrott JA & Skinner MK 1997 Direct actions of kit-ligand on theca cell growth and differentiation during follicle development. *Endocrinology* **138**: 3819-3827.

Pawson AJ & McNeilly AS 2005 The pituitary effects of GnRH. *Animal Reproduction Science* **88**: 75-94.

Pearce JJ, Penny G, Rossant J 1999 A mouse cerberus/Dan-related gene family. *Developmental biology* **209**: 98-110.

Perks CM, Denning-Kendall PA, Gilmour RS & Wathes DC 1995 Localization of messenger ribonucleic acids for insulin-like growth factor I (IGF-I), IGF-II, and the type 1 IGF receptor in the ovine ovary throughout the estrous cycle. *Endocrinology* **136**: 5266-5273.

Pernasetti F, Vasilyev VV, Rosenberg SB, Bailey JS, Huang HJ, Miller WL & Mellon PL 2001 Cell-specific transcriptional regulation of follicle-stimulating hormone-beta by

activin and gonadotropin-releasing hormone in the LbetaT2 pituitary gonadotrope cell model. *Endocrinology* **142**: 2284-2295.

Persson U, Izumi H, Souchelnytskyi S, Itoh S, Grimsby S, Engstrom U, Heldin CH, Funa K & ten Dijke P 1998 The L45 loop in type I receptors for TGF-beta family members is a critical determinant in specifying Smad isoform activation. *FEBS letters* **434**: 83-87.

Pereira RC, Economides AN & Canalis E 2000 Bone morphogenetic proteins induce gremlin, a protein that limits their activity in osteoblasts. *Endocrinology* **141**: 4558-4563.

Phillips DJ & de Kretser DM 1998 Follistatin: a multifunctional regulatory protein. *Frontiers in neuroendocrinology* **19**: 287-322. Review

Phillips CL, Lin LW, Wu JC, Guzman K, Miloted A & Miller WL 1988 17β-estradiol and progesterone inhibit transcription of the genes encoding the subunits of ovine follicle-stimulating hormone. *Molecular Endocrinology* **2**: 641-649.

Piccolo S, Sasai Y, Lu B & de Robertis EM 1996 Dorsoventral patterning in Xenopus: inhibition of ventral signals by direct binding of chordin to BMP-4. *Cell* **86**: 589-598.

Piccolo S, Agius E, Leyns L, Bhattacharyya S, Grunz H, Bouwmeester T & De Robertis EM 1999 The head inducer Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signals. *Nature* **397**: 707-710.

Pierre A, Pisselet C, Dupont J, Mandon-Pepin B, Monniaux D, Monget P & Fabre S 2004 Molecular basis of bone morphogenetic protein-4 inhibitory action on progesterone secretion by ovine granulosa cells. *Journal of molecular endocrinology* **33**: 805-817.

Pouponnot C, Jayaraman L, Massague J 1998 Physical and functional interaction of SMADs and p300/CBP. *The Journal of biological chemistry* **273**: 22865-22868.

Prendergast KA, Burger LL, Aylor KW, Haisenleder DJ, Dalkin AC & Marshall JC 2004 Pituitary follistatin gene expression in female rats: evidence that inhibin regulates transcription. *Biology of Reproduction* **70**: 364-370.

Prokova V, Mavridou S, Papakosta P, Kardassis D 2005 Characterization of a novel transcriptionally active domain in the transforming growth factor beta-regulated Smad3 protein. *Nucleic acids research* **33**: 3708-3721.

Q

R

Reguly T & Wrana JL 2003 In or out? The dynamics of Smad nucleocytoplasmic shuttling. *TRENDS in Cell Biology* **13**: 216-220.

Rispoli LA & Nett TM 2005 Pituitary gonadotropin-releasing hormone (GnRH) receptor : structure, distribution and regulation of expression. *Animal Reproductive Science* **88**: 57-74.

Rivier C, Rivier J & Vale W 1986 Inhibin-mediated feedback control of follicle-stimulating hormone secretion in the female rat. *Science* **234**: 205-208.

Roberts V, Meunier H, Vaughan J, Rivier J, Rivier C, Vale W & Sawchenko P 1989 Production and regulation of inhibin subunits in pituitary gonadotropes. *Endocrinology* **124**: 552-554.

Roberts VJ, Peto CA, Vale W & Sawchenko PE 1992 Inhibin/activin subunits are costored with FSH and LH secretory granules of the rat anterior pituitary gland. *Neuroendocrinology* **56**: 214-224.

Robertson DM, Farnworth PG, Clarke L, Jacobsen J, Cahir NF, Burger HG & de Kretser DM 1990 Effects of bovine 35 kDa FSH-suppressing protein on FSH and LH in rat pituitary cells in vitro: comparison with bovine 31 kDa inhibin. *The Journal of endocrinology* **124**: 417-423.

Rosenzweig BL, Imamura T, Okadome T, Cox GN, Yamashita H, ten Dijke P, Heldin CH & Miyazono K 1995 Cloning and characterization of a human type II receptor for bone morphogenetic proteins. *Proceedings of the National Academy of Sciences USA* **92**: 7632-7636.

S

Safwat N, Ninomiya-Tsuji J, Gore AJ & Miller WL 2005 Transforming Growth Factor Beta Activated Kinase1 (TAK1) is a Key Mediator of Ovine Follicle Stimulating Hormone Beta Subunit Expression. *Endocrinology* **146**: 4814-4824.

Scharstuhl A, Diepens R, Lensen J, Vitters E, van Beuningen H, van der Kraan P & van den Berg W 2003 Adenoviral overexpression of Smad-7 and Smad-6 differentially regulates TGF-beta-mediated chondrocyte proliferation and proteoglycan synthesis. *Osteoarthritis Cartilage* **11**: 773-782.

Scherer A & Graff JM 2000 Calmodulin differentially modulates Smad1 and Smad2 signaling. *The Journal of biological chemistry* **275**: 41430-41438.

Schirman-Hildesheim TD, Bar T, Ben-Aroya N & Koch Y 2005 Differential Gonadotropin-Releasing Hormone (GnRH) and GnRH Receptor Messenger Ribonucleic Acid Expression Patterns in Different Tissues of the Female Rat across the Estrous Cycle. *Endocrinology* **146**: 3401-3408.

Schneyer AL, Rzucidlo DA, Sluss PM & Crowley WF Jr 1994 Characterization of unique binding kinetics of follistatin and activin or inhibin in serum. *Endocrinology* **135**: 667-674.

Schubert RL & Puett D 2003 Single-chain human chorionic gonadotropin analogs containing the determinant loop of the beta-subunit linked to the alpha-subunit. *The journal of molecular endocrinolology* **31**: 157-68.

Schwanzel-Fukuda M & Pfaff DW 1989 Origin of luteinizing hormone-releasing hormone neurons. *Nature* **338**: 161-164.

Sekiya T, Oda T, Matsuura K & Akiyama T 2004 Transcriptional regulation of the TGF-beta pseudoreceptor BAMBI by TGF-beta signaling. *Biochemical and biophysical research communications* **320**: 680-684.

Seminara SB, Hayes FJ, Crowley WF Jr 1998 Gonadotropin-releasing hormone deficiency in the human (idiopathic hypogonadotropic hypogonadism and Kallmann's syndrome): pathophysiological and genetic considerations. *Endocrine Reviews* **19**: 521-539. Review.

Shi Y, Wang YF, Jayaraman L, Yang H, Massague J, Pavletich NP 1998 Crystal structure of a Smad MH1 domain bound to DNA: insights on DNA binding in TGF-beta signaling. *Cell* **94**: 585-594.

Shi Y & Massagué J 2003 Mechanisms of TGF-β signaling from cell membrane to the nucleus. *Cell* **113**: 685-700.

Shimasaki S, Koga M, Buscaglia ML, Simmons DM, Bicsak TA & Ling N 1989 Follistatin gene expression in the ovary and extragonadal tissues. *Molecular Endocrinology* **3**: 651-659.

Shimasaki S, Zachow RJ, Li D, Kim H, Iemura S, Ueno N, Sampath K, Chang RJ & Erickson GF 1999 A functional bone morphogenetic protein system in the ovary. *Proceedings of the National Academy of Sciences USA* **96**: 7282-7289.

Shimasaki S, Moore RK, Otsuka F & Erickson GF 2004 The bone morphogenetic protein system in mammalian reproduction. *Endocrine Reviews* **25**: 72-101. Review.

Shupnik MA, Gharib SD & Chin WW 1988 Estrogen suppresses rat gonadotropin gene transcription in vivo. *Endocrinology* **122**: 1842-1846.

Shupnik MA & Rosenzweig BA 1991 Identification of an estrogen-responsive element in the rat LH beta gene. DNA-estrogen receptor interactions and functional analysis. *The Journal of biological chemistry* **266**: 17084-17091.

Shupnik MA & Fallest PC 1994 Pulsatile GnRH regulation of gonadotropin subunit gene transcription. *Neuroscience and biobehavioral reviews* **18**: 597-599.

Simon AM, Goodenough DA, Li E & Paul DL 1997 Female infertility in mice lacking connexin 37. *Nature* **385**: 525-529.

Simonsson M, Heldin CH, Ericsson J & Gronroos E 2005 The balance between acetylation and deacetylation controls Smad7 stability. *Journal of Biological Chemistry* **280**: 21797-21803.

Sion B, Chanat E, Duval J & Thieulant ML 1988 Peptides co-released with luteinizing hormone by perifused pituitary cell aggregates. *Molecular and cellular endocrinology* **60**: 151-61.

Skinner DC, Caraty A, Malpaux B & Evans NP 1997 Simultaneous measurement of gonadotropin-releasing hormone in the third ventricular cerebrospinal fluid and hypophyseal portal blood of the ewe. *Endocrinology* **138**: 4699-4704.

Solloway MJ, Dudley AT, Bikoff EK, Lyons KM, Hogan BL & Robertson EJ 1998 Mice lacking Bmp6 function. *Developmental genetics* **22**: 321-339.

Solloway MJ & Robertson EJ Early embryonic lethality in Bmp5;Bmp7 double mutant mice suggests functional redundancy within the 60A subgroup. *Development* **126**: 1753-1768.

Souchelnytskyi S, Tamaki K, Engstrom U, Wernstedt C, ten Dijke P & Heldin CH 1997 Phosphorylation of Ser465 and Ser467 in the C terminus of Smad2 mediates interaction with Smad4 and is required for transforming growth factor-beta signaling. *The Journal of biological chemistry* **272**: 28107-28115.

Souchelnytskyi S, Nakayama T, Nakao A, Moren A, Heldin CH, Christian JL & ten Dijke P 1998 Physical and functional interaction of murine and Xenopus Smad7 with bone morphogenetic protein receptors and transforming growth factor-beta receptors. *Journal of Biological Chemistry* **273**: 25364-25370.

Souza CJ, Campbell BK, Webb R & Baird DT 1997 Secretion of inhibin A and follicular dynamics throughout the estrous cycle in the sheep with and without the Booroola gene (FecB). *Endocrinology* **138**: 5333-5340.

Souza CJ, MacDougall C, MacDougall C, Campbell BK, McNeilly AS & Baird DT 2001 The Booroola (FecB) phenotype is associated with a mutation in the bone morphogenetic receptor type 1 B (BMPR1B) gene. *Journal of Endocrinology* **169**: R1-R6.

Souza CJ, Campbell BK, McNeilly AS & Baird DT 2002 Effect of bone morphogenetic protein 2 (BMP2) on oestradiol and inhibin A production by sheep granulosa cells, and

localization of BMP receptors in the ovary by immunohistochemistry. *Reproduction* **123** 363-369.

Souza CJ, Campbell BK, McNeilly AS & Baird DT 2003 Bone morphogenetic proteins and folliculogenesis: lessons from the Booroola mutation. *Reproduction (Cambridge, England) Supplement* **61**: 361-370. Review.

Spady TJ, Shayya R, Thackray VG, Ehrensberger L, Bailey JS & Mellon PL 2004 Androgen regulates follicle-stimulating hormone beta gene expression in an activin-dependent manner in immortalized gonadotropes. *Molecular Endocrinology* **18**: 925-940.

Stanley E, Biben C, Kotecha S, Fabri L, Tajbakhsh S, Wang CC, Hatzistavrou T, Roberts B, Drinkwater C, Lah M, Buckingham M, Hilton D, Nash A, Mohun T & Harvey RP 1998 DAN is a secreted glycoprotein related to Xenopus cerberus. *Mechanisms of development* **77**: 173-184.

Stockell-Hartree A & Renwick AG 1992 Molecular structures of glycoprotein hormones and functions of their carbohydrate components. *The Biochemical Journal* **287**: 665-679. Review.

Stojilkovic SS, Reinhart J & Catt KJ 1994 Gonadotropin-releasing hormone receptors: structure and signal transduction pathways. *Endocrine Reviews* **15**: 462-499. Review.

Strahl BD, Huang HJ, Sebastian J, Ghosh BR & Miller WL 1998 Transcriptional activation of the ovine follicle-stimulating hormone beta-subunit gene by gonadotropin-releasing hormone: involvement of two activating protein-1-binding sites and protein kinase C. *Endocrinology* **139**: 4455-4465.

Suszko MI, Lo DJ, Suh H, Camper SA & Woodruff TK 2003 Regulation of the rat follicle-stimulating hormone beta-subunit promoter by activin. *Molecular Endocrinology* **17**: 318-332.

Suszko MI, Balkin DM, Chen Y & Woodruff TK 2005 Smad3 mediates activin-induced transcription of follicle-stimulating Hormone beta-subunit gene. *Molecular Endocrinology* **19**: 1849-1858.

T

Takase M, Imamura T, Sampath TK, Takeda K, Ichijo H, Miyazono K & Kawabata M 1998 Induction of Smad6 mRNA by Bone Morphogenetic Proteins. *Biochemical and Biophysical Research Communications* **244**: 26-29.

Takeda K, Oida S, Ichijo H, Iimura T, Maruoka Y, Amagasa T & Sasaki S 1994 Molecular cloning of rat bone morphogenetic protein (BMP) type IA receptor and its expression

during ectopic bone formation induced by BMP. *Biochemical and biophysical research communications* **204**: 203-209.

Takeda M, Otsuka F, Suzuki J, Kishida M, Ogura T, Tamiya T & Makino H 2003 Involvement of activin/BMP system in development of human pituitary gonadotropinomas and nonfunctioning adenomas. *Biochemical and biophysical research communications* **306**: 812-818.

Takuma N, Sheng HZ, Furuta Y, Ward JM, Sharma K, Hogan BL, Pfaff SL, Westphal H, Kimura S & Mahon KA 1998 Formation of Rathke's pouch requires dual induction from the diencephalon. *Development* **125**: 4835-4840.

Tamaki K, Souchelnytskyi S, Itoh S, Nakao A, Sampath K, Heldin CH & ten Dijke P 1998 Intracellular signaling of osteogenic protein-1 through Smad5 activation. *Journal of cellular physiology* **177**: 355-363.

Taragnat C, Bernier A & Fontaine J 1998 Gonadotrophin storage patterns in the ewe during the oestrous cycle or after long-term treatment with a GnRH agonist. *The Journal of endocrinology* **156**: 149-157.

Thomas GB, Martin GB, Ford JR, Moore PM, Campbell BK & Lindsay DR 1988 Secretion of LH, FSH and oestradiol-17 beta during the follicular phase of the oestrous cycle in the ewe. *Australian journal of biological sciences* **41**: 303-308.

Thomas SG & Clarke IJ 1997 The positive feedback action of estrogen mobilizes LH-containing, but not FSH-containing secretory granules in ovine gonadotropes. *Endocrinology* **138**: 1347-1350.

Tisdall DJ, Quirke LD, Smith P & McNatty KP 1997 Expression of the ovine stem cell factor gene during folliculogenesis in late fetal and adult ovaries. *Journal of molecular endocrinology* **18**: 127-135.

Tobin VA, Pompolo S & Clarke IJ 2001 The percentage of pituitary gonadotropes with immunoreactive oestradiol receptors increases in the follicular phase of the ovine oestrous cycle. *Journal of neuroendocrinology* **13**: 846-854.

Topper JN, Cai J, Qiu Y, Anderson KR, Xu YY, Deeds JD, Feeley R, Gimeno CJ, Woolf EA, Tayber O, Mays GG, Sampson BA, Schoen FJ, Gimbrone MA Jr & Falb D 1997 Vascular MADs: two novel MAD-related genes selectively inducible by flow in human vascular endothelium. *Proceedings of the National Academy of Sciences of the United States of America* **94**: 9314-9319.

Tougard C, Tixier-Vidal A 1988 Lactotropes and gonadotropes. *In*: The physiology of reproduction, Knobil E, Neill J coord., Raven Press limited, New York, 1994, 1711-1747.

Treier M, Gleiberman AS, O'Connell SM, Szeto DP, McMahon JA, McMahon AP & Rosenfeld MG 1998 Multistep signaling requirements for pituitary organogenesis in vivo. *Genes & development* **12**: 1691-1704.

Tsonis CG, Quigg H, Lee VW, Leversha L, Trounson AO, Findlay JK 1983 Inhibin in individual ovine follicles in relation to diameter and atresia. *Journal of Reproduction and Fertility* **67**: 83-90.

Tsukazaki T, Chiang TA, Davison AF, Attisano L & Wrana JL 1998 SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor. *Cell* **95**: 779-791.

Tsutsumi M, Zhou W, Millar RP, Mellon PL, Roberts JL, Flanagan CA, Dong K, Gillo B & Sealfon SC 1992 Cloning and functional expression of a mouse gonadotropin-releasing hormone receptor. *Molecular endocrinology* **6**: 1163-1169.

Turgeon JL, Kimura Y, Waring DW & Mellon PL 1996 Steroid and pulsatile gonadotropin-releasing hormone (GnRH) regulation of luteinizing hormone and GnRH receptor in a novel gonadotrope cell line. *Molecular endocrinolology* **10**: 439-450.

Turzillo AM & Nett TM 1997 Effects of bovine follicular fluid and passive immunization against gonadotropin-releasing hormone (GnRH) on messenger ribonucleic acid for GnRH receptor and gonadotropin subunits in ovariectomized ewes. *Biology of reproduction* **56**: 1537-1543.

U

Ueno N, Ling N, Ying SY, Esch F, Shimasaki S & Guillemin R 1987 Isolation and partial characterization of follistatin: a single-chain Mr 35,000 monomeric protein that inhibits the release of follicle-stimulating hormone. *Proceedings of the National Academy of Sciences of the United States of America* **84**: 8282-8286.

Ulloa-Aguirre A, Midgley AR Jr, Beitins IZ & Padmanabhan V 1995 Follicle-stimulating isohormones: characterization and physiological relevance. *Endocrine Reviews* **16**: 765-787. Review.

V

Vale W, Rivier J, Vaughan J, McClintock R, Corrigan A, Woo W, Karr D, Spiess J 1986 Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid. *Nature* **321**: 776-779.

Vitt UA, Hayashi M, Klein C & Hsueh AJ 2000 Growth differentiation factor-9 stimulates proliferation but suppresses the follicle-stimulating hormone-induced differentiation of cultured granulosa cells from small antral and preovulatory rat follicles. *Biology of Reproduction* **62**: 370-377.

Vitt UA, Hsu SY & Hsueh AJ 2001 Evolution and classification of cystine knot-containing hormones and related extracellular signaling molecules. *Molecular Endocrinology* **15**: 681-694. Review

W

Wandji SA, Srsen V, Voss AK, Eppig JJ, Fortune JE 1996 Initiation in vitro of growth of bovine primordial follicles. *Biology of Reproduction* **55**: 942-948.

Wang QF, Farnworth PG, Findlay JK & Burger HG 1988 Effect of purified 31K bovine inhibin on the specific binding of gonadotropins-releasing hormone to rat anterior pituitary cells in culture. *Endocrinology* **123**: 2161-2166.

Wang T, Li BY, Danielson PD, Shah PC, Rockwell S, Lechleider RJ, Martin J, Manganaro T & Donahoe PK 1996 The immunophilin FKBP12 functions as a common inhibitor of the TGF beta family type I receptors. *Cell* **86**: 435-444.

Watanabe T, Uchiyama Y & Grube D 1991 Topology of chromogranin A and secretogranin II in the rat anterior pituitary: potential marker proteins for distinct secretory pathways in gonadotrophs. *Histochemistry* **96**: 285-293.

Welt C, Sidis Y, Keutmann H & Schneyer A 2002 Activins, Inhibins, and Follistatins: from endocrinology to signaling. A paradigm for the new millennium. *Experimental Biology and Medecine (Maywood)* **227**: 724-752. Review.

Wiater E & Vale W 2003 Inhibin is an antagonist of bone morphogenetic protein signaling. *Journal of Biological Chemistry* **278**: 7934-7941.

Wildt L, Hausler A, Marshall G, Hutchison JS, Plant TM, Belchetz PE & Knobil E 1981 Frequency and amplitude of gonadotropin-releasing hormone stimulation and gonadotropin secretion in the rhesus monkey. *Endocrinology* **109**: 376-385.

Wilson T, Wu XY, Juengel JL, Ross IK, Lumsden JM, Lord EA, Dodds KG, Walling GA, McEwan JC, O'Connell AR, McNatty KP & Montgomery GW 2001 Highly prolific Booroola sheep have a mutation in the intracellular kinase domain of bone

morphogenetic protein IB receptor (ALK-6) that is expressed in both oocytes and granulosa cells. *Biology of Reproduction* **64**: 1225-1235.

Winnier G, Blessing M, Labosky PA & Hogan BL 1995 Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. *Genes & development* **9**: 2105-2116.

de Winter JP, ten Dijke P, de Vries CJ, van Achterberg TA, Sugino H, de Waele P, Huylebroeck D, Verschueren K & van den Eijnden-van Raaij AJ 1996 Follistatins neutralize activin bioactivity by inhibition of activin binding to its type II receptors. *Molecular and Cellular Endocrinology* **116**: 105-114.

Wise ME, Nieman D, Stewart J & Nett TM 1984. Effect of number of receptors for gonadotropin-releasing hormone on the release of luteinizing hormone. *Biology of Reproduction* **31**: 1007–1013.

Wrana JL, Attisano L, Wieser R, Ventura F & Massagué J 1994 Mechanism of activation of the TGF-beta receptor. *Nature* **370**: 341-347.

Wrana JL, Attisano L 1996 MAD-related proteins in TGF-beta signalling. *Trends in genetics: TIG* **12**: 493-496.

Wu X, Chen L, Brown CA, Yan C & Matzuk MM 2004 Interrelationship of growth differentiation factor 9 and inhibin in early folliculogenesis and ovarian tumorigenesis in mice. *Molecular Endocrinology* **18**: 1509-1519.

Wun WS & Thorneycroft IH 1987 Estradiol positive feedback on the rat anterior pituitary gland in vitro. *Molecular and cellular endocrinology* **54**: 165-169.

X

Xiao S, Robertson DM & Findlay JK 1992 Effects of activin and follicle-stimulating hormone (FSH)-suppressing protein/follistatin on FSH receptors and differentiation of cultured rat granulosa cells. *Endocrinology* **131**: 1009-1016.

Xing Y, Myers RV, CAo D, lin W, Jiang M, Bernard MP & Moyle MR 2004 Glycoprotein hormone assembly in the endoplasmic reticulum. III. The seatbelt and its latch site determine the assembly pathway. *The Journal of Biochemistry* **279**: 35449-35457.

Xu RH, Lechleider RJ, Shih HM, Hao CF, Sredni D, Roberts AB & Kung H 1999 Functional analysis of human Smad1: role of the amino-terminal domain. *Biochemical and biophysical research communications* **258**: 366-373.

Y

Yakymovych I, Heldin CH & Souchelnytskyi S 2004 Smad2 phosphorylation by type I receptor: contribution of arginine 462 and cysteine 463 In the C terminus of Smad2 for specificity. *The Journal of biological chemistry* **279**: 35781-35787.

Yamada Y, Yamamoto H, Yonehara T, Kanasaki H, Nakanishi H, Miyamoto E & Miyazaki K 2004 Differential activation of the luteinizing hormone beta-subunit promoter by activin and gonadotropin-releasing hormone: a role for the mitogen-activated protein kinase signaling pathway in LbetaT2 gonadotrophs. *Biology of Reproduction* **70**: 236-243.

Yamamoto N, Akiyama S, Katagiri T, Namiki M, Kurokawa T & Suda T 1997 Smad1 and smad5 act downstream of intracellular signalings of BMP-2 that inhibits myogenic differentiation and induces osteoblast differentiation in C2C12 myoblasts. *Biochemical and biophysical research communications* **238**: 574-580.

Yamashita H, ten Dijke P, Huylebroeck D, Sampath TK, Andries M, Smith JC, Heldin CH & Miyazono K 1995 Osteogenic protein-1 binds to activin type II receptors and induces certain activin-like effects. *The Journal of Cell Biology* **130**: 217-226.

Yan C, Wang P, DeMayo J, DeMayo FJ, Elvin JA, Carino C, Prasad SV, Skinner SS, Dunbar BS, Dube JL, Celeste AJ & Matzuk MM 2001 Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. *Molecular Endocrinology* **15**: 854-866.

Yao D, Dore JJ Jr, Leof EB 2000 FKBP12 is a negative regulator of transforming growth factor-beta receptor internalization. *Journal of Biological Chemistry* **275**: 13149-13154.

Ying SY, Becker A, Swanson G, Tan P, Ling N, Esch F, Ueno N, Shimasaki S & Guillemin R 1987 Follistatin specifically inhibits pituitary follicle stimulating hormone release in vitro. *Biochemical and biophysical research communications* **149**: 133-139.

Ying SY. Inhibins, activins, and follistatins: gonadal proteins modulating the secretion of follicle-stimulating hormone. *Endocrine Reviews* **9**: 267-293. Review.

Yu WH, Karanth S, Walczewska A, Sower SA & McCann SM 1997 A hypothalamic follicle-stimulating hormone-releasing decapeptide in the rat. *Proceedings of the National Academy of Sciences of the United States of America* **94**: 9499-9503.

Yu WH, Karanth S, Mastronardi CA, Sealfon S, Dean C, Dees WL & McCann SM 2002 Lamprey GnRH-III acts on its putative receptor via nitric oxide to release follicle-stimulating hormone specifically. *Experimental biology and medicine (Maywood, N.J.)* **227**: 786-793.

Z

Zhang H & Bradley A 1996 Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development. *Development* **122**: 2977-2986.

Zhang Y, Musci T & Derynck R 1997 The tumor suppressor Smad4/DPC 4 as a central mediator of Smad function. *Current biology* **7**: 270-276.

Zhou J, Kumar TR, Matzuk MM & Bondy C 1997 Insulin-like growth factor I regulates gonadotropin responsiveness in the murine ovary. *Molecular Endocrinology* **11**: 1924-1933.

Zhu H, Kavsak P, Abdollah S, Wrana JL & Thomsen GH 1999 A SMAD ubiquitin ligase targets the BMP pathway and affects embryonic pattern formation. *Nature* **400**: 687-693.

Zimmerman LB, de Jesus-Escobar JM & Harland RM 1996 The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell* **86**: 599-606.

Zimmerman CM, Kariapper MS & Mathews LS 1998 Smad proteins physically interact with calmodulin. *The Journal of biological chemistry* **273**: 677-680.

Zwijsen A, Verschueren K & Huylebroeck D 2003 New intracellular components of bone morphogenetic protein/Smad signaling cascades. *FEBS letters* **546**: 133-139.

Introduction

Les hormones gonadotropes, l'hormone lutéinisante (LH) et l'hormone folliculo-stimulante (FSH) sont synthétisées par les cellules gonadotropes de l'hypophyse antérieure. Une fois libérées dans le sang, elles agissent sur les gonades pour réguler la gamétogenèse et la stéroïdogénèse. Chez la femelle, elles jouent un rôle critique et complémentaire lors de la phase terminale de la croissance folliculaire. FSH stimule la croissance de follicules et la sélection du follicule dominant, agissant sur les cellules de granulosa. LH induit l'ovulation et la formation du corps jaune grâce à son action sur les cellules de la granulosa et celles de la thèque interne. Le signal donné par les hormones gonadotropes doit être finement régulé pour permettre un contrôle précis des étapes de la croissance folliculaire jusqu'à l'ovulation (McNeilly *et al.* 1992). Ainsi, les niveaux sécrétés de LH et de FSH varient au cours du cycle. De plus, la libération de LH se fait de manière pulsatile reflétant le profil de sécrétion du facteur hypothalamique, GnRH (Gonadotropin Releasing Hormone). Ceci a été démontré grâce à des mesures dans le sang porte hypothalamo-hypophysaire proche du site de libération de GnRH et du sang périphérique chez le mouton (Caraty *et al.* 1982, Clarke & Cummins 1982). Lorsque des mesures de FSH sont réalisées dans le sang périphérique, les profils de sécrétion ne sont pas clairement pulsatiles. Cependant, des études récentes chez le mouton montrent une synchronie entre la libération de GnRH et celle de la FSH lors de mesures dans le sang porte hypothalamo-hypophysaire (Padmanabhan *et al.* 1997) ou dans le sinus caverneux (Clarke *et al.* 2002). De plus, d'autres pulses de FSH sont présents, indépendants de ceux reflétant les pulses du GnRH. Ces différences de sécrétion entre LH et FSH suggèrent des mécanismes ou des facteurs de régulation différents. En plus de cette nature de sécrétion, les concentrations de LH et de FSH varient au cours du cycle. Chez la brebis comme chez la ratte, les profils de sécrétions suivent le même profil. Les concentrations plasmatiques de LH sont faibles et augmentent au moment du pic pré-ovulatoire en même temps que celles de FSH. Mais, alors que les concentrations en LH sont faibles en dehors du pic pré-ovulatoire, les concentrations de FSH fluctuent tout au long du cycle. Le rapport des concentrations LH/FSH détermine les phases du cycle. En effet, les concentrations en FSH élevées lors de la phase folliculaire sont nécessaires pour la croissance folliculaire et la maturation des follicules ovariens (Niswender *et al.* 1975). La forte concentration en oestradiol sécrétés par les follicules en croissance déclenche alors le pic pré-ovulatoire de LH et de FSH en fin de phase folliculaire. Juste après l'ovulation, les concentrations en LH redeviennent faibles alors qu'un second pic de FSH survient (Mann *et al.* 1990). Lors de la phase lutéale, les concentrations en

LH restent faibles alors que celles de FSH fluctuent épisodiquement. Malgré la forte augmentation de LH au cours du pic pré-ovulatoire, les niveaux d'expression de l' ARNm LHβ ne sont pas modifiés pendant le cycle oestrien (Currie & McNeilly 1995, Crawford *et al.* 2000, Fafioffe *et al.* 2004). A contrario, l'expression de l'ARNm FSHβ reflète la libération de FSH (McNeilly *et al.* 2003, Fafioffe *et al.* 2004).

Cette différence de profil de sécrétion des hormones gonadotropes souligne l'importance de mécanismes spécifiques et/ou de facteurs de régulation. Plusieurs niveaux sont concernés : lors de la synthèse, lors du stockage et/ou lors de la sécrétion.

Au niveau structural, les hormones gonadotropes sont des glycoprotéines constituées par l'association non covalente de deux sous-unités, une commune α et une spécifique, β. Une fois synthétisées et leur peptide signal clivé, ces sous-unités sont modifiées par l'ajout de chaînes oligo-saccharidiques spécifiques. Chez l'espèce ovine, les sous-unités α et FSHβ comportent deux chaînes N-gycosylées alors que la LHβ n'en comporte qu'une (Sockell-Hartree & Renvick 1992, Bousfield *et al.* 1994). En outre, la nature des sucres varie d'une hormone à l'autre. Ceci explique une différence dans les demi-vies des hormones. Par exemple, la LH humaine injectée chez le Rat a une demi-vie de 15min (Burgon *et al.* 1996) alors que celle de la FSH humaine injectée chez le singe est de 1h30 (Klein *et al.* 2002).

Le deuxième niveau concerné par un profil différentiel de sécrétion des hormones gonadotropes est celui du stockage des hormones une fois synthétisées. LH et FSH sont toutes deux synthétisées par les cellules gonadotropes de l'hypophyse antérieure qui constituent environ 10% des cellules hypophysaires (Childs *et al.* 1987, Taragnat *et al.* 1998). Des études ultrastructurales ont montré plusieurs types morphologiques cellulaires chez le rat (Childs *et al.* 1980, Tougard & Tixier-Vidal 1988, 1994), un seul type cellulaire est observé dans les autres espèces porcines et ovines (Dacheux 1978, Currie & McNeilly 1995). Par ailleurs, des études immunohistochimiques ou d'hybridation *in situ* ont montré que des populations cellulaires gonadotropes contenant LH et FSH (cellules bi-hormonales) ou seulement LH ou FSH (cellules mono-hormonales). Chez des rattes cyclées, la proportion de cellules contenant LH est doublée lors du pic pré-ovulatoire sans que le nombre de cellules gonadotropes soit modifié suggérant que les cellules monohormonales FSH aient participé à la synthèse de LH (Childs *et al.* 1987). Chez des brebis cyclées, la proportion de cellules gonadotropes diminue suite au pic pré-ovulatoire des hormones gonadotropes en conséquence d'une diminution du

nombre de cellules bihormonales (Taragnat *et al.* 1998). Ces variations de proportions des sous-populations lors du cycle oestrien peuvent être dépendantes des facteurs de régulations tels que le GnRH, les stéroïdes. En effet, des études chez la ratte ou chez la brebis montre que le nombre de cellules bihormonales est modifié par une stimulation au GnRH (Childs 1985, Molter-Gérard *et al.* 1999) ou par l'oestradiol (Molter-Gérard *et al.* 2000). De plus, au niveau sub-cellulaire, des granules de sécrétions contiennent l'une ou les deux hormones gonadotropes (Inoue & Kurosumi 1984). Alors que LH est stockée dans des granules denses aux électrons (Currie & McNeilly 1995, Crawford *et al.* 2002), FSH est présente dans des granules moins denses (Crawford *et al.* 2002). Cette différence de stockage est associée avec une différence de profil de sécrétion. LH est préférentiellement libérée par une voie régulée par GnRH et quelque FSH peut être associée avec LH. Cependant, la plupart de la FSH est libérée par une voie dite constitutive où chaque nouvelle molécule de FSH synthétisée est aussitôt libérée dans le sang. La voie régulée de LH est associée à des molécules appelées granines qui sont nécessaires pour l'exocytose. Dans les granules de sécrétion, LH est associée avec la secretogranine II (SgII) (Sion *et al.* 1988, Crawford *et al.* 2002) et FSH avec la chromogranine A (CgA) (Watanabe *et al.* 1991). Cependant, dans la lignée de cellules gonadotropes murines, $L\beta T_2$, la libération de la FSH n'est pas corrélée avec celle de la CgA contrairement à la LH qui est libérée simultanément avec SgII en réponse au GnRH et à l'activine (Nicol *et al.* 2002, 2004).

La synthèse et la sécrétion des hormones gonadotropes est modulée par des facteurs de régulation, hypothalamiques, gonadiques ou hypophysaires.

Le GnRH, facteur hypothalamique, est le premier stimulus nécessaire dans la fonction de reproduction. Plusieurs formes structurales sont connues à ce jour mais dans la majorité des espèces vertébrées, deux ou trois variants (GnRH-I, GnRH-II et GnRH-III) sont détectés et ceci dans des populations neuronales distinctes (Millar et al. 2004). GnRH-I est le facteur hypothalamique (appelé par la suite GnRH) alors que GnRH-II est une forme ubiquitaire. GnRH est libéré de manière pulsatile et module la biosynthèse et la libération des hormones gonadotropes (Fink 1988). Des mutations génétiques de GnRH ou une perte de fonction de son récepteur induisent un niveau faible des concentrations circulantes de LH et de FSH que ce soit chez la souris ou chez l'humain (Mason *et al.* 1986, Seminara *et al.* 1998, Kottler *et al.* 1999). L'importance du GnRH a été démontrée grâce à un blocage de son action par des techniques de disconnection hypothalamo-hypophysaire, d'immunisation passive ou d'injection par des antagonistes du GnRH diminuant ainsi les concentrations des hormones

gonadotropes et de leurs messagers (Clarke *et al.* 1983, Caraty *et al.* 1984, Lalloz *et al.* 1988). Cependant, les effets sont différents sur la synthèse et la libération de LH et de FSH. Le blocage de l'effet du GnRH résulte en une inhibition rapide de la sécrétion de LH démontrant un lien direct GnRH/LH alors que les concentrations de FSH diminuent plus lentement (Culler & Negro-Vilar 1986, Turzillo & Nett 1997, Molter-Gérard *et al.* 1999). Comment un même peptide peut-il agir sur une même cellule tout en modulant différemment la libération de LH et FSH ? Il a été montré que la fréquence des pulses de GnRH affectait de manière différente la libération des hormones gonadotropes. De fortes fréquences de pulses de GnRH augmentent le nombre de récepteurs au GnRH à la surface des cellules gonadotropes alors que de faibles fréquences diminuent leur nombre correspondant au profil observé lors du cycle oestrien (Clarke *et al.* 1987, Brooks *et al.* 1993, Kaiser *et al.* 1997, Fafioffe *et al.* 2004, Schirman-Hildesheim *et al.* 2005). De plus, une forte fréquence de GnRH, semblable à ceux de la phase folliculaire, stimule l'expression des ARNm α et LHβ et la libération de la LH alors qu'une faible fréquence, mimant ceux de la phase lutéale, favorise la production de FSH (Dalkin *et al.* 1989, Kaiser *et al.* 1997, Molter-Gérard *et al.* 1999, Farnworth 2000, Burger *et al.* 2002).

Par ailleurs, des études montrent que dans le sang porte hypophysaire, une certaine proportion de pulses de FSH n'est pas associée avec des pulses de GnRH (Padmanabhan *et al.* 1997). Ces pulses sont maintenus en présence d'un antagoniste du GnRH (Padmanabhan *et al.* 2003) suggérant le rôle d'autres facteurs de régulation spécifiques de la sécrétion de FSH (Padmanabhan & Sharma 2001, Pawson & McNeilly 2005). De plus, l'existence d'un facteur permettant la libération de FSH (FSH-RF) a été suggéré et son hypothèse a été soutenue par une expérience de lésion de l'aire hypothalamique dorsale antérieure montrant l'abolition des pulses de FSH mais pas ceux de LH (Lumpkin *et al.* 1989). L'un des variants de GnRH, GnRH-III pourrait être ce facteur (Yu *et al.* 1997, 2002). Néanmoins, cette hypothèse n'a pas été validée pour le moment.

D'autre part, des résultats montrent l'importance des facteurs gonadiques et hypophysaires sur la régulation différentielle de la sécrétion des hormones gonadotropes. Les effets des stéroïdes (progestérone et oestradiol) sur la libération de LH et FSH sont généralement positifs mais peuvent être négatifs en fonction du moment du cycle oestrien. Lors de la phase folliculaire, les follicules sécrètent de l'oestradiol qui augmente la fréquence des pulses GnRH/LH provoquant ainsi le pic pré-ovulatoire. En phase lutéale, le corps jaune sécrète de la progestérone diminuant les fréquences des pulses de GnRH (Karsh *et al.* 1997). L'effet positif des oestrogènes s'explique par la stimulation du nombre de récepteurs au

GnRH (Clarke *et al.* 1988, Gregg *et al.* 1990, Ghosh *et al.* 1996, Cowley *et al.* 1998) ainsi que la libération de GnRH elle-même (Moenter *et al.* 1990). L'effet négatif des stéroïdes a été démontré lors de gonadectomie car dans ce cas, les ARNm LHβ, FSHβ et les concentrations en FSH et LH sont stimulés. De plus, l'addition d'oestrogènes abolit cette stimulation (Corbani *et al.* 1984, Shupnik *et al.* 1988). Pour LH, l'effet des oestrogènes est hypothalamique comme le démontre l'ajout d'un antagoniste du GnRH qui rétablit les concentrations des hormones gonadotropes (Dalkin *et al.* 1993, Shupnik & Fallest 1994). Cependant, les oestrogènes peuvent aussi agir au niveau hypophysaire. Chez les animaux dont l'hypothalamus est déconnecté de l'hypophyse et donc ne présentant plus de libération de LH, la restauration des pulses de LH est obtenue par un analogue du GnRH. Dans ces conditions, l'injection d'oestradiol induit une suppression rapide des concentrations de LH due à une réduction de l'amplitude des pulses de LH et de FSH (Clarke & Cummins 1984, Molter-Gérard *et al.* 2000). De plus, cet effet inhibiteur est aussi observé sur la FSH lorsque des cellules hypophysaires ovines sont traitées par de l'oestradiol pendant 24h (Phillips *et al.* 1988, Nett *et al.* 2002, Faure *et al.* 2005).

En plus de l'action du GnRH et des stéroïdes, des protéines sécrétées par les gonades, les activines et les inhibines agissent préférentiellement sur la synthèse et la libération FSH. Elles sont constituées de deux sous-unités (α et β pour les inhibines et deux β pour les activines). Les inhibines A et B comportant respectivement les sous-unités βA et βB inhibent la sécrétion de la FSH (De Kretser & Robertson 1989, Vale *et al.* 1990) alors que les activines A (βA-βA), B (βB-βB) et AB (βA-βB) stimulent la production de FSH (Ling *et al.* 1986, Vale *et al.* 1986). La follistatine, protéine sans rapport structural avec les activines et les inhibines, lie les activines et bloque leurs activités (Ueno *et al.* 1987, Ying *et al.* 1987, Nakamura *et al.* 1990, Farnworth *et al.* 1995). L'injection d'un anticorps polyclonal anti-inhibine chez la ratte provoque une stimulation de la libération de FSH et des messagers FSHβ sans affecter celle de LH (Rivier *et al.* 1986, Dalkin *et al.* 1993). L'effet opposé a été obtenu en injectant un anticorps anti-activin sans modifier la sécrétion de LH (Corrigan *et al.* 1991). Ces expériences démontrent l'importance des inhibines et des activines sur la régulation spécifique de la sécrétion de FSH.

Par ailleurs, des études ont montré que d'autres protéines appartenant à la même famille du TGFβ que les activines et les inhibines, les Bone Morphogenetic Proteins (BMPS) sont décrites comme des facteurs importants dans la fonction de reproduction. En effet, des mutations naturelles de ces facteurs ou de leurs récepteurs entraînent des cas

d'hyperprolificité chez les brebis (Davis *et al.* 1991, Mulsant *et al.* 2001, Souza *et al.* 2001, Wilson *et al.* 2001). Alors que le rôle de ces BMPs au niveau ovarien est bien documenté, peu de données sont disponibles au niveau hypophysaire excepté un rôle dans la mise en place de l'hypophyse et la détermination des cellules gonadotropes lors de l'embryogénèse. Dans ce contexte, notre étude à viser à évaluer le rôle potentiel des BMPs dans la régulation différentielle de la sécrétion des hormones gonadotropes.

Résultats

Chapitre I :

Les ARNm des BMP-2, BMP-4, BMP-7 et GDF-9 ont été détectés dans l'hypophyse ovine par RT-PCR. Les transcrits des récepteurs BMP-RIA, BMP-RIB et BMP-RII sont exprimés mais seuls BMP-RIA et BMP-RII, pas BMPR-IB, colocalisent avec les cellules gonadotropes sur des coupes d'hypophyses. Pour déterminer l'effet des BMPs, BMP-4, BMP-6, BMP-15 et GDF-9 sur la sécrétion de FSH, différentes doses (de 3.10^{-11} à 3.10^{-9} M) sont ajoutées au milieu de culture des cellules hypophysaires ovines dissociées et platées. Quarante-huit heures plus tard, les surnageants sont récoltés et FSH et LH sont dosées grâce à un ELISA de type sandwich. Les ARN cellulaires sont extraits pour analyser les ARNm de FSHβ et de LHβ par RT-PCR en temps réel (Taqman). BMP-4 entraîne une inhibition dose-dépendante de la libération de FSH par les cellules hypophysaires qui atteint 41,1 ± 7,8 % pour une dose de 3.10^{-9}M (n=10, $p<0.05$) sans affecter celle de la LH. Cette inhibition s'accompagne d'une diminution des ARNm de FSHβ. Cette même inhibition est retrouvée avec les autres BMPs testées.

Pour déterminer le rôle des BMPs vis-à-vis des régulateurs connus de FSH et LH, les cellules hypophysaires ont été co-traitées avec GnRH (10^{-8}M), l'oestradiol (10^{-13}M) ou l'activine A (10^{-9}M). Le traitement des cellules hypophysaires avec BMP-4 modère la stimulation de la libération de FSH induite par GnRH (10^{-8}M). Par ailleurs, BMP-4 amplifie l'action négative de l'oestradiol (10^{-13}M) sur l'expression du messager FSHβ et sur la libération de FSH. Le co-traitement des cellules hypophysaires avec BMP-4 et activine (10^{-9}M) annule les effets respectivement inhibiteur et stimulateur de ces molécules sur l'expression des ARNm de FSHβ et la libération de FSH. Ces résultats indiquent que les BMPs exercent une action inhibitrice sur la synthèse et la libération de FSH. Cette action

s'oppose à celle de l'activine. De plus, les BMPs diminuent l'effet stimulateur de GnRH et amplifient l'action inhibitrice de l'œstradiol.

Chapitre II :

Dans le premier chapitre, nous avons démontré que BMP-4 était capable d'inhiber la sécrétion de FSH et d'antagoniser les effets stimulants de l'activine dans des cellules hypophysaires ovines en culture. Nous avons recherché si l'effet de BMP-4 était dépendant ou non de la voie de signalisation de l'activine. Les voies de signalisation des BMPs et de l'activine sont similaires. En effet, après liaison aux récepteurs spécifiques, ceux-ci entraînent la phosphorylation de R-Smads (Smad-1, Smad-5 et Smad-8 pour BMPs ou de Smad-2 et Smad-3 pour l'activine). Une fois activées, ces R-Smads se lient à une Smad commune aux deux voies de signalisation (Smad-4) et le complexe transloque au noyau pour activer le(s) gène(s) cible(s). Nous avons montré par Western blot que la protéine Smad-1 était phosphorylée après un traitement d'une heure par BMP-4 indiquant que la voie de signalisation spécifique des BMPs était activée. Pour tester si l'inhibition de la sécrétion de FSH par le traitement BMP-4 était due à l'activation des voies de signalisation utilisant les R-Smads, nous avons surexprimé Smad-7, un inhibiteur de la phosphorylation des récepteurs BMP et/ou activine. Des expériences préliminaires de surexpression de LacZ (100pfu/cellule) montrent que 90% des cellules hypophysaires ovines étaient infectées. La surexpression de Smad-7 (100pfu/cellule) a été vérifiée grâce à une immunohistochimie utilisant l'anticorps anti-Smad-7. De plus, le niveau de phosphorylation de Smad-1 est réduit de manière dose-dépendante. De manière intéressante, la surexpression de Smad-7 reverse l'inhibition des niveaux de messagers FSHβ ainsi que de la libération de la FSH due au traitement par BMP-4. Il faut aussi souligner que la surexpression de Smad-7 marque une inhibition des messagers FSHβ et de la libération de la FSH au niveau basal. Ce résultat suggère un effet endogène stimulant qui aurait été bloqué par la surexpression de Smad-7. Nous faisons l'hypothèse qu'il s'agit du rôle de l'activine qui est bien connue pour agir de manière endogène sur la sécrétion de FSH via la voie de signalisation Smad-2/Smad-3 (Bernard 2004). Dans nos conditions de cultures, la forme phosphorylée de Smad-2 est détectée en l'absence de tout traitement. De plus, la surexpression de Smad-7 bloque la stimulation par l'activine de l'expression du messager FSHβ. En conséquence, nous ne pouvons pas conclure si les BMPs agissent via Smad-1 ou en en interagissant avec la voie de signalisation de l'activine pour inhiber la sécrétion de FSH. Néanmoins, nous avons utilisé la follistatine comme inhibiteur de la voie de

signalisation de l'activine. Lorsque les cellules hypophysaires ovines sont co-traitées avec BMP-4 (10^{-9}M) et follistatine (0,03 à $1,3x10^{-9}$M), une inhibition plus forte que celle obtenue avec chaque facteur est observée. Par ailleurs, la présence conjointe de BMP-4 et d'activine ne modifie pas les niveaux de phosphorylation de Smad-2 par rapport à un seul facteur. Pour tester si BMP-4 pouvait agir sur des facteurs communs avec la voie de l'activine, les niveaux des messagers de Smad-4 et de Smad-7 sont mesurés après un traitement par BMP-4, activine ou les deux facteurs sur des cellules hypophysaires ovines mises en culture. BMP-4 ne modifie pas l'expression du messager de Smad-4 mais stimule celui de Smad-7. L'ensemble de ces résultats suggère que l'effet de BMP-4 sur la sécrétion de la FSH se fait dans un premier temps via la voie Smad-1 et dans un deuxième temps en modulant l'expresssion de l'ARNm de Smad-7 qui pourrait inhiber la voie de signalisation de l'activine requise pour la synthèse et la libération de FSH. D'autres expériences en cours vise à surexprimer Smad-6, un inhibiteur spécifique de la voie BMP, bloquant l'interaction entre Smad-1 phosphorylée et Smad-4.

Chapitre III :

Nos résultats ont été obtenus sur des cultures primaires de cellules hypophysaires ovines. L'hypophyse antérieure est constituée de plusieurs types cellulaires dont les cellules gonadotropes qui représentent seulement 10% des cellules. Pour déterminer le rôle des BMPs sur les cellules gonadotropes, une lignée murine de cellules gonadotropes a été utilisée.

BMP-6, BMP-7, BMP-RIA et BMP-RII ont été détectés par RT-PCR suggérant un rôle paracrine de ces BMPs sur les cellules gonadotropes murines. Lorsque des cellules $L\beta T_2$ sont traitées par BMP-4 seul, aucun effet sur les niveaux de messagers de FSHβ, de LHβ ni de libération de FSH et de LH ne sont observés. Par contre, en présence simultanée de GnRH et d'activine, BMP-4 est capable d'amplifier l'effet stimulateur de GnRH et de l'activine sur la synthèse et la libération de FSH. De manière intéressante, BMP-4 inhibe l'effet stimulateur de GnRH et de l'activine sur les niveaux du messager LHβ et sur la libération de LH.

Pour déterminer si les voies BMPs et activine pouvaient interagir, la phosphorylation de Smad-1 et de Smad-2 a été suivie par Western blot. Que ce soit en présence ou non de GnRH, aucune modification de l'intensité du signal n'est visible.

Comme BMP-4 est capable de moduler de manière différentielle la sécrétion des hormones gonadotropes en présence du GnRH dans la lignée gonadotrope murine, $L\beta T_2$, nous avons étudié l'effet de BMP-4 sur l'expression du messager du récepteur du GnRH. Le

résultat obtenu suit le profil de celui de la LH suggérant que BMP-4 serait capable en inhibant l'expression du messager du récepteur du GnRH de mimer une situation analogue à une diminution des fréquences de pulses de GnRH via une baisse du nombre de récepteurs au GnRH, privilégiant ainsi la sécrétion de FSH.

Conclusion et discussion

Nous avons montré la présence de plusieurs BMPs et de leurs récepteurs dans l'hypophyse ovine et dans la lignée gonadotrope murine, $L\beta T_2$. Les BMPs régulent de manière différentielle la sécrétion des hormones gonadotropes en fonction des espèces étudiées. Nos résultats montrent que BMP-4 et d'autres BMPs (BMP-6, BMP-15 et GDF-9) sont capables d'inhiber préférentiellement la sécrétion de FSH sans inhiber celle de LH chez l'ovin alors que BMP-4 amplifie l'effet stimulateur de l'activine et du GnRH sur la sécrétion de FSH et inhibe la production de LH stimulée par l'activine et le GnRH dans la lignée murine de cellules gonadotropes. De plus, BMP-4 est capable d'interagir avec des facteurs régulateurs de la sécrétion des hormones gonadotropes tels que le facteur hypothalamique, GnRH, les facteurs gonadiques et hypophysaires, l'oestradiol et l'activine. Dans cette discussion, nous avons traité les questions suivantes :

1- Quel rôle les BMPs jouent-ils dans la régulation différentielle de la synthèse et de la libération des hormones gonadotropes ?

2- Comment les BMPs interagissent-ils avec les autres modulateurs de la sécrétion des hormones gonadotropes ?

3- Existe-t-il un rôle physiologique pour les BMPs sur la régulation différentielle de la sécrétion de LH et de FSH ?

1- Quel rôle les BMPs jouent-ils dans la régulation différentielle de la synthèse et de la libération des hormones gonadotropes ?

Les messagers de BMP-2, BMP-4 et BMP-7 ont été détectés dans l'hypophyse ovine et ceux de BMP-6 et BMP-7 dans la lignée gonadotrope murine, $L\beta T_2$. Ces résultats corroborent ceux de Souza et collaborateurs (2003) pour l'ovin et d'Otsuka et Shimasaki (2002) pour les $L\beta T_2$. De plus, nous avons montré la présence d'un facteur exprimé spécifiquement dans l'ovocyte, GDF-9 (Yan *et al.* 2001). Les résultats obtenus soulignent une différence d'expression entre l'ovin et la souris. Les messagers des récepteurs BMP-RIA,

BMPR-IB et BMP-RII ont été détectés dans l'hypophyse ovine mais seuls les récepteurs de type IA et II ont été colocalisés sur les cellules gonadotropes ovines. De plus, ces mêmes récepteurs ont été détectés dans la lignée de cellules gonadotropes murines. Comme cela a été décrit précédemment, une race de brebis hyperprolifique, Mérinos Booroola, possède le récepteur de type IB muté. Il serait donc intéressant de déterminer sur quel type cellulaire ce récepteur est exprimé et ainsi, de doser l'hormone concernée. Comme les ligands sont présents, nous avons émis l'hypothèse d'un rôle paracrine. Le traitement des cellules hypophysaires en culture avec noggin, un antagoniste des BMPs, n'a pas permis de conclure quand à leurs rôles. Il serait intéressant de doser les BMPs circulants une fois un dosage disponible ou d'incuber les cellules hypophysaires ovines avec un anticorps anti-BMPs.

Nous avons montré par ailleurs que les BMPs agissaient de manière différente sur la sécrétion des hormones gonadotropes chez l'ovin et dans la lignée cellulaire murine. Chez l'ovin, l'effet de BMP-4 inhibe spécifiquement la synthèse et la libération de la FSH sans affecter ni la production de LH ni celle de la prolactine. Cet effet sur la sécrétion de FSH a été aussi retrouvé avec BMP-6, BMP-15 et GDF-9. Ces résultats étaient surprenants car d'autres études montraient plutôt un rôle stimulateur de BMP-6, BMP-7 et de BMP-15 sur la sécrétion de FSH chez les rongeurs (Huang *et al.* 2001, Otsuka & Shimasaki 2002). Nous avons donc voulu tester l'effet de BMP-4 sur nos cultures de cellules provenant de la lignée gonadotrope murine. Aucun effet de BMP-4 seul ne fut détectable mais BMP-4 était capable de moduler en présence simultanée d'activine et de GnRH la synthèse et la sécrétion des hormones gonadotropes, d'une part en amplifiant leur effet stimulateur sur la FSH et d'autre part, en inhibant leur effet stimulateur sur la production de LH. Ces résultats montrent bien que les BMPs peuvent réguler de manière différentielle la synthèse et la libération des hormones gonadotropes en fonction de l'espèce étudiée.

Bien que nos résultats montrent que BMP-4 soit capable de phosphoryler Smad-1 dans les deux espèces, nous ne pouvons pas déterminer si cette activation est liée à l'effet obtenu sur la production des hormones gonadotropes. Par ailleurs, le blocage de la voie de signalisation spécifique des BMPs par la surexpression d'un inhibiteur, Smad-7, montre une réversion de l'effet inhibiteur de BMP-4 sur la synthèse et la libération de la FSH. Mais comme Smad-7 peut aussi moduler la voie de signalisation de l'activine, nous ne pouvons exclure que l'effet de BMPs passe via la voie de l'activine. Des résultats supplémentaires suggèrent pourtant une indépendance des deux voies de signalisation car l'addition simultanée avec BMP-4 de follistatine, antagoniste de l'activine, inhibe plus fortement la libération de FSH qu'en présence des facteurs seuls. D'autres expériences permettront de déterminer le

type de voie de signalisation engendrée par BMP-4. En effet, BMPs peuvent interagir avec d'autres voies comme celles des mitogen-activated protein kinase (MAPK), Ca2+/calmoduline, CDK ou JAK/STAT (pour revues : von Bubnoff & Cho 2001, Miyazono *et al.* 2001, Nohe *et al.* 2004).

Quelque soit la (les) voie(s) de signalisation activée(s) par BMP-4 pour moduler la sécrétion des hormones gonadotropes, il semble important de déterminer à quel niveau la régulation s'effectue. Des études du promoteur de FSHβ et/ou de LHβ nous apporteraient des éléments de réponse. De plus, BMPs peut agir sur la stabilité du messager et des expériences en utilisant l'actinomycine élucideraient ce point.

2- Comment les BMPs interagissent-ils avec les autres modulateurs de la sécrétion des hormones gonadotropes ?

BMP-4 module la régulation différentielle de la sécrétion des hormones gonadotropes. Cette régulation est le jeu de plusieurs facteurs, hypothalamique (GnRH), gonadiques (les stéroïdes et les facteurs non-stéroïdiens comme les activines et les inhibines) et hypophysaires (les activines).

GnRH est connu pour stimuler la production de LH et de FSH. Dans nos cultures hypophysaires ovines, BMP-4 a inhibé cet effet stimulateur sur la libération de FSH sans modifier la stimulation de LH par le GnRH. Cependant, la stimulation par le GnRH n'a pas été obtenue de manière consistante. Une amélioration du protocole est envisagée. Par ailleurs, en utilisant le protocole mis en place par Nicol et collaborateurs (2002, 2004), nous avons reproduit une situation où un pulse de GnRH par jour (équivalent à une faible fréquence de pulses de GnRH) favorisaient avec l'activine la synthèse et la libération de FSH dans la lignée de cellules murines. Dans ces conditions et de manière complètement opposée à l'ovin, BMP-4 amplifiait l'effet stimulateur de l'activine et du GnRH sur la sécrétion de FSH. Par ailleurs, dans ces conditions, BMP-4 inhibait la stimulation de la sécrétion de LH par l'activine et le GnRH. De manière intéressante, BMP-4 inhibait l'expression du messager du récepteur du GnRH mimant ainsi une situation analogue à une réduction des pulses de GnRH.

Chez l'ovin, l'étude de l'effet simultané de l'oestradiol connu pour inhiber la production de FSH et de celui de BMP-4 a montré une inhibition encore plus forte qu'en présence des deux facteurs seuls. Dans un autre système, celui des prolactinomes humains, BMP-4 est décrit pour stimuler la prolifération des cellules. Dans ces conditions, l'oestradiol et BMP-4 ont des effets additifs (Paez-Pereda *et al.* 2003). Dans ce modèle, Smad-1 et Smad-4 interagissent physiquement avec le récepteur de l'oestradiol en présence de BMP-4 (Paez-

Pereda *et al.* 2003, Wu *et al.* 2003). Il serait donc intéressant de déterminer si de tels mécanismes interviennent dans les cellules hypophysaires ovines en analysant par exemple les éléments de réponse du récepteur des oestrogènes.

Les facteurs gonadiques appartenant à la famille du TGFβ, les inhibines et les activines, agissent préférentiellement sur la sécrétion de FSH. Les inhibines inhibent la transcription du messager FSHβ et la libération de la FSH alors que les activines les stimulent (De Kretser & Robertson 1989, Vale *et al.* 1990, Ling *et al.* 1986, Vale *et al.* 1986). Les sous-unités des inhibines et des activines ont été détectées chez le rat par immunolocalisation dans l'ovaire et l'hypophyse antérieure (Meunier *et al.* 1988). De plus, l'incubation de cellules hypophysaires de rat avec un anticorps anti-activine B réduit expression du messager FSHβ et les concentrations en FSH sans modifier la production de LH (Corrigan *et al.* 1991). Dans notre travail, des cellules hypophysaires ovines ont été traitées simultanément par de l'activine A et BMP-4. Ce co-traitement a eu pour résultat d'antagoniser les effets stimulateurs de l'activine et les effets inhibiteurs de BMP-4. Pour comprendre à quel niveau ces deux protéines pouvaient interagir, nous sommes intéressés aux voies de signalisation. Aucune modification n'a été détectée sur les niveaux de phosphorylation des protéines Smad-1 et Smad-2, respectivement spécifiques des voies de signalisation BMPs et activines. L'interaction a vraisemblablement lieu à un niveau post- R-Smads. Une étude approfondie des éléments de réponse aux Smads sur le promoteur de FSHβ ou LHβ devrait nous apporter des informations supplémentaires.

3- Existe-t-il un rôle physiologique pour les BMPs sur la régulation différentielle de la sécrétion de LH et de FSH ?

BMPs affectent de manière différente la sécrétion des hormones gonadotropes. Est-ce que cet effet a un sens physiologique et est-il reproductible au cours du cycle oestrien et lors d'expériences *in vivo* ?

Des expériences *in vivo* pourraient être mises en place en injectant par stéréotaxie des BMPs ou des inhibiteurs au niveau de l'hypophyse et d'analyser les variations des concentrations en hormones gonadotropes. Une seconde approche qui a été développée par Davis et collaborateurs (2001) serait d'infecter les cellules hypophysaires par stéréotaxie avec un adénovirus.

Des expériences préliminaires ont montré que l'expression du messager de BMP-4 pouvait être stimulé par l'activine mais non affecté par un traitement par l'oestradiol. Pour déterminer si l'effet de BMPs peut varier au cours du cycle, il faudrait obtenir un dosage des BMPs qui n'est pas disponible à l'heure actuelle. De plus, une étude des antagonistes pourrait nous indiquer la disponibilité des BMPs. Pour tester si les cellules gonadotropes sont réceptives tout au long du cycle à l'effet des BMPs, des cellules prélevées à différents stades du cycle pourraient être traitées par les BMPs.

En conclusion, pour la première fois, nous avons mis en évidence un effet inhibiteur spécifique sur la synthèse et la libération de la FSH chez l'ovin. En revanche, ces mêmes molécules modulent différemment la sécrétion des hormones gonadotropes chez la souris. L'ensemble de ce travail souligne l'importance des BMPs au niveau hypophysaire.

Abstract

Bone Morphogenetic Proteins (BMPs): new modulators of the follicle-stimulating hormone (FSH) synthesis and release in the ewe

In females, the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) produced by gonadotropes are required for the terminal follicular growth and the regulation of ovulation number. The patterns of the synthesis and release of LH and FSH diverge in several physiological situations. However, the mechanisms involved in LH and FSH differential regulation are still unclear. Gonadotropin synthesis and release are orchestrated by interplay of hypothalamic, gonadal and pituitary factors. Although GnRH and the gonadal steroids are common to FSH and LH hormone regulation, they act differentially on their production. In addition to GnRH and steroids, some members of the Transforming Growth Factor-Beta (TGFβ) superfamily, particularly activins and inhibins, are involved in the specific regulation of FSH production, stimulating and inhibiting respectively FSH synthesis and release. Inhibins and activins are gonadic factors but activins are also described to act as paracrine factors. Recently, other molecules of the TGF-β superfamily, the Bone Morphogenetic Proteins (BMPs) were shown to play a role in Reproduction, acting at least at the ovary level.

To extend our knowledge on FSH synthesis regulation, we investigated the potential role of BMPs in the ewe pituitary. The two types of BMP receptors (BMPR-IA and BMPR-II) co-localised with gonadotrope cells and their ligands (BMP-2, BMP-4, BMP-7 and GDF-9) mRNAs were detected in the pituitary suggesting a paracrine action of BMPs. BMPR-IB was not detected on gonadotropes. Interestingly, in three breeds of ewes, BMP-4 as well as BMP-6, BMP-15 and GDF-9 inhibited specifically the FSH synthesis and release, but not the LH production from ewe pituitary cells. Moreover, BMP-4 antagonized the stimulatory effect of activin and amplified the inhibitory action of 17β-estradiol.

To determine at which level BMP-4 was able to act on FSH differential secretion, we investigated the Smads signaling pathways. BMP-4 activated the phosphorylation of Smad-1/-5 without affecting Smad-2 phosphorylation level triggered by activin. The blockade by inhibitory Smad, Smad7 showed the role of the Smad signaling in BMP-4 action. Blocking the activin signaling by follistatin suggested independence between activin and BMP-4 signaling pathways.

In the mouse gonadotrope cell line ($L\beta T_2$), BMPs and their receptors mRNAs were detected. In contrast to ewe, BMP-4 alone was not able to modulate the gonadotropin secretion. However, BMP-4 amplified the stimulatory effect of activin and GnRH on FSH secretion. Moreover, BMP-4 inhibited the stimulatory effect of activin and GnRH on LH production by inhibiting the expression of GnRH receptor. These differences of effect due to the species underlined the necessity of studying different models to enhance our understanding of reproductive function.

Collectively, these data open up a novel concept that a functional BMP system plays a crucial role in regulating gonadotropin synthesis and release. Further investigations should determine by which intracellular mechanisms BMPs act on FSH secretion in ewe and in mouse. Furthermore, the physiological importance of BMPs within the pituitary in the overall regulation of FSH synthesis and release has to be established.