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Molecular mechanisms regulating follicular recruitment and selection

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Ovarian follicular growth and development is an integrated process encompassing both extraovarian signals, such as gonadotrophins and metabolic hormones, and intraovarian factors. Follicular development has been classified into gonadotrophin-independent and -dependent phases. In the latter, FSH provides the primary drive for follicular recruitment and LH is required for continued development of follicles to the preovulatory stage. A transient increase in circulating FSH precedes the recruitment of a group of follicles, and these recruited follicles are characterized by expression of mRNAs encoding P450scc and P450arom in granulosal cells. As follicles mature, there is a transfer of dependency from FSH to LH, which may be part of the mechanism(s) involved in selection of follicles for continued growth. Indeed, changes in the pattern of expression of mRNA for gonadotrophin receptors and steroid enzymes within follicular cells appear to be closely linked to changes in peripheral concentrations of gonadotrophins. The mechanism of selection of dominant follicles still requires clarification, but seems to be linked to the timing of mRNA expression encoding LHr and 3β-hydroxysteroid dehydrogenase (3βHSD) in granulosal cells. Additional intraovarian systems, including the ovarian IGF and activin/inhibin systems, also exert a role. For example, it appears that the development of follicular dominance in cows is associated with the FSH-dependent inhibition of the expression of mRNA encoding insulin-like growth factor binding protein 2 (IGFBP-2) in granulosal cells. In conclusion, the integration of these endocrine signals and intraovarian factors within follicles determines whether follicles continue to develop and become dominant or are diverted into apoptotic pathways leading to atresia.

Introduction

Ovarian follicular growth is a developmental process during which follicles sequentially acquire a number of characteristics, each of which is an essential prerequisite for further development. The number of follicles that reach the ovulatory stage is regulated in a species- and breed-specific manner. However, many key mechanisms involved in this developmental process are still not understood, including (i) factors regulating the initiation of primordial follicle growth, (ii) control of antrum formation, (iii) mechanisms controlling follicle recruitment, selection and dominance, and (iv) the process of follicular atresia, the end-point of > 99% of follicles. However, molecular techniques have been used in domestic species over recent years to elucidate the patterns of expression of local follicular factors involved in this differentiative process.

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This review will concentrate on some primary factors involved in follicular recruitment, selection and dominance. Recent work investigating patterns in gene expression will be reviewed, as this has provided new insights into possible key mechanisms. A brief outline of the patterns of follicular growth and the relationship with gonadotrophins and metabolic hormones will be described, since the control mechanisms involve the interaction of systemic hormones and intrafollicular factors.

Definitions of Stages of Follicular Development

For this review the following generally accepted definitions have been used:
Recruitment – gonadotrophins stimulation of a pool of rapidly growing follicles.
Selection – a process whereby one or more of these recruited follicles are selected to continue to develop further.
Dominance – the mechanism(s) by which the dominant follicle(s), the number of which is species- and breed-specific, undergoes rapid development in an environment where growth and development of other follicles are suppressed.

Early Follicular Growth

Growth of antral follicles to 2–4 mm in cattle and 1–2 mm in sheep is thought to be independent of gonadotrophins. Follicles can grow to this size in either the absence of gonadotrophins or the presence of very low concentrations of gonadotrophins (Campbell *et al.*, 1995; Webb and Armstrong, 1998). However, recent studies investigating follicular growth patterns using transrectal ultrasonography show that small follicles (approximately 2 mm) do exhibit waves of growth, as discussed later, suggesting that they are responsive to gonadotrophins.

Gene expression in preantral and early antral follicles

Gonadotrophin receptors. FSH receptor (FSHr) mRNA is localized specifically to both mural and cumulus granulosal cells and can be detected in follicles with only one or two layers of granulosal cells (Xu *et al.*, 1995a). The role of the FSHr in preantral and early antral follicular growth is unknown. However, gonadotrophins do not seem to be required for the activation of bovine primordial follicles during culture of bovine ovarian cortical slices (Wandji *et al.*, 1996). In contrast, late preantral follicles in culture show increased growth in response to FSH (Ralph *et al.*, 1995). We have recently developed a system in which bovine preantral follicles can be sustained in long-term culture and proceed to develop an antrum (Gutierrez *et al.*, 1997a). In this system follicles were responsive to the stimulatory effects of both FSH and a number of growth factors including IGF-I and EGF.

Expression of LH receptor (LHr) mRNA is localized to thecal cells during the preantral and early antral stages of growth, and expression is detected when the theca interna forms around the granulosal cells (Xu *et al.*, 1995a,b; Bao *et al.*, 1997a).

Steroidogenic enzymes. mRNA for steroidogenic enzymes, cytochrome P450 side-chain cleavage (P450scc), cytochrome P450 17α-hydroxylase (P450c17), and 3β-hydroxysteroid dehydrogenase (3β-HSD) are expressed soon after formation of the theca interna. Expression of steroid acute regulatory protein (StAR) mRNA has also been detected in thecal cells (Bao *et al.*, 1997b). Expression of mRNA for these enzymes tends to increase with growth of these early antral follicles (Xu *et al.*, 1995a,b; Bao *et al.*, 1997a,c). Cytochrome P450 aromatase (P450arom) is localized solely to granulosal cells, but expression cannot be found in non-recruited follicles < 4 mm in diameter. Apparently, the main steroid hormones produced by preantral and early antral follicles are pregnenolone, progesterone and androgen from

thecal cells. This finding is in agreement with earlier work that measured *in vitro* steroid production and steroid concentrations in follicular fluid (see Webb and Gauld, 1987; Skyer *et al.*, 1987).

The role of gonadotrophins in the induction of mRNA encoding gonadotrophin receptor and steroidogenic enzymes in preantral and early antral follicles is unknown. However, mRNA expression of FSHr in granulosal cells and expression of LHr, P450scc and P450c17 in thecal cells was not different in follicles < 4 mm in diameter in heifers with normal oestrous cycles compared with heifers in which follicles were arrested at approximately 4 mm in diameter due to GnRH agonist inhibition of FSH and LH secretion (H. A. Garverick, J. G. Gong, B. Baxter, D. G. Armstrong, B. K. Campbell and R. Webb, unpublished).

Waves of Follicular Growth in Cattle and Sheep

Two or three major phases of growth of large follicles occur during the bovine oestrous cycle, and the ovulatory follicle is selected at about 3 days before ovulation (see Ginther *et al.*, 1996; Webb and Armstrong, 1998). Each wave of follicular development is characterized by the simultaneous emergence of medium-sized (> 4–8 mm in diameter) follicles from a pool of smaller follicles. A dominant follicle emerges and continues to develop, while the others undergo atresia. The dominant follicle remains dominant for a few days, until it too becomes atretic and regresses, to be replaced within approximately 5 days by the next dominant follicle from the next follicular wave. If luteal regression takes place, the dominant follicle, free from the restrictive hormonal milieu imposed by the corpus luteum upon the hypothalamus–pituitary gland, will continue to develop (up to 20 mm in diameter) and will trigger the hormonal cascade leading to ovulation. Follicular waves appear to be constitutive, because they are present before puberty, throughout most of pregnancy and during the post-partum period, as well as during the oestrous cycle (Ginther *et al.*, 1996; Webb and Armstrong, 1998).

In sheep, transrectal ultrasonography has proved more difficult to perform and interpret than in cattle because of problems of anatomical access and the smaller size and greater number of ovulatory follicles. Although evidence from both histological and ink marking studies support the occurrence of follicular waves (see Campbell *et al.*, 1995), a number of studies using ultrasonography have reported random emergence of ovulatory-sized follicles throughout the sheep oestrous cycle (Schrick *et al.*, 1993; Ravindra and Rawlings, 1997). Recent results, using the ovarian autotransplant model during seasonal anoestrus (Souza *et al.*, 1996) and during the follicular (Souza *et al.*, 1997a,b) and luteal (Souza *et al.*, 1998) phases of the oestrous cycle, indicate that large antral follicles in sheep exhibit wave-like cycles (Fig. 1). All of these studies indicate that there is a period of functional dominance characterized by high oestradiol and inhibin A secretion, shorter than the period of morphological dominance, although dominant follicles are not the only source of inhibin A. Therefore, in sheep, follicular size alone is not an adequate parameter to assign dominance.

The overall pattern of follicle turnover in sheep during the luteal phase appears to be similar to that in cows, but there are clear species differences. In sheep, the wave interval is just 4–5 days, perhaps reflecting the smaller diameter of the dominant follicle, so that the ovulatory wave in sheep is likely to be either the third or the fourth, rather than either the second or the third, as in cattle. Furthermore, sheep can have more than one dominant follicle per wave, depending on the ovulation rate of the breed (Fig. 1), compared to usually one dominant follicle per wave in cows, indicating that follicular dominance is not so intense in sheep (Driancourt *et al.*, 1991).

Follicular Waves Associated with Patterns of Hormone Secretion

Although it is clear that waves of dominant follicle development occur in both sheep and cattle, the endocrine and local mechanisms associated with this pattern of development have not been fully elucidated. It is well established, in both cattle (Adams *et al.*, 1992) and sheep (Figs 1 and 2), that the emergence of a follicular wave is preceded by a transient increase in FSH and that the secretion of FSH is regulated by oestradiol and inhibin (Campbell *et al.*, 1995). During the first follicular wave

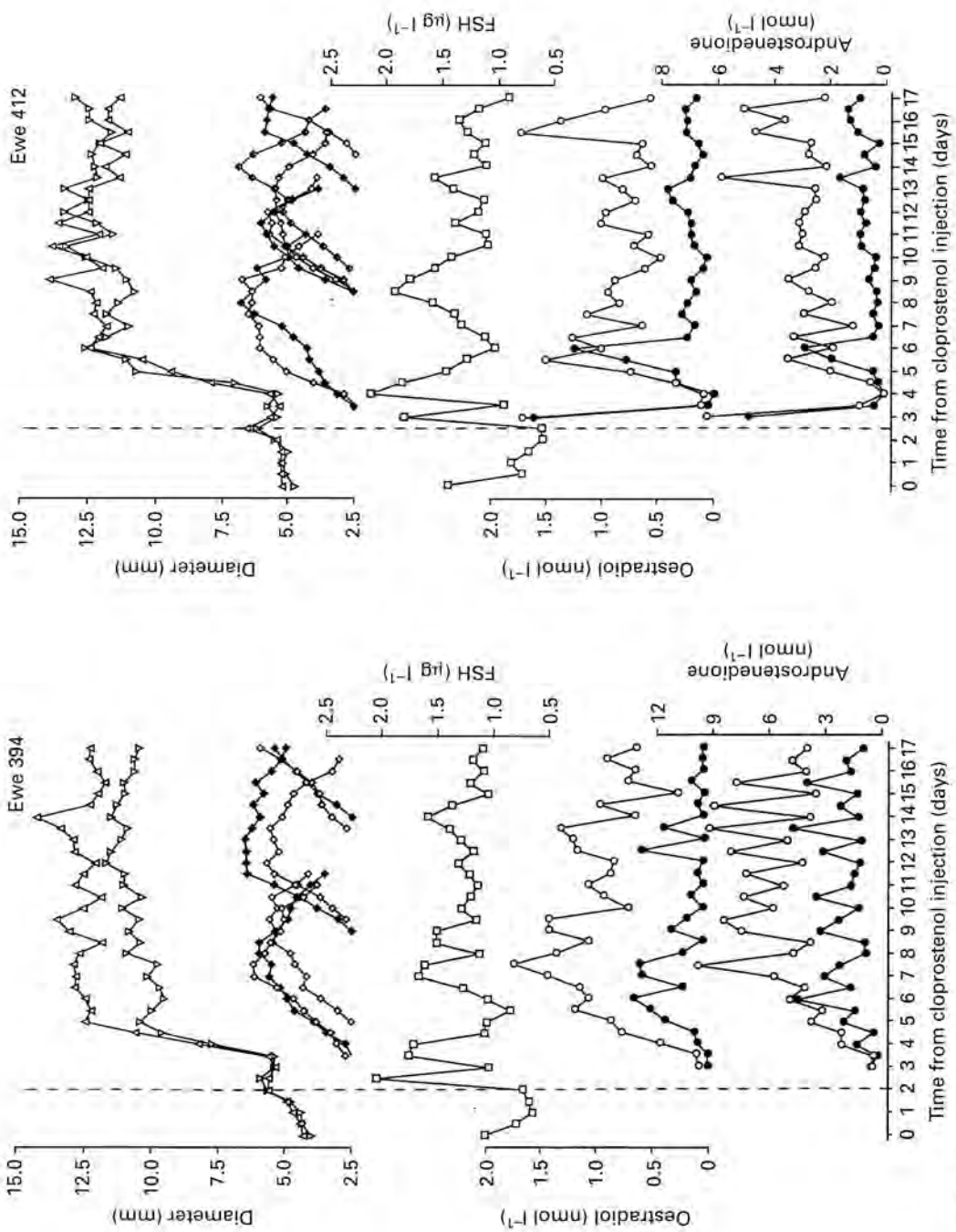

Fig. 1. For legend see facing page.

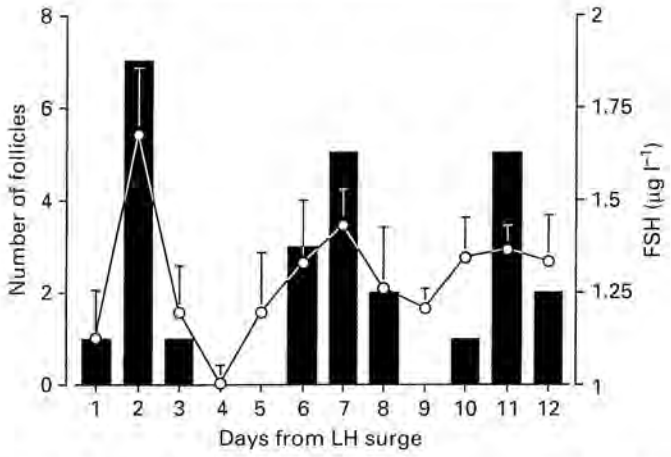

Fig. 2. Relationship between emergence of dominant follicles (solid bars) and mean concentration of FSH (open circles) in jugular venous blood (± SEM, $n = 5$) during the luteal phase of the ovine oestrous cycle. Adapted from Souza *et al.* (1998).

there is compelling evidence to support this explanation: secretion of both oestradiol (Knight, 1996; Souza *et al.*, 1997a, 1997b, 1998) and inhibin A (Souza *et al.*, 1997b, 1998) are positively related to follicular growth and inversely related to FSH. However, over subsequent waves, these relationships are less evident. Ovarian oestradiol secretion is dependent on both the presence of a large oestrogenic follicle in the ovary and appropriate LH stimulation (Campbell *et al.*, 1995).

During the first follicular wave, LH pulse frequency is high and ovarian oestradiol secretion reflects this. However, during subsequent follicular waves LH pulse frequency declines as a result of luteal progesterone. Hence secretion of oestradiol no longer predicts the secretory capacity of the dominant follicle accurately (Souza *et al.*, 1998). Indeed, wave 2 and 3 dominant follicles in sheep secrete the same amount of androstenedione, but less oestradiol, when challenged with LH. Therefore, it seems likely that a period of exposure to high frequency LH pulses is required before follicles acquire the ability to secrete normal amounts of oestradiol (Souza *et al.*, 1998). As indicated, in contrast to oestradiol, inhibin A, measured in the sheep autotransplant model, is not solely derived from the dominant follicle(s) and is secreted in relatively high and constant amounts throughout the luteal phase. Thus the contribution of multiple follicles to ovarian secretion of inhibin A explains the lack of association between inhibin A and development of the dominant follicle during the mid–late luteal phase. However, as inhibin A secretion remains high during this phase of the ovarian cycle, when oestradiol secretion is low, it appears that inhibin A is the major regulator of FSH during the mid–late luteal phase. Thus, although it is clear that oestradiol and inhibin A control FSH secretion during the first follicular wave, the precise regulation of the FSH fluctuations associated with waves 2 and 3 awaits studies on the pattern of secretion of dimeric inhibin B.

We have recently developed an experimental model to investigate the relative importance of FSH and LH during different stages of follicular development. In this model, heifers are

Fig. 1. Dynamics of ovulatory follicles and/or corpus luteum (triangle) and dominant follicles (diamonds) from the three waves of follicular development during the luteal phase (top panel) and concentration of FSH (squares) in jugular venous plasma and concentration of oestradiol and androstenedione in ovarian venous plasma during the luteal phase from two representative ewes. The results show basal steroid concentrations (filled circles) and the steroid concentrations following a GnRH-challenge (250 ng i.v.) (open circles). The dotted line indicates the time of the onset of the LH surge. Adapted from Souza *et al.* (1998).

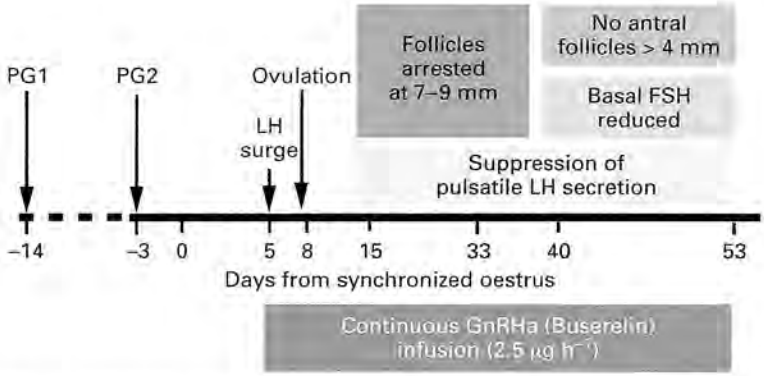

Fig. 3. Schematic summary of ovarian follicular dynamics, as measured by ultrasound per rectum, and association with patterns of gonadotrophin secretion in heifers treated with a GnRH agonist. Two injections of prostaglandin $F_{2\alpha}$ (PG1 and PG2) were given to synchronize oestrous cycles. Note that growth was arrested initially at 7–9 mm diameter and then at < 4 mm in diameter.

continuously infused with GnRH agonist (Fig. 3). We found that the growth of follicles is arrested at 7–9 mm in diameter when pulsatile LH secretion is suppressed (Gong *et al.*, 1995, 1996). When the secretion of basal FSH was also reduced, no antral follicles > 4 mm in diameter were observed (Gong *et al.*, 1996). Using this model in conjunction with FSH, LH, or LH and FSH infusion, we have shown that FSH is initially required for the development of gonadotrophin responsive follicles (Campbell *et al.*, 1995, 1998a; Gong *et al.*, 1997). However, large antral follicles can transfer their dependence on gonadotrophins from FSH to LH (Campbell *et al.*, 1995; Gong *et al.*, 1996b). Furthermore, adequate LH pulsatile support appears to be required to maintain the ovulatory competence of the preovulatory follicles under decreased FSH concentrations (Campbell *et al.*, 1995a). Indeed increased pulsatile secretion of LH has also been associated with the extended lifespan of dominant follicles (Sirois and Fortune, 1990). How do these changes in patterns of hormone release and follicular growth correlate with changes within the follicles themselves?

Expression of mRNA for Gonadotrophin Receptors and Steroidogenic Enzymes during Follicular Growth

Gene expression during recruitment

In cattle, follicular recruitment is generally thought to be gonadotrophin dependent and is considered to occur when follicles are stimulated to grow beyond 4 mm in diameter. Recruitment is also characterized by initiation of growth of a cohort of up to seven follicles that continue to grow to 8–9 mm in diameter. Thereafter usually one follicle in the cohort diverges rapidly from the others and continues to mature.

Gonadotrophin receptors. FSHr and LHr remain localized to granulosal and thecal cells, respectively, during recruitment and growth of the cohort (Fig. 4; Xu *et al.*, 1995a,b; Bao *et al.*, 1997a,c). During this time there is little change in expression of mRNA encoding FSHr and LHr (Table 1).

Steroidogenic enzymes. The initiation of simultaneous mRNA expression of P450scc and P450arom in granulosal cells of most follicles of 4–6 mm in diameter is associated with follicular recruitment in cattle (Xu *et al.*, 1995a,b; Bao *et al.*, 1997a). During later stages when the recruited follicles reach

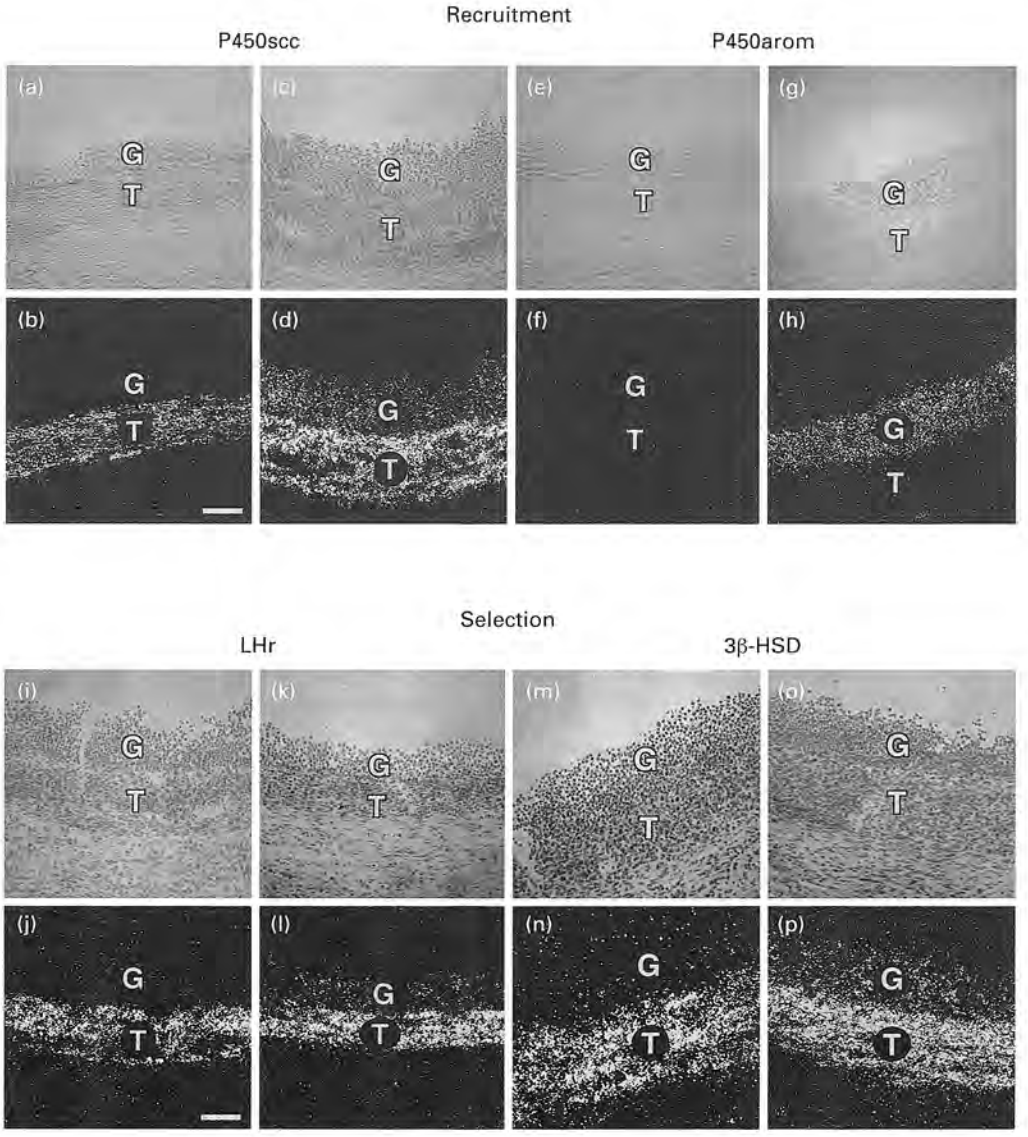

Fig. 4. *In situ* hybridization of luteinizing hormone receptor (LHr) and steroidogenic enzymes, cytochrome P450 side-chain cleavage (P450scc), cytochrome P450 aromatase (P450arom) and 3β-hydroxysteroid dehydrogenase (3β-HSD) mRNAs in cryosections of bovine ovarian follicles collected around the times of recruitment and selection of follicles during the first follicular wave. (a,b) Bright- and dark-field views of a 5 mm healthy follicle with no specific hybridization of P450scc in granulosal cells (not recruited). (c,d) Bright- and dark-field views of a healthy 6 mm recruited follicle collected at 24 h with specific hybridization for P450scc in both thecal and granulosal cells. (e,f) Bright- and dark-field views of a 5 mm healthy follicle with no specific hybridization of P450arom in granulosal cells (not recruited). (g,h) Bright- and dark-field views of a healthy 6 mm recruited follicle collected at 24 h with specific hybridization of P450arom in both thecal and granulosal cells. (i,j) Bright- and dark-field views of a healthy 5 mm follicle with hybridization of LHr localized to thecal cells. (k,l) Bright- and dark-field views of a 9 mm healthy follicle with specific hybridization of LHr to both thecal and granulosal cells. (m,n) Bright- and dark-field views of a 4 mm healthy follicle with specific hybridization of 3β-HSD localized to thecal cells. (o,p) Bright- and dark-field views of a 9 mm healthy follicle with hybridization of 3β-HSD to both thecal and granulosal cells. G: granulosal cells; T: thecal cells. Scale bars represent 50 μm.

Table 1. Expression of messenger RNA encoding gonadotrophin receptors and steroidogenic enzymes during recruitment and selection of bovine ovarian follicles

	Not recruited[a]		Recruited[a]		Selected	
	Granulosa	Theca	Granulosa	Theca	Granulosa	Theca
Gonadotrophin receptors						
FSH	+	–	+	–	++	–
LH	–	+	–	+	+*	++
Steroidogenic enzymes						
P450scc	–	+	+*	+	++	++
P450c17	–	+	–	+	–	++
P450arom	–	–	+*	–	++	–
3β-HSD	–	+	–	+	+*	++
Steroidogenic acute regulatory protein (StAR)	–	+	–	+	–	++

[a] Differences in the intensity of expression have not been tested between not recruited and recruited follicles.
* Indicates when mRNA expression is first detected.
+ or ++ denotes amount of expression.

6–9 mm in diameter, all follicles in the cohort express P450scc and P450arom mRNA in the granulosal cells. During this time some of the 4–5 mm follicles that were apparently recruited do not express P450scc and P450arom. The number of follicles that express P450scc and P450arom during the early stages of recruitment is similar to the number of follicles that continue growth during later stages of recruitment. Therefore, recruitment of follicles that continue growth beyond 4–6 mm in diameter may be associated with expression of mRNA P450scc and P450arom in granulosal cells (Table 1; Fig. 4).

Expression of P450scc and P450arom mRNA is likely to be important for continued growth, since all follicles that continue to grow beyond 4–6 mm in diameter, after the initial stages of recruitment, expressed mRNA for P450scc and P450arom (Bao *et al.*, 1997c). It is at this stage of growth that follicles develop the capability to produce significant quantities of oestradiol. This is consistent with previous reports that follicles less than 5 mm in diameter do not produce oestradiol (Skyer *et al.*, 1987). Induction of P450scc and P450arom mRNA is probably due to a transient increase in circulating FSH that precedes initiation of each wave of follicular growth (Adams *et al.*, 1992; Fig. 4). LH may not be involved in follicular recruitment or mRNA P450scc and P450arom expression, since LHr mRNA is not detected in granulosal cells during recruitment. If LH is involved, the effect is likely to be an indirect one through stimulation of thecal androgen synthesis. In addition, follicles grow to 7–9 mm in diameter when LH, but not FSH, is inhibited (Fig 3; Gong *et al.*, 1996).

Gene Expression during Selection

When the cohort of follicles in cattle reach 8–9 mm in diameter, there is rapid divergence whereby one follicle increases rapidly in size, becomes larger than the other follicles and becomes the dominant follicle (Ginther *et al.*, 1996). Divergence of the selected follicle seems to occur about 36 to 48 h after initiation of a follicular wave. In cattle, divergence of the selected follicle appears to be associated with initiation of mRNA expression of LHr and 3β-HSD in granulosal cells (Xu *et al.*, 1995b; Bao *et al.*, 1997c; Fig. 4). Whether selection of the dominant follicle or granulosal cell mRNA LHr or 3β-HSD expression occurs first, or whether they occur simultaneously, is unclear. Evans and Fortune (1997) reported an increase in both size and oestradiol secretion in one follicle of the cohort before detection of LHr and 3β-HSD mRNA expression in granulosal cells. Similarly, Bodensteiner *et al.* (1996) reported an increase in oestradiol concentration in the selected dominant follicle before an increase in the numbers of gonadotrophin receptors. Regardless, all dominant follicles express

mRNA for LHr, 3β-HSD and P450arom (Xu et al, 1995a; Bao *et al.*, 1997a,c). However, in this study, the number of receptors for LH included those in the thecal cells as well as those in the granulosal cells. In addition, divergence (identification) of the selected follicle occurs when circulating concentration of FSH, which has been decreasing from shortly after recruitment, reaches its nadir. Thus, the follicle that is the most functionally developed can survive in an environment of decreasing FSH concentration. Hence the first follicle to develop LHr in granulosal cells would be able to respond to LH, as well as FSH, and to survive in an environment unable to support the other follicles (Gong *et al.*, 1996; Figs 3 and 4). In rodents, induction of the LHr in granulosal cells is dependent on the action of FSH and oestradiol (Segaloff *et al.*, 1990). Thus, the follicle with the highest concentration of oestradiol would be the first follicle to develop LHr in the granulosal cells and hence, allow granulosal cells of the selected follicle to become responsive to LH, as well as FSH, and survive in the face of declining serum FSH.

Continued growth of selected follicles is generally accompanied by increased expression of gonadotrophin receptors, steroidogenic enzymes and StAR, and selected and dominant follicles have higher mRNA expression than subordinate and atretic follicles. Despite the increases in mRNA expression of follicles during development, selection probably cannot be determined by the differential mRNA expression of either LHr, P450scc, P450c17, 3β-HSD or StAR in thecal cells, FSHr and P450arom in granulosal cells, or P450scc in granulosal and thecal cells (Table 1). This is because more than one follicle, of approximately the same size, expresses similar amounts of these mRNAs indicating that either the current techniques are too insensitive or selection depends on the expression of other upstream factors that remain to be determined.

Gene Expression during Dominance and Atresia

Follicular dominance

Dominant follicles continue to grow for a few days after selection. Expression of mRNA for the gonadotrophin receptors, steroidogenic enzymes and StAR generally increase in thecal and granulosal cells during the growing phase, and follicles produce greater amounts of oestradiol (Xu *et al.*, 1995a,b; Bao *et al.*, 1997a,c). Thus, dominant follicles acquire increased capability to produce steroids during their development, supporting previously published work investigating follicular steroid production (see Campbell *et al.*, 1995; Webb and Armstrong, 1998). In addition, the patterns of mRNA expression are in agreement with previous work in sheep and cattle that has found that LH can support dominant follicle development. However, if the LH pulse frequency is too low, for example during the middle of the luteal phase, dominant follicle growth will not continue. After luteolysis, mRNA expression for P450scc, P450c17 and 3βHSD, but not P450arom, increases (Tian *et al.*, 1995). Concurrently, follicular fluid concentrations of androstenedione and oestradiol increase, under the influence of increased pulsatile LH release. The significant increase in expression of mRNAs encoding the steroidogenic enzymes and follicular fluid steroid concentrations are likely due to increased pulse frequency of LH secretion during the preovulatory period (Campbell *et al.*, 1995). The increase in LH pulse frequency may increase mRNA expression for the steroidogenic enzymes necessary for synthesis of androgen precursors for oestradiol production. Thecal cell production of androgens in cattle may be the rate-limiting step for follicular oestradiol production (Badinga *et al.*, 1992), but the fact that the oestradiol : androgen ratio remains approximately one throughout the follicular phase in sheep (Campbell *et al.*, 1990) would appear to make this unlikely unless there are key species differences.

Follicular atresia

If luteolysis does not occur during the growing phase of the dominant follicle, the fate is atresia. Expression of gonadotrophin receptor mRNAs, steroidogenic enzymes and StAR decrease rapidly with atresia, and a decline in expression occurs earlier than morphological signs of atresia are

observed (Xu *et al.*, 1995a,b). Atresia of dominant follicles appears to be initiated between days 4 and 6 in the non-ovulatory follicular wave. Expression of mRNAs for FSHr in granulosal cells, LHr in thecal cells, P450scc in granulosal and thecal cells, and P450c17 in thecal cells decreases markedly between days 4 and 6 of the follicular wave. Interestingly, expression of mRNAs for LHr and P450arom in granulosal cells is still high on day 6, but declines by day 8 of the follicular wave (Xu *et al.*, 1995a,b). Atresia of unselected follicles from the cohort appears to be similar to atresia of dominant follicles.

Additional Extraovarian Regulators of Follicular Growth

Although follicular development is primarily regulated by gonadotrophins, other systemic factors have been shown to alter follicular growth patterns. Pharmacological administration of recombinant GH increased the number of antral follicles without altering gonadotrophin concentrations (Gong *et al.*, 1991; de la Sota *et al.*, 1993; Gong *et al.*, 1993). Moreover, a reduction in GH and IGF-I concentrations after immunization of prepubertal heifers against GHRH inhibited the development of follicles > 7 mm in diameter (Cohick *et al.*, 1996). However, pharmacological manipulation of GH concentration may not reflect its physiological action. Recently we demonstrated that flushing heifers (200% maintenance of a low fibre diet) stimulated an increase in the number of small (< 4 mm) follicles, despite reduced GH concentrations. However, there was high insulin concentration compared with controls (Gutierrez *et al.*, 1997b) indicating that GH may not act directly to alter follicular development. Indeed, direct administration of GH into the ovarian artery did not stimulate ovarian steroid secretion in the autotransplanted sheep (Campbell *et al.*, 1995) and the identification of follicular GH receptors has proved difficult (Lucy *et al.*, 1993). GH may act through differential responses of IGF-I and insulin. IGF-I is a potent stimulator of steroidogenesis and proliferation of both granulosal and thecal cells *in vitro* (Campbell *et al.*, 1996, 1998b; Gutierrez *et al.*, 1997c). Insulin also stimulates proliferation and steroidogenesis of granulosal and thecal cells *in vitro* (Campbell *et al.*, 1998b; Gutierrez *et al.*, 1997d). The bioavailability of IGFs is regulated by their association with a family of specific IGFBPs which in turn are affected by nutrition (Webb and Armstrong, 1998). Hence, systemic metabolic factors can influence follicular recruitment and selection. However, in addition to this extraovarian IGF system there is also an intraovarian system that may function in concert to alter the response of follicles to gonadotrophins.

Intraovarian Regulation of Follicular Growth

A range of follicular growth factors are now known to be involved in the regulation of follicular growth, including the TGF-β superfamily, FGFs, EGF and TGFα and cytokines as well as the IGFs. Many of the intraovarian growth factor signalling systems act through tyrosine kinase receptors that regulate granulosal and thecal cell differentiation in a coordinated manner through interaction with gonadotrophin–cAMP-stimulated mechanisms. The integration of the endocrine and intraovarian mechanisms provide the necessary signals that either stimulate follicular growth or divert the follicle into apoptotic pathways resulting in follicular atresia. This section will concentrate on components of two of the most intensively studied families, namely the IGF and activin–inhibin systems.

Ovarian IGF system

The IGF system, occupying a central position within the 'network' of intraovarian signals, includes the IGF ligands (IGF-I and -II), at least six IGF-binding proteins (IGFBP-1 to -6), type 1 and type 2 IGF receptors and specific IGFBP proteases. To date, expression of mRNAs encoding IGFBP-2 to -5 have been found in bovine follicles and expression of IGFBP-2, -4 and -5 in ovine follicles (Armstrong and Webb, 1997; Webb and Armstrong, 1998).

Insulin-like growth factors I and II. IGFs stimulate granulosal and thecal cell proliferation and differentiation and have been identified as follicular survival factors. There is considerable species variation in the patterns of mRNA expression of IGF ligands and BPs during folliculogenesis. We have detected the expression of mRNA encoding IGF-II in thecal tissue of bovine ovarian follicles (Armstrong and Webb, 1997) and a similar spatial distribution has been described in sheep (Perks *et al.*, 1995). The expression of mRNA encoding IGF-I in ruminants remains controversial. Leeuwenberg *et al.* (1995) detected IGF-I mRNA in ovine granulosal and thecal tissue, and Yuan *et al.* (1998) detected IGF-I mRNA in bovine granulosal cells. In contrast, Perks *et al.* (1995) failed to detect the expression of mRNA encoding IGF-I in ovine follicles. Similarly, we were unable to detect expression of IGF-I mRNA in bovine follicles by *in situ* hybridization (Fig. 5). In support of this last observation we recently demonstrated that non-luteinized bovine granulosal cells do not produce IGF-I in serum-free cultures (Gutierrez *et al.*, 1997c).

IGF-binding proteins. The bioactivity of IGFs are controlled by their association with IGFBPs. As with the IGFs, the spatial expression of these binding proteins within ovarian follicles is species specific (Armstrong and Webb, 1997). In cows (Armstrong *et al.*, 1998) and sheep (Besnard *et al.*, 1996), expression of mRNA encoding IGFBP-4 and -2 is restricted to thecal and granulosal tissue, respectively. The spatial and temporal patterns of expression of mRNA encoding components of the IGF system in the bovine follicle are summarized in Figs 5 and 6.

In ovarian cell culture systems examined so far, IGFBPs attenuate the actions of IGFs (Mason *et al.*, 1992; Monget *et al.*, 1993; Spicer *et al.*, 1997). A decrease in follicular IGFBP production would therefore be expected to enhance the biological activity of locally produced IGFs, resulting in increased follicular response to gonadotrophins. The observed decrease in the concentration of IGFBP-2, -4 and -5 in follicular fluid during the development of dominance supports this hypothesis (Armstrong *et al.*, 1996). In cows (Armstrong *et al.*, 1998) and sheep (Besnard *et al.*, 1996) the decrease in IGFBP-2 concentration in follicular fluid during follicular growth was shown to be due to a loss of expression of mRNA encoding IGFBP-2 in granulosal cells in dominant follicles. Using serum-free bovine granulosal cell cultures, we have shown that FSH, at physiological concentrations, inhibits expression of mRNA encoding IGFBP-2 (Armstrong *et al.*, 1998). These results indicate that a key feature in the development of follicular dominance in cattle is the FSH-dependent inhibition of the expression of mRNA encoding IGFBP-2 in granulosal cells (Fig. 6). The resultant increase in IGF bioactivity in these follicles would increase FSH responsiveness of their granulosal cells.

Activin–inhibin system

The TGF-β superfamily comprises a range of proteins, including members of the activin–inhibin system, with the potential to act as intraovarian regulators. mRNAs encoding TGF-β are expressed in thecal cells from both mammalian and non-mammalian species (Armstrong and Webb, 1997). In cows, TGFβs inhibit granulosal and thecal cell proliferation while enhancing gonadotrophin-stimulated steroidogenesis (Roberts and Skinner, 1991).

Inhibin–activin family. Expression of mRNA for members of the inhibin–activin family appears to be initiated in a sequential and co-ordinated way during early follicle development. In sheep, mRNA expression of B-inhibin–activin subunit appears concomitant with expression of FSHr at 1–2 layers of cuboidal granulosal cells followed by inhibin α-subunit and follistatin at more than 2–4 layers of granulosa cells and finally βA-inhibin–activin subunit during early antral development (Eckery *et al.*, 1996). As ovine follicles progress from small (< 2 mm), to medium-sized (2–4 mm) and to large (> 4 mm) there is a progressive increase in P450c17 expression in the theca and inhibin α and βA-subunit, LH-receptor and P450arom expression in the granulosal cells. In contrast expression for βB-inhibin–activin subunit in granulosal cells and LH receptor in thecal cells remains relatively constant (B. K. Campbell, L. M. Harkness, D. G. Armstrong, H. A. Garverick and R. Webb,

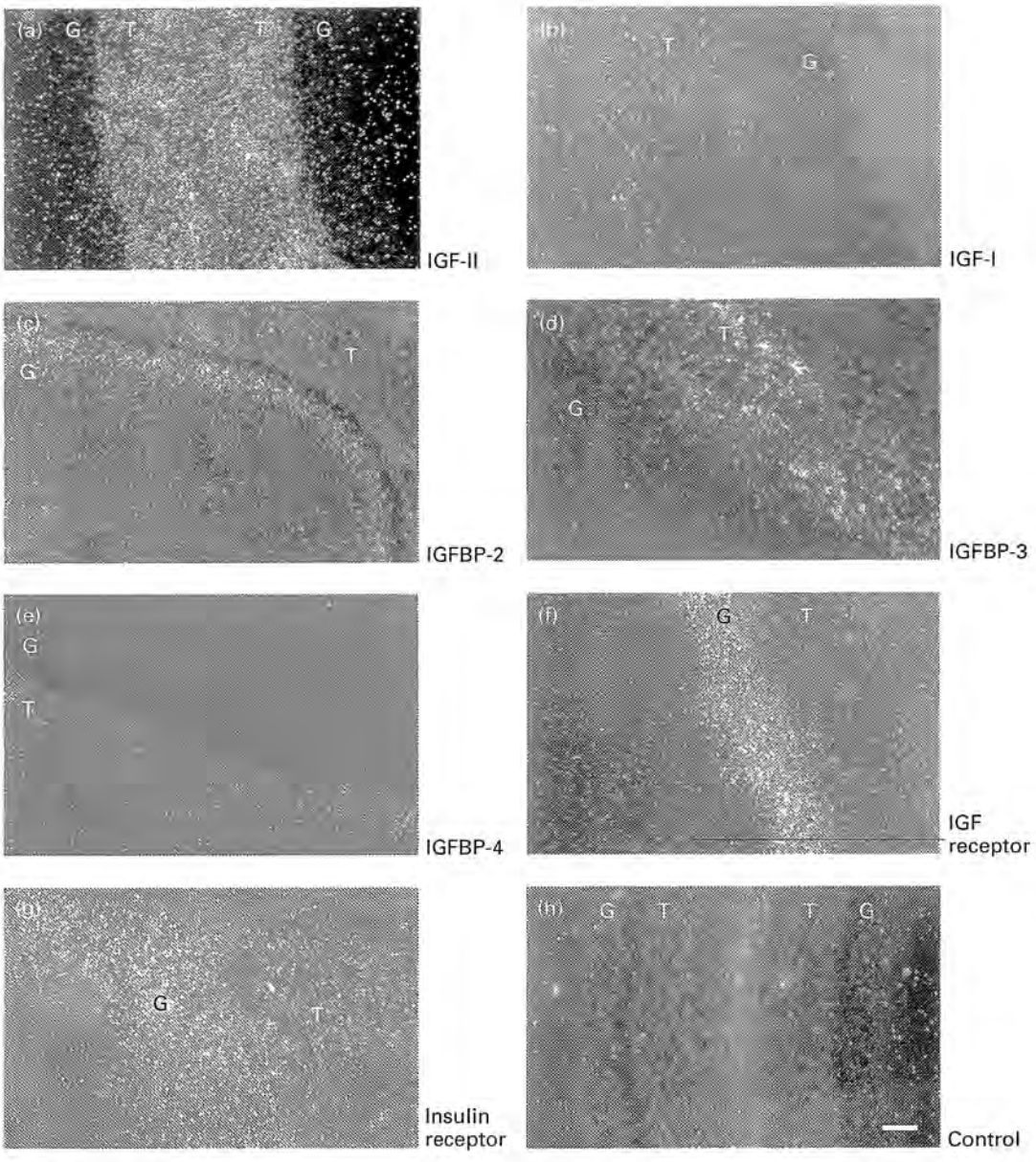

Fig. 5. *In situ* hybridization of mRNAs encoding (a) IGF-II, (b) IGF-I, (c) IGFBP-2, (d) IGFBP-3, (e) IGFBP-4, (f) type 1 IGF receptor, (g) insulin receptor and typical control section probed with sense IGF-II RNA in bovine follicles. G and T represent granulosal and thecal cells, respectively. Scale bar represents 100 μm.

unpublished). The association between P450arom and βA-subunit expression is particularly interesting as both these factors are expressed precociously in medium-sized follicles in sheep carrying the *FecB* gene (Fig. 7). This is a major gene that results in a marked increase in prolificacy associated with the ovulation of significantly more follicles at a smaller size. The correlation between

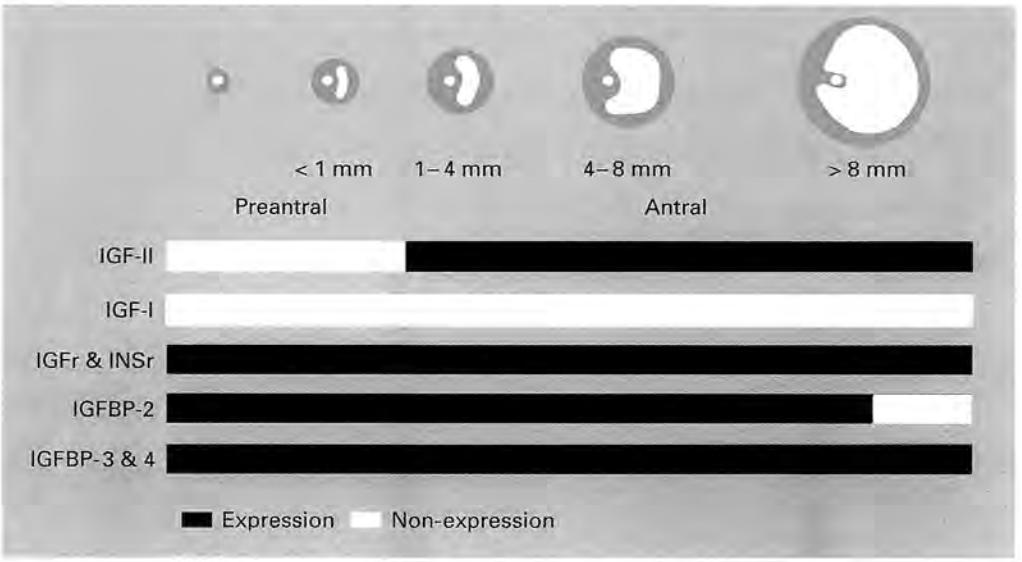

Fig. 6. The relationship between the temporal patterns of expression of mRNAs encoding components of the IGF system during bovine follicular development.

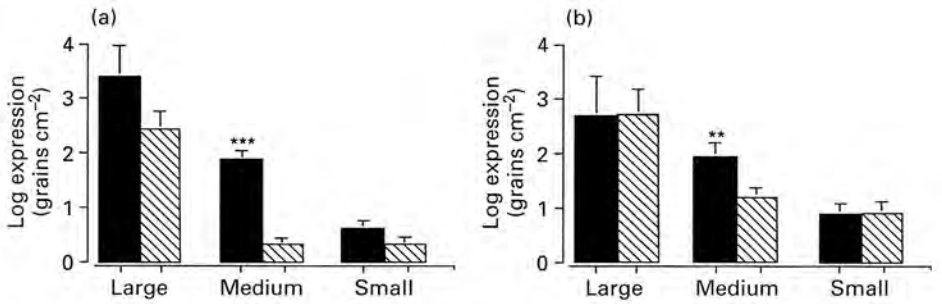

Fig. 7. Expression of mRNA, determined by *in situ* hybridization, for (a) cytochrome P450 aromatase and (b) the inhibin βA subunit in the granulosal cells of large (> 4 mm), medium-sized (2–4 mm) and small (< 2 mm) ovarian follicles of sheep with (black columns) and without (hatched columns) the *FecB* gene. Ovaries were recovered on day 4 of the oestrous cycle. Asterisks indicate significant differences (** $P < 0.01$; *** $P < 0.001$).

P450arom and βA-subunit expression and terminal follicle development is in agreement with results from experiments *in vitro*. These experiments demonstrated that FSH-stimulated differentiation of granulosal cells, from small follicles, results in a dose-responsive induction of inhibin A and oestradiol (Campbell *et al.*, 1997). Furthermore, inhibin A has been shown to modulate both oestradiol production by granulosal cells and androgen production by thecal cells *in vitro* (Knight, 1996; Campbell and Webb, 1995). Thus, inhibin βA-subunit expression would appear to be an essential component of the differentiative cascade.

Conclusions

Follicular recruitment, selection and the development of dominance involve the integration of systemic and intra-follicular mechanisms. Gonadotrophins provide the primary drive, particularly during the final stages of follicular development, although other extraovarian signals, including metabolic factors, can influence patterns of follicular growth. Indeed, it seems that, in cattle, FSH stimulates follicle growth up to 7 mm in diameter, followed by a requirement for LH in the final stages of follicular growth and maturation. Recent evidence on the pattern of gene expression in follicles has also demonstrated that there are a significant number of protein or peptide factors produced by follicles. The precise timing of mRNA expression, and hence production of these local factors, appears to be involved in the mechanisms of recruitment, selection and dominance. For example, it appears that a key feature in the development of follicular dominance in cows is the FSH-dependent inhibition of the expression of mRNA encoding IGFBP-2 in granulosal cells. The information from these molecular studies together with results using physiologically relevant *in vitro* granulosal and thecal cell culture systems demonstrate that these locally produced factors can amplify, attenuate or mediate the effects of circulating gonadotrophins on granulosal and thecal cell function. The optimum integration of these control systems determines both the response of individual follicles to gonadotrophins and whether it continues to develop to become dominant or is diverted into apoptotic pathways leading to eventual atresia.

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