



## The endocrine and local control of ovarian follicle development in the ewe

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### Abstract

Follicle development can be divided into gonadotrophin-independent, gonadotrophin-responsive and gonadotrophin-dependent phases. The basic level of control of folliculogenesis lies with the actions of an extensively observed but poorly understood range of somatic and germ cell derived local factors that constitute an intrafollicular developmental cascade that regulates the proliferative and differentiative phases of follicle development. As follicle development progresses, the follicle become increasingly responsive to the actions of the pituitary gonadotrophins FSH and LH and during these phases of development local factors act to modulate the actions of gonadotrophins so that the fate of each follicle depends on an intra-follicular balance between local factors which augment or attenuate gonadotrophic actions. Gonadotrophins are therefore not a prerequisite for the continued growth of gonadotrophin-responsive pre-antral follicles, but FSH does appear to stimulate development and these actions are modulated by local growth factors, such as IGFs, AMH and BMPs. Naturally occurring mutations in sheep for a number of these factors or their receptors have provided insights into their roles during both the early and terminal stages of follicle development and we have recently produced extensive evidence supporting a major role for members of the BMP system in regulating follicle selection mechanisms through increased activation of gonadotrophic augmentors (BMP-6), decreased activation of attenuators (BMP-15, GDF-9, AMH) or a combination of these two mechanisms resulting in the deregulation of the normal follicle selection mechanisms. The terminal stages of follicle development, however, remain primarily under the control of the pituitary gonadotrophins, with both FSH and LH having essential and inter-related roles in regulating final maturation and selection of the ovulatory follicle and the oocyte which it contains.

**Keywords:** follicle, intraovarian, FSH, LH, AMH, BMP.

### Introduction

The sheep ovary functions as both an endocrine and gametogenic organ responsible for the production of an appropriate number of developmentally competent oocytes, at an optimum time to ensure fertilisation and

subsequent implantation and establishment of pregnancy that will result in the birth of offspring when environmental conditions are optimal to ensure lamb survival. To accomplish this feat, a series of intricate homeostatic control mechanisms have evolved which transduce environmental and physiological information, either directly or via the hypopituitary axis, to control reproductive activity. Over the 20 years, advances in our understanding of the cell biology of the somatic and germ cell components of ovarian follicles have shown that follicle development can be divided into two distinct developmental phases, separated by a prolonged intermediate phase. Thus the phase from primordial follicle initiation to the late preantral phase in which follicle development appears to be controlled by expression of a range of local factors is termed gonadotrophin-independent; the phase from the small antral to large antral stage is termed gonadotrophin-dependent as follicles of this size do not occur in the absence of critical threshold concentrations of the pituitary gonadotrophins, LH and FSH; and the intermediate gonadotrophin-responsive phase in which follicles will respond to the actions of gonadotrophins but do not require them for normal growth and development. In this review I will examine recent advances in this area with respect to advances in the understanding of the mechanisms controlling follicle development in monovulatory species, concentrating primarily on the sheep.

### Gonadotrophin-independent phase

Primordial follicles represent the source from which follicles will be recruited for growth throughout life, with paired ovaries of an individual containing around 100,000-250,000 of these follicles at birth (Turnbull *et al.*, 1977). Once follicles have been initiated to grow, the granulosa cells proliferate to form multilaminar structures (pre-antral follicles) which subsequently form a fluid filled space (antrum) and a well differentiated theca layer. Follicular development in sheep and cattle takes around 4-5 months with the majority of this time (3-4 months) being spent in the pre-antral stages of development (Turnbull *et al.*, 1977; Cahill, 1981; Driancourt *et al.*, 1985).

The gonadotrophin-independent phase of follicle development is the least studied, due primarily to the technical difficulty in isolating and conducting interventionist studies on such small structures



embedded in a tough matrix of ovarian cortex. Accordingly, histological and more recently immunohistochemical and *in situ* hybridisation approaches have been the main experimental tools utilised to study early folliculogenesis and these approaches have yielded a large body of observational data describing the growth characteristics (Turnbull *et al.*, 1977; Cahill, 1981; Driancourt *et al.*, 1985) and patterns of expression of a wide range of local factors throughout this stage of folliculogenesis (McNatty *et al.*, 1999, 2000). Whilst yielding essential information with respect to the identity and pattern of expression of local factors, the observational nature of these determinations means that it is not possible to identify the key development checkpoints controlling this developmental process nor formulate and test hypotheses relating to these mechanisms. Our understanding of the control of gonadotrophin-independent follicle development has therefore been largely based on the occurrence of naturally occurring mutations in which this process has been disrupted and more recently the development of *in vitro* (Gutierrez *et al.*, 2000; Picton *et al.*, 2003, 2004; Walters *et al.*, 2006) and *in vivo* (Campbell *et al.*, 2004) models which allow, to some degree, manipulation of the endocrine and local environment.

In Inverdale (FecX<sup>L</sup>) and Hanna (FecX<sup>H</sup>) ewes, separate point mutations in the bone morphogenetic protein (BMP15) gene on the X chromosome occur corresponding to sites in the mature peptide coding region of the BMP15 growth factor (Galloway *et al.*, 2000). A remarkable characteristic of these mutations is that those which are heterozygous for the FecX<sup>L</sup> or FecX<sup>H</sup> mutation have higher than normal ovulation rates and litter sizes whereas the homozygotes are sterile (Davis *et al.*, 2001). Similarly, in Cambridge and Belclare ewes, mutations in both BMP-15 and the closely related GDF-9 lead to similar ovarian pathology in animals with one or two copies of the mutation (Hanrahan *et al.*, 2004). Histological evaluation of ovarian tissue from sterile FecX homozygotes has shown that ovarian follicles do not normally grow beyond the primary stage of development and that whilst the oocytes continue to grow, in the absence of granulosa cell proliferation, the oocytes are unable to be supported by the residual granulosa cells and eventually degenerate (Braw-Tal *et al.*, 1993). Similar abnormal ovarian pathologies have been observed in animals actively immunised against both BMP-15 and GDF-9 (Juengel *et al.*, 2004b) suggesting that both these factors have similar actions in sheep. Further, this result suggests that active immunisation offers an efficacious route to conduct interventionist studies on the role of other local factors in the control of early follicle development.

The development of serum-free culture systems for both thin strips of ovarian cortex in which primordial follicle initiation and growth can be

observed, and isolated pre-antral follicles which can be stimulated to grow into the antral phase, has provided experimental systems in which interventionist studies can be conducted (Gutierrez *et al.*, 2000; Picton *et al.*, 2003, 2004; Walters *et al.*, 2006). One of the major findings from this approach to date, is that it appears that the control of primordial follicle initiation is under inhibitory control. Thus, when pieces or strips of ovarian cortex are placed in culture, the rate of primordial follicle initiation is higher than would normally be expected from *in vivo* estimations (Wandji *et al.*, 1996; Picton *et al.*, 2004). Thus, it appears likely that removing the primordial follicles from the inhibitory influence of larger developing follicles results in a dysregulation of the initiation process. However, the culture environment also seems to play a part in the initiation process as systems which utilise very high doses of insulin (Wandji *et al.*, 1996) report much higher rates of initiation than systems which utilise low doses (Picton *et al.*, 2004). The identity of this inhibitory influence is unknown but one possible candidate, suggested from studies in knock-out mice, is anti-mullerian hormone (AMH). The ovaries of 4-month-old AMH null mice contain almost threefold more small nonatretic growing follicles and less primordial follicles than their wild-type littermates (Durlinger *et al.*, 2002), indicating that in the absence of AMH primordial follicles are recruited at a faster rate. *In vitro* culture of neonatal ovaries in the presence of AMH has confirmed the inhibitory effect of AMH on primordial follicle recruitment (Durlinger *et al.*, 2002). The pattern of AMH expression in sheep is similar to that observed in mice (Durlinger *et al.*, 2002) and humans (Weenen *et al.*, 2004) with expression first being observed in the columnar granulosa cells of primary follicles immediately after differentiation from the flattened pregranulosa cells of primordial follicles (BK Campbell unpublished observations). Expression is highest in granulosa cells of gonadotrophin-responsive preantral and small antral follicles and gradually diminishes in the subsequent stages of follicle development. At the time of writing, the literature contained two references to studies which supported a role for AMH in the suppression of primordial follicle initiation in the human (Carlsson *et al.*, 2006) or bovine (Gigli *et al.*, 2005) whereas another study reports the opposite (Schmidt *et al.*, 2005). The first of these papers examined the effect of recombinant rat AMH on primordial follicle growth in patches of cultured human ovarian cortex in culture whereas in the second utilized patches of bovine cortex grafted under the chorioallantoic membrane (CAM) of gonadectomised chick embryos. Both these results support the knock-out mouse data but both examine a relatively short period (7-8 days) and are confounded by either wide-spread initiation of primordial follicles when untreated control tissue is placed in culture (Carlsson *et al.*, 2006) or the specificity of effects induced by gonadectomy (Gigli *et*



*al.*, 2005). Further studies are therefore required to define a functionally significant role for AMH in controlling the gonadotrophin-independent stages of follicle development in monovulatory species and defining how AMH interacts with the wide range of other local factors, such as BMPs, activins, inhibins, KIT and KIT ligand, basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF), that have also been shown to be expressed during the initial stages of gonadotrophin-independent follicle development (Driancourt *et al.*, 2000; Knight and Glister, 2001; Smitz and Cortvrindt, 2002; Webb *et al.*, 2003).

### Gonadotrophin-responsive phase

The pituitary gonadotrophins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) are non-covalently linked heterodimeric glycoproteins that consist of a common  $\alpha$ -subunit and a hormone specific  $\beta$  subunit (Pierce and Parsons, 1981). Distinct genes encode these gonadotrophin subunits which are synthesised as precursor proteins that are processed, assembled and secreted by the pituitary gonadotrophs of the anterior pituitary (Gharib *et al.*, 1990). The release of the pituitary gonadotrophins by the gonadotrophs is under the acute trophic control of the hypothalamic decapeptide gonadotrophin-releasing hormone (GnRH) and the responsiveness of gonadotrophs, in addition to the level and pulsatile pattern of release of GnRH, is in turn controlled by the concentration of ovarian steroids and peptides in the peripheral circulation via intricate endocrine feedback loops (McNeilly *et al.*, 2003). Both LH and FSH exert their effects on ovarian somatic cells via specific membrane bound receptors that exhibit alternate patterns of expression. FSH receptors occur exclusively on the granulosa cells of ovarian follicles from primary through to the preovulatory stages of follicle development (Gharib *et al.*, 1990; McNatty *et al.*, 1999). Similarly, LH receptors first develop on the cells of the theca interna at the tertiary stage of development and this pattern of expression is maintained through to the preovulatory stage (Richards, 1994). In addition, it is well established that the granulosa cells of large oestrogenic antral follicles also develop LH receptors (Richards *et al.*, 1998; Webb *et al.*, 1999).

The nomination of a definitive stage of development to mark the beginning of the gonadotrophin-responsive phase has proved difficult. Although FSHr mRNA is detected in follicles with only one or two layers of granulosa cells (McNatty *et al.*, 1999, 2000) there is little evidence that physiological levels of FSH will modulate follicle development at this stage. We have used the ovarian autograft model as a means to assess the functional start of the gonadotrophin-responsive phase. In this model system, early follicle development is synchronised through the loss of growing follicles during graft re-vascularisation

and we have examined the effect of this process under hyper, hypo or normogonadotrophic conditions (Campbell *et al.*, 2004; BK Campbell, unpublished). These data show that the reinitiation of follicle development following autografting during the primary and secondary phases of follicle development are not affected by the concentration of FSH in the peripheral circulation and it is not until the multi-laminar tertiary stage of development that effects are observed. Thus, in sheep in which FSH levels are maintained at very low levels by a combination treatment of GnRH-agonist and oestradiol, growth of tertiary, pre-antral and small antral follicles is markedly slower with much lower levels of expression of proliferative markers such as PCNA and Ki67 (Fig. 1) with normogonadotrophic individuals being intermediate between these extremes (BK Campbell, unpublished). Thus it appears likely that the functional beginning of the gonadotrophin-responsive phase lies around the beginning of the multi-laminar tertiary follicular phase and this seems to agree with the results of *in vitro* studies which have shown that the ability of isolated sheep follicles to develop and grow in culture in response to media containing physiological doses of FSH is much greater at diameters greater than 180  $\mu$ m (Picton *et al.*, 2003). Importantly, this is also around the stage of development at which expression of LH receptor (LHr) mRNA is first detected when the theca interna forms around the granulosa cells (McNatty *et al.*, 1999, 2000). This is supported by a range of steroidogenic enzymes, these include cytochrome P450 side chain cleavage (P450scc), cytochrome P450 17 $\alpha$ -hydroxylase (P450c17), and 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD) mRNA's which are first expressed soon after formation of the theca interna (Bao and Garverick, 1998), with cytochrome P450 aromatase (P450arom) being localised solely to granulosa cells. Steroid enzyme protein information and activity agree with mRNA expression patterns confirming that the gonadotrophin-responsive stage of follicle development is functionally steroidogenic with the ability to produce progesterone, androgens and oestradiol in small but significant quantities (McNatty *et al.* 1999, 2000; Webb *et al.* 2003). In addition, these follicles also express high levels of mRNA and protein for inhibin/activin subunits (McNatty *et al.*, 1999; Campbell and Baird, 2001) and AMH (Fig. 2).

As indicated above, members of the insulin/IGF system have also been implicated as potential modulators of gonadotrophin-independent follicle development and in support of this hypothesis, Armstrong *et al.* (2002) demonstrated that bovine pre-antral follicles express mRNAs encoding both IGFBP-2, -3 and type 1 IGF receptor. Additional functional investigation into the role of gonadotrophins and their interaction with other extra-ovarian factors *in vivo* have utilised ovarian autografts treated with bovine somatotrophin (BST). These studies, showed that BST had a marked effect on the growing follicle population and that there was a clear interaction with

gonadotrophic status so that under normogonadotrophic conditions in hemi-ovariectomised sheep, BST treatment had a negative effect on the relative proportion of secondary/tertiary, pre-antral and antral follicles, whereas under hypergonadotrophic conditions there were less secondary and tertiary follicles, but more late pre-antral and antral follicles in BST-treated animals (BK Campbell, D Armstrong, E Telfer, unpublished observations). Whilst confirming our previous data (Campbell *et al.*, 2004) that gonadotrophin levels influence the rate of pre-antral follicle development, these findings also support the hypothesis that the IGF system represents a key

determinant of successful follicle and oocyte development during stages of pre-antral follicle development. Our current view is that exposure of the developing follicle and oocyte to the potent proliferative and differentiative actions of the IGFs is modulated through the abundant local expression of IGF-BP2 during the pre-antral stages of development (Webb *et al.*, 2003) and findings from follicle culture studies support this hypothesis (Walters *et al.*, 2006). These findings therefore demonstrate possible interactions between gonadotrophins and other endocrine factors and local growth factors in the control of the gonadotrophin-responsive stages of follicle development.

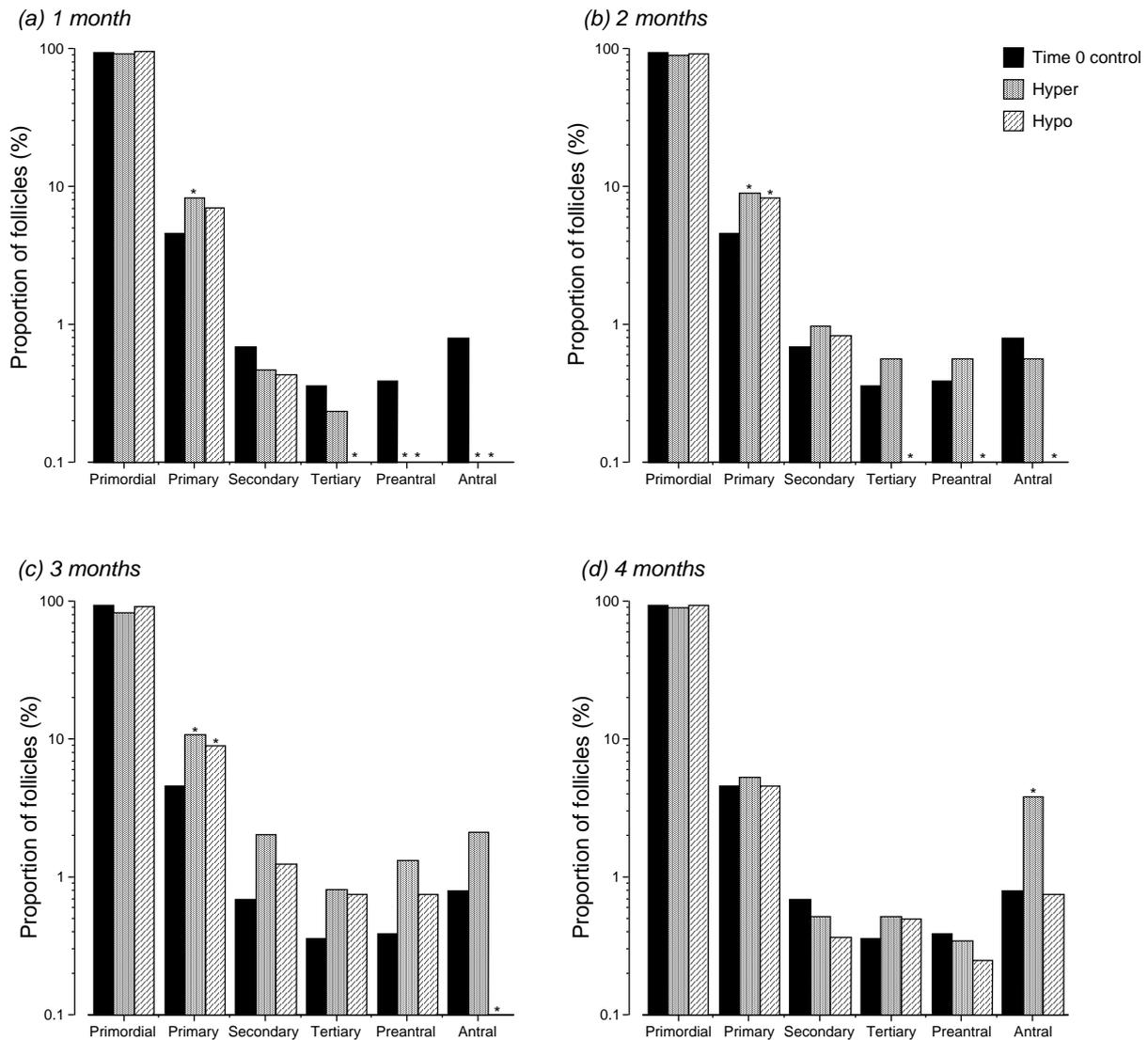


Figure 1. Proportion of follicles at different maturational stages in ovarian cortical patches recovered at the time of ovariectomy (closed bar; normal time zero controls) and in untreated hypergonadotrophic autografts (hatched bar; Hyper) or autografts treated with implants to render them hypogonadotrophic (Diagonal hatched bar; Hypo) recovered 1 (a), 2 (b), 3 (c) or 4 (d) months after autografting. Values represent proportion of the total number observed at each time point within treatment groups. \* P < 0.05; \*\* P < 0.01 and \*\*\* P < 0.001 compared to normal time zero controls (Campbell *et al.*, 2004).

The end of the gonadotrophin-responsive phase in sheep, is marked by a peak in the mitotic index of the granulosa cells within small antral follicles at a diameter of around 1.2 mm (Turnbull *et al.*, 1977) and a marked decline thereafter to diameters of around 2-2.5 mm which marks the beginning of the gonadotrophin-dependent phase in this species (see below). This dramatic change is accompanied by a marked increase in the rate of follicular atresia and increasing levels of steroidogenic enzymes and endocrine factors as the somatic cells begin to differentiate (McNatty *et al.*, 1999). Under experimental

conditions in which FSH concentrations are maintained below threshold (see below) such as following hypophysectomy (Dufour *et al.*, 1979), GnRH-immunisation (McNeilly *et al.*, 1984) or GnRH analogue treatment (Picton *et al.*, 1990a; Campbell *et al.*, 1998), further follicle development ceases at around 2-2.5 mm in diameter and there is evidence that follicles of this diameter tend to “stack-up” under these conditions, with the ovaries of suppressed individuals, containing a large number of these small antral follicles at different stages of atresia (Picton *et al.*, 1990a; Dufour *et al.*, 1999).

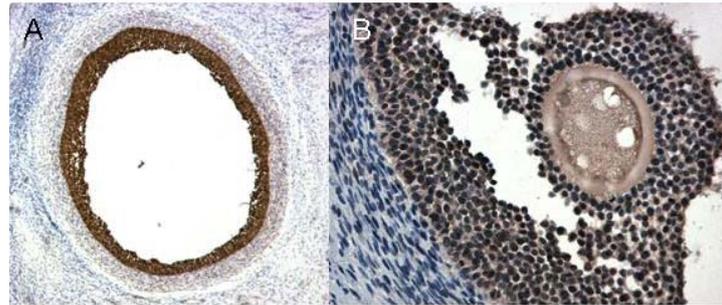


Figure 2. Abundant AMH immunoreactivity in the mural granulosa cell layer of small healthy gonadotrophin-responsive follicles in sheep: A. Serotec Cl 5/6 raised against 25 kDa rhAMH; B. in-house monoclonal 14C1 raised against conserved region N-terminus of the full size 140 kDa molecule (BK Campbell and AS Skinner, unpublished results).

### Gonadotrophin-dependent phase of development

The control of the terminal stages of folliculogenesis lies primarily with the pituitary gonadotrophins, FSH and LH, combined with the differential expression of both somatic cell and oocyte derived growth factors that modulate the action of gonadotrophins at key points during the process of follicle development (Campbell *et al.*, 1995; Webb *et al.*, 1999, 2003).

#### Gonadotrophic control

Through the utilization of a variety of gonadotrophin suppression model systems, naturally occurring mutations in humans and gene knockout approaches in mice, it has been convincingly demonstrated that both FSH and LH act in concert to control antral follicle development. As its name implies, FSH plays a pivotal role in the stimulation of both follicle growth and differentiation (McNeilly *et al.*, 1992; Campbell *et al.*, 1998). FSH alone (Picton *et al.*, 1990a; Campbell *et al.*, 1998), but not LH (Picton *et al.*, 1990b; Picton and McNeilly, 1991), can stimulate the growth of follicles to a preovulatory size in ewes made hypogonadotrophic by long term treatment with GnRH analogues. In cattle, short term FSH infusion of GnRH-agonist suppressed heifers induced changes in the mRNA expression of major developmental markers that were identical to those observed in recruited follicles during the first follicular wave in normal animals (Garverick *et al.*, 2002). Further, specific depression of

FSH by treatment with follicular fluid (McNeilly, 1984; Baird *et al.*, 1990), inhibin (Campbell *et al.*, 1996), or withdrawal of FSH support in GnRH-antagonist depressed ewes (Campbell *et al.*, 1999), results in a rapid decline and atresia of ovulatory sized follicles. The pivotal role of FSH in antral follicle development has been confirmed in other species by gene knockout in mice (Kumar *et al.*, 1997) and naturally occurring mutations in humans (Simpson, 2008).

During normal folliculogenesis and oocyte maturation it is clear that LH also plays a crucial role. In most species, it is well established that in a normal cycle the final maturation and development of antral follicles, and their oocytes, to ovulation is dependent on an increase in the pulsatile secretion of LH (Baird, 1983; Hillier, 2001). Further, there is abundant evidence that LH can influence the development and maintenance of antral follicles, both directly and indirectly. A direct role of LH in antral follicle development has been shown in transgenic mice (Lei *et al.*, 2001; Zhang *et al.*, 2001; Mann *et al.*, 2003; Ma *et al.*, 2004), GnRH-agonist suppressed sheep (Picton *et al.*, 1990b; Picton and McNeilly, 1991) and in humans (Filicori *et al.*, 2002) and it has long been postulated that an LH ceiling concentration exists which is deleterious to antral follicle development (Hillier, 1994). Further, recent studies we have performed in GnRH-antagonist suppressed sheep also suggests the existence of an LH threshold concentration (Campbell *et al.*, 2007). In addition to direct effects on follicle development, LH can also indirectly affect follicle development through its steroidogenic role (McCracken *et al.*, 1969;

Baird *et al.*, 1981; Peluso *et al.*, 1984; Walters and Schallenger, 1984). Thus, the level of ovarian oestrogen secretion is dependent not only on the presence of an oestrogenic follicle in the ovary but also the pattern of LH that this follicle is exposed to (Campbell *et al.* 1999; 2007). Therefore, in a normal cycle, LH is able to influence the level and pattern of pituitary FSH release through its action in controlling the level of ovarian oestradiol secretion. Further, recent

evidence from GnRH-antagonist suppressed sheep suggests that LH can also influence the level of inhibin A secretion (Fig.3) providing an additional mechanism whereby LH can indirectly influence the development of FSH-responsive follicles (Campbell *et al.*, 2007). Taken together these observations suggest that both FSH and LH play a role in the control of selection of the ovulatory follicle(s) and its development to a preovulatory size.

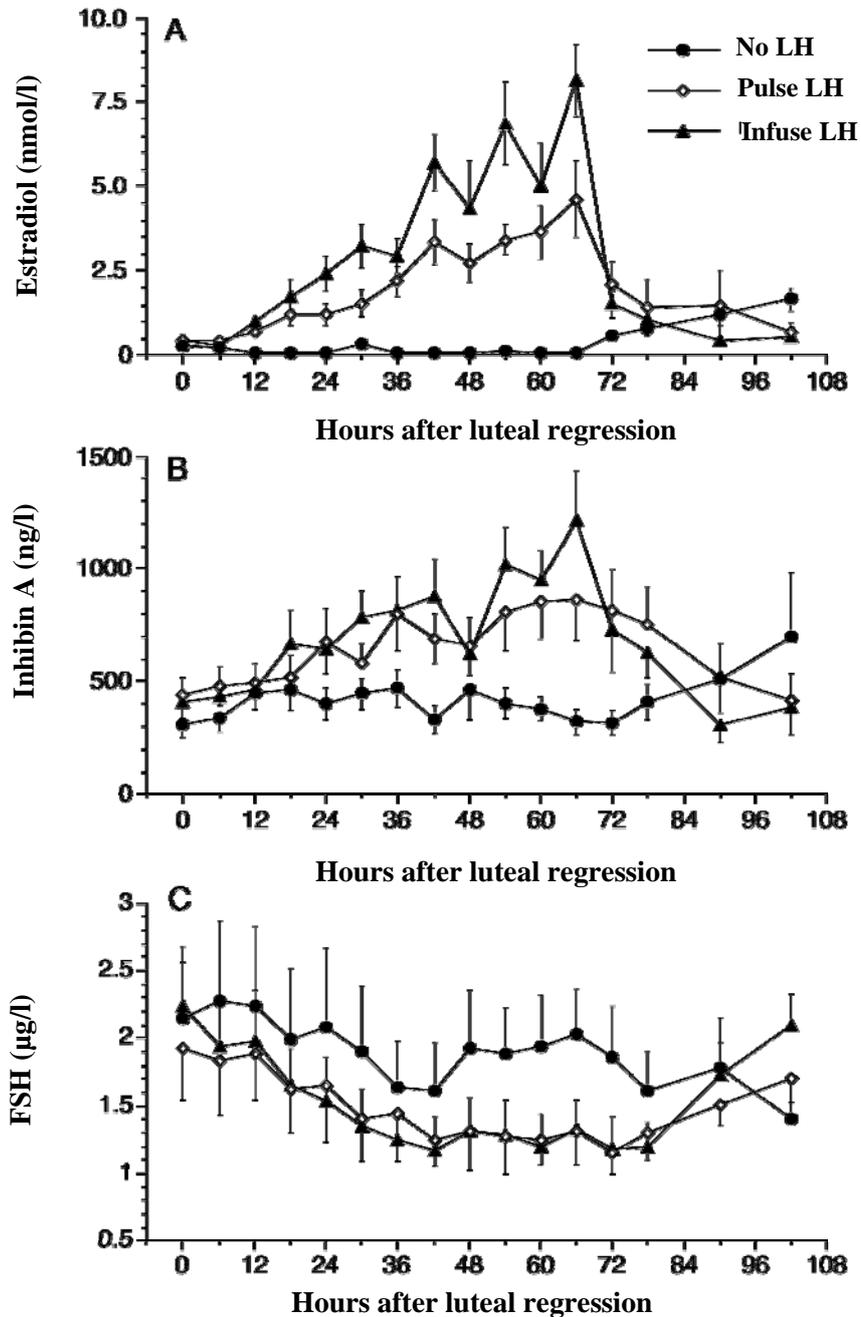


Figure 3. Ovarian venous estradiol (A) and inhibin A (B) and jugular venous FSH (C) concentrations in ewes which received either no LH (n = 8; closed circles), pulsed LH (n = 8; open diamonds) or constant LH (n = 8; closed triangles) for 60 h after induction of luteal regression followed by an ovulatory stimulus at that time. Values are means  $\pm$  SEM (Campbell *et al.*, 2007).



### The modulation of gonadotrophic actions by local factors

Although the latter stages of follicle development are primarily regulated by the pituitary gonadotrophins (FSH and LH) there is now strong evidence that this process relies heavily on complex actions and interactions between locally produced hormones and growth factors (Knight and Glister, 2003; Webb and Campbell, 2007). These systems include the insulin/IGF system (Webb *et al.*, 1999), the inhibin/activin system (Campbell and Baird, 2001; Campbell *et al.*, 2003b) and the bone morphogenic system (Souza *et al.*, 2001, 2002; Campbell *et al.*, 2006). In addition, recent studies also suggest that the oocyte, rather than being purely a passenger within the follicle, secretes numerous factors that affect follicle development and ovarian function. Known oocyte-secreted factors include growth differentiation factor-9 (GDF-9; Juengel *et al.*, 2004a), bone morphogenetic protein -6 (BMP-6; Knight and Glister 2003; Campbell *et al.*, 2006) and BMP-15 (Galloway *et al.*, 2000), as well as factor in the germline alpha (FIG- $\alpha$ ; Huntriss *et al.* 2002), NOBOX (Huntriss *et al.*, 2006) and kit receptor (Driancourt *et al.*, 2000). Gap junction-mediated communication between the oocyte and the surrounding somatic cells is essential for the coordinated development of both cell types and this link is maintained throughout follicle growth, during which time somatic cells provide the oocyte with metabolic substrates and meiosis-arresting signals (Themmen, 2005).

The orderly, stage specific expression of these somatic and oocyte-derived factors at the correct time, or "intrafollicular cascade", is thought to be essential for the development of the follicle to an ovulatory size, the production of an ovulatory signal and the release of a fully developmentally competent oocyte in response to that signal. During the gonadotrophin responsive and dependent stages of follicle development it has been postulated that local factors regulate the sensitivity of follicular somatic cells to gonadotrophins and are therefore considered to be central to the mechanism of follicle selection and the control of ovulation rate. Our mechanistic models of the control of follicle selection (see Fig 4A) therefore postulate that these factors act to either attenuate or augment the stimulatory actions of gonadotrophins on follicle development so that the fate of individual follicles relies on the balance between these conflicting local actions (Campbell *et al.*, 1995; Webb and Campbell, 2007). From this model, it is therefore possible to postulate an increase in ovulation rate through either an increase in circulating gonadotrophin concentrations, an increase in the activity of augmentors of gonadotrophic actions or a decrease in the activity of attenuators of gonadotrophic actions (Fig. 4A). In the following section, recent investigations into the ovarian BMP system in sheep will be used to illustrate this mechanistic model.

### The ovarian BMP system in sheep

TGF- $\beta$  superfamily members such as BMP, signal via a heteromeric receptor complex consisting of a type I and a type II receptor serine/threonine kinase. Upon ligand binding the type II receptor recruits the non-ligand binding type I receptor into the complex, resulting in phosphorylation of signaling pathway effector proteins called Smads (Rey *et al.*, 2003). The receptor regulated SMADs are grouped into two subsets; Smad2 and 3 are activated by TGF $\beta$  and activin whereas Smad1, 5 and 8 are activated by BMPs. In addition Smad4 serves as a common partner for all receptor regulated Smads whereas Smad6 and 7 are inhibitory Smads that interfere with Smad-receptor and Smad-Smad interactions (Massague, 2000). At present, the identity of the ligands that signal through the BMPRI1B receptor in the sheep ovary are uncertain. Studies using non-ovarian cell types and lines have demonstrated that there are a limited number of ligands in the TGFbeta family which after binding to a type II receptor use BMPRI1B. These include BMPs 2, 4, 6, 7, 15 and AMH (Shi and Massague, 2003; Miyazono *et al.*, 2005). In contrast, it appears likely that GDF-9 utilises TGFBR1 (ALK5) as its type II receptor (Mazerbourg *et al.*, 2004) although as it now known that BMP-15 and GDF-9 form heterodimers in sheep (McIntosh *et al.*, 2008) the signalling pathways utilised by these molecules in this species is unclear. A similar promiscuity is also evident in potential type II receptors as although the type II AMH receptor (AMH RII) is unique and does not bind other TGF- $\beta$  superfamily members, BMPs 2, 4, 6, 7, 15 and GDF-9 can utilise BMPRI1 as their type II receptor (Massague, 2000; Juengel *et al.*, 2004a; Mazerbourg *et al.*, 2004). In sheep we have demonstrated expression of both BMPRI1B and BMPRI1A type I receptors and the type II BMP receptors in ovarian somatic cells in sheep across all stages of folliculogenesis (Souza *et al.*, 2002). We and others have also demonstrated specific expression of BMP-6, BMP-15, GDF-9, and AMH (Fig. 2) in either the oocyte or somatic cells of ovarian follicles in this species (Juengel *et al.*, 2004a; Campbell *et al.*, 2006). AMHR2 expression has not been described in the sheep ovary although we have observed responses of sheep granulosa cells to AMH *in vitro* (Campbell *et al.*, 2005). It therefore appears that all components of this regulatory system are expressed in the sheep ovary although unequivocal evidence indicating both mRNA and protein expression in ovarian cells types in sheep has been observed for BMP-6 (oocyte, granulosa cells: (Campbell *et al.*, 2006; Juengel *et al.*, 2006), AMH (granulosa cells), GDF-9 (oocyte: Juengel *et al.*, 2004a) and BMP-15 (oocyte: Juengel *et al.*, 2004a).

As indicated in Fig. 4, BMP-6 appears to be an example of a factor which augments the action of gonadotrophins during the terminal stages of follicle development. Cell culture studies in sheep have shown that BMP-6 results in an increase in FSH-induced oestradiol and inhibin A production (Campbell *et al.*, 2006), while decreasing FSH-induced progesterone

production (Campbell *et al.*, 2006), by granulosa cells. In terms of thecal cell function, high doses of BMP-6 (5-50 ng/ml) inhibited LH-stimulated androstenedione (A4) production by TC whereas lower doses (0.005-0.05 ng/ml) stimulated TC proliferation and total androstenedione production (Campbell *et al.*, 2006). This differential sensitivity of somatic cells is consistent with the pattern of BMP-6 protein expression with high intensity staining in the oocyte and membrane granulosa cell layer with much fainter staining in the theca cell layer. Further there was a significant increase in BMP-6 protein expression with increasing follicle size in both granulosa and theca cell layers (Campbell *et al.*, 2006). These observational and *in vitro* findings have recently been supported by whole animal studies. We have

therefore shown that direct ovarian infusion of BMP-6 into wild-type ewes with ovarian autotransplants results in transient but significant ( $P < 0.05$ ) increases in ovarian inhibin A, androstenedione and oestradiol secretion, an advance in the time of the LH surge (Fig. 5) and the precocious ovulation of smaller ovulatory follicles which gave rise to smaller corpora lutea (Campbell *et al.*, 2008). Importantly, these changes parallel the precocious maturation of ovulatory follicles observed in FecB mutant animals in which we have also observed an enhanced response to BMP-6 *in vitro* (Campbell *et al.*, 2006). Overall, these findings provide persuasive evidence that BMP-6 acts as a major local factor that acts to augment the actions of gonadotrophins.

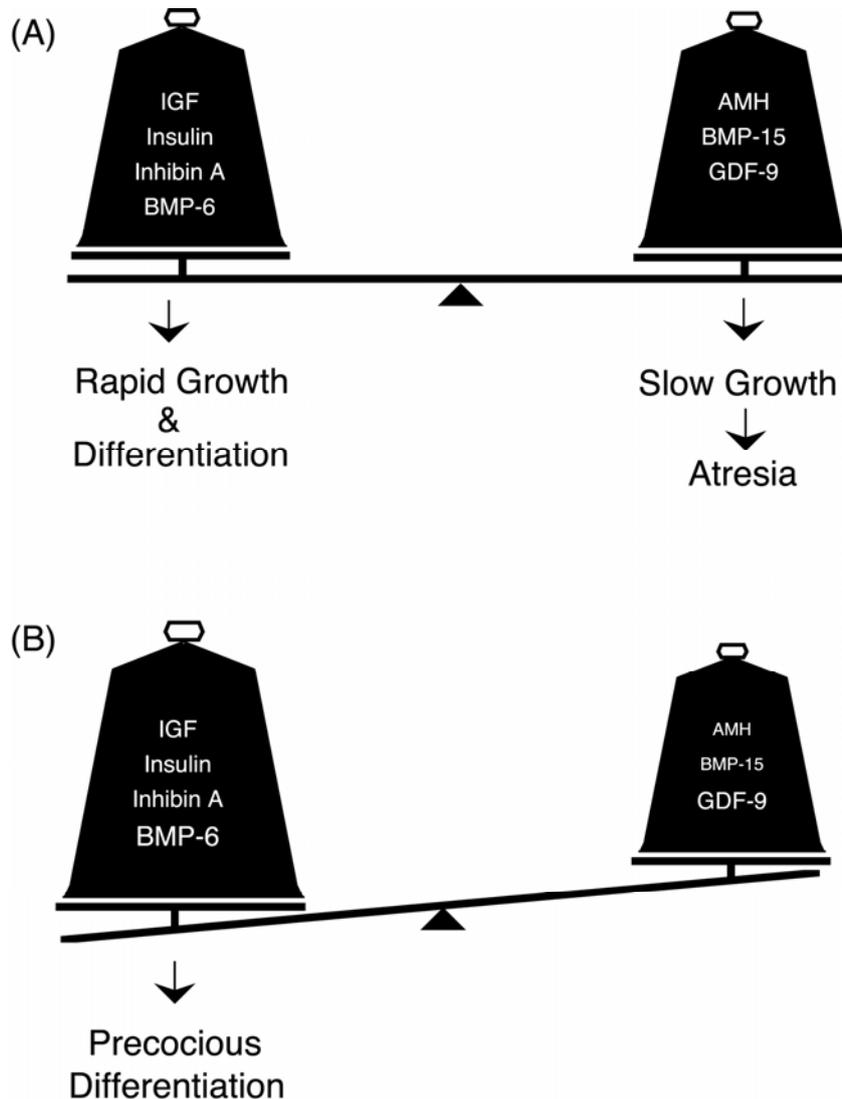


Figure 4. Simplified model illustrating the concept that the fate of each gonadotrophin-dependent follicle relies on the intra-follicular balance between factors that augment the action of gonadotrophins to stimulate follicular growth and differentiation and those that attenuate the action of gonadotrophins and which will slow growth and ultimately lead to atresia of the follicle (A). Hypothesis that the precocious differentiation of multiple ovulatory follicles in FecB mutants may result from either the increased activity of augmentors of gonadotrophins (e.g., BMP-6), the decreased activity of attenuators of gonadotrophic actions (e.g., AMH or BMP-15) or the combined action of both systems (B). Each of these factors has been shown to be capable of signalling through the BMPRI1B (Alk6) receptor.

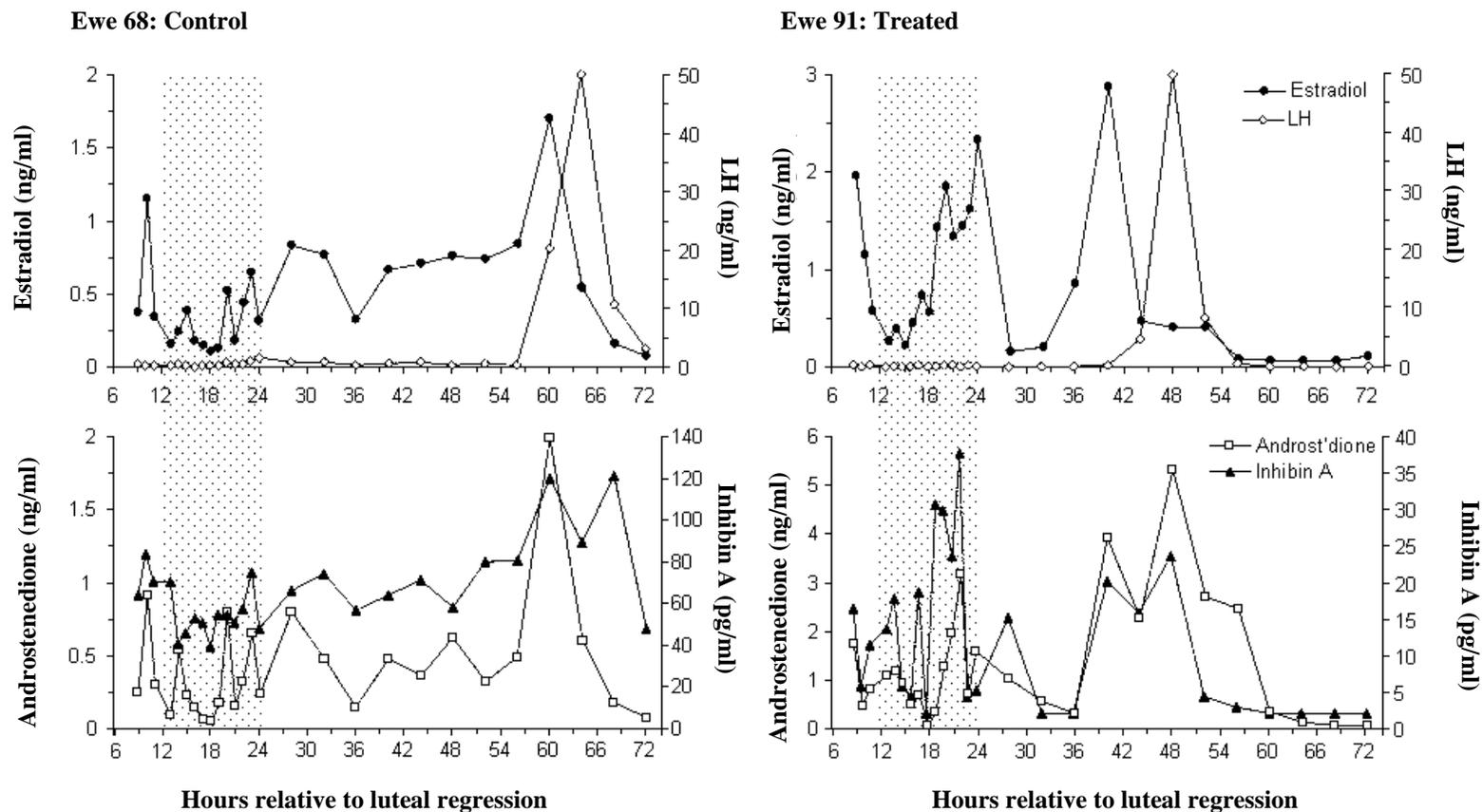


Figure 5. Individual hormone profiles over the period of intensive blood sampling for two experimental animals, one vehicle treated control (Ewe 68) and one ewe that received BMP-6 (Ewe 91) at a rate of 2  $\mu\text{g/h}$  for 12 h (hatched area) during the early follicular phase, between 12-24 h after induction of luteal regression. Upper panel for each ewe shows concentrations of LH (open diamond) and estradiol (closed circle) whereas as lower panel shows the concentration of androstenedione (open square) and inhibin A (closed triangle) in ovarian venous plasma. Note the acute changes in ovarian hormone secretion toward the last 6h of infusion in the treated animal and the subsequent advance in the time of the preovulatory surge of LH, preceded by normal peaks of estradiol, androstenedione and inhibin A (Campbell *et al.*, 2008).



A member of this family which appears to have inverse actions, attenuating the differentiative actions of gonadotrophins, is AMH. Evidence from null AMH mice indicate that in addition to its effect on follicle initiation (see above), AMH may also effect follicle selection. Studies on AMH in sheep in my laboratory have revealed that the predominant form of AMH in follicular fluid across follicle sizes is the high molecular weight 140 kDa form. Immunohistochemistry showed highest levels of AMH expression in the granulosa cells of small antral follicles and an inverse relationship between AMH and P450-aromatase expression in large antral follicles ( $P < 0.001$ ). Supplementation with rhAMH *in vitro* resulted in a dose responsive inhibition of FSH-stimulated oestradiol production by granulosa cells ( $P < 0.01$ ) but had no effect on theca cells. Direct ovarian infusion of 25kDa AMH had no effect on ovarian steroidogenesis but active immunization against the 140kDa form resulted in a significant ( $P < 0.01$ ) increase in the number of both small and large antral follicles. Collectively these data support the hypothesis that AMH acts as an inhibitor of gonadotrophin-stimulated follicle development but suggests that the different molecular forms may have differential bioactivities (Campbell *et al.*, 2009).

Further members of the BMP system that appears to be attenuators of gonadotrophic actions are BMP-15 and GDF-9. As detailed above, Inverdale, Hanna, Cambridge and Belclare ewes with naturally occurring point mutations in BMP-15 and GDF-9 have higher than normal ovulation rates and litter sizes than wild-type animals (Davis *et al.*, 2001). Further, controlled immunisation against either of these factors results in similar increases in ovulation rate (Juengel *et al.*, 2004b). Culture studies in both rodents (Otsuka *et al.*, 2001) and sheep (Campbell *et al.*, 2003a) have shown that both these factors act as inhibitors of FSH-induced estradiol production by cultured granulosa cells in sheep and other species. Inhibitory effects of GDF-9 and BMP-15 on thecal steroidogenesis have also been observed, but only at very high doses (Campbell *et al.*, 2005). Comparison of the actions of AMH, GDF-9 and BMP-15 on granulosa cells from wild-type and BMPRI1B receptor mutant (FecB) animals showed marked differences in differentiative responses to FSH with mutant animals being more resistant to the inhibitory actions of each of these factors (BK Campbell, S Shimasaki, DT Baird, unpublished observations).

The available evidence therefore support a model whereby the fate of gonadotrophin-dependent follicles is dependent on the intrafollicular balance between factors that augment the action of gonadotrophins to stimulate follicular growth and differentiation and those that attenuate the action of gonadotrophins and which will ultimately lead to apoptosis and atresia of the follicle. Thus the increased ovulation rate in naturally occurring mutations such as the FecB, FecX or FecG could be accounted for by

increased activation of an augmentor (BMP-6), decreased activation of attenuators (BMP-15, AMH) or a combination of these two mechanisms (Fig. 4B) resulting in the deregulation of the normal follicle selection mechanisms.

### Conclusion

The evaluation of naturally occurring mutations in local factors or their receptors, the development of physiological cell culture model systems and interventionist whole animal model systems have allowed us to further define the role and interactions between local autocrine and paracrine factors and the pituitary gonadotrophins in regulating folliculogenesis in monovulatory species. It is clear that a number of these factors (e.g., AMH, BMP-15, GDF-9) have major regulatory roles during both the gonadotrophin-independent and dependent stages of follicle development and a mechanistic model has been developed for the terminal stages of development supporting the concept that the fate of each follicle depends on the balance between stimulatory and inhibitory factors that modulate the role of gonadotrophins. The challenge for the future will be the determination of the interaction between these intra-ovarian factors and the identification of key developmental check points with the ultimate aim of understanding the causes and devising improved means for the treatment of infertility.

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