

Control of early ovarian follicular development

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Early follicular growth refers to the development of an ovarian follicle from the primordial to early antral phase. In sheep and cows these phases of growth can be classified by the configuration of granulosa cells in the largest cross-section of the follicle as types 1 (primordial), 1a (transitory) 2 (primary), 3 and 4 (preantral) and 5 (early antral). Follicles classified as type 1 may be highly variable within each species with respect to number of granulosa cells and diameter of oocyte. Much of the variation in granulosa cell composition of type 1 follicles may occur at formation and this may account for the variability in granulosa cell composition throughout subsequent stages of growth. There appear to be important differences among species (for example sheep and cattle) in the number and function of granulosa cells relative to the diameter of the oocyte during the initiation of follicular growth. There is evidence that most, if not all, of the growth phases from types 1 to 5 are gonadotrophin-independent and that follicles develop in a hierarchical manner. In sheep, cows and pigs, numerous growth factor, growth factor receptor and gonadotrophin receptor mRNAs and peptides (for example c-kit, stem cell factor, GDF-9, β_B and β_A activin/inhibin subunit, α inhibin subunit, follistatin, FGF-2, EGF, EGF-R, TGF $\beta_{1,2}$ and β_3 , FSH-R and LH-R) are expressed in a phase of growth (for example types 1-5)-specific and cell-specific manner. However, the roles of many of these factors remain to be determined.

Introduction

The aim of this review is to describe the localization of growth factors and receptors for both growth factors and gonadotrophins during the development of an ovarian follicle from the primordial to early antral phase of growth. Particular emphasis is focused on current understanding of early follicular growth in domestic ruminants. Where appropriate some references to the extensive literature in other species is included.

Classification of Early Follicular Growth Stages

A classification system for small bovine follicles was described by Braw-Tal and Yossefi (1997). In this classification system preantral follicles and the smallest antral follicles are classified as types 1-5: type 1 refers to primordial follicles (one layer of 'flattened' granulosa cells, granulosa cell), type 1a to transitory follicles (one layer of cells that are a mixture of 'flattened' and cuboidal granulosa cell), type 2 to primary follicles (one or two layers of cuboidal granulosa cells), type 3 to small preantral (two to four layers of granulosa cells), type 4 to large preantral (four to six layers of granulosa cells) and type 5 to small antral follicles (more than five layers of granulosa cells). The inclusion of type 1a follicles acknowledges the finding that in cows 82.5% of the follicle population with one layer of

Table 1. Classification and characterization of small ovine follicles

Follicle (type)	<i>n</i>	Layers of granulosa cells (shape)	Number of granulosa cells in largest cross-section	Total number of granulosa cells in follicle	Oocyte diameter (µm)	Follicle diameter (µm)
Primordial (1)	195	1 (all flattened)	4 ^a (1,11)	16 ^a (3,52)	34.6 ^a (22.8,52.3)	40.8 ^a (28.1,60.5)
Transitory (1a)	53	1 (one or more cuboidal)	9 ^b (4,18)	39 ^b (9,136)	40.6 ^b (27.3,53.0)	50.8 ^b (37.3,64.0)
Primary (2)	109	1-2 (all cuboidal)	19 ^c (3,49)	128 ^c (30,520)	52.1 ^c (31.0,80.0)	75.2 ^c (49.7,118.8)
Small preantral (3)	38	> 2-4	64 ^d (19,152)	637 ^d (127,2174)	72.9 ^d (40.6,92.0)	128.5 ^d (63.5,191.0)
Large preantral (4)	18	> 4-6	187 ^e (116,308)	2104 ^e (1090,3464)	87.8 ^e (76.9,100.2)	194.1 ^e (164.2,256.3)
Small antral (5)	23	> 5	475 ^f (199,1128)	11649 ^f (3425,51447)	118.8 ^f (90.9,141.6)	326.9 ^f (191.5,450.0)

Values are geometric means (and ranges). Values in columns not sharing a common alphabetical superscript are significantly different ($P < 0.05$, ANOVA on log_e transformed data). Data from Lundy *et al.* (1999).

Table 2. Expression of growth factors and receptors during early follicular growth in sheep

Cell type	C-kit	SCF	β _a activin/ inhibin	FSHR	α-Inhibin	Follistatin	β _b activin/ inhibin	TGFβ 1 2/3	FGF ₂ ⁺	LHR
Granulosa										
Follicle type										
1		● ○					○			
1a		● ○	● ○				○			
2		● ○	● ○	● ○			○	○	○	○
3		● ○	● ○	● ○	● ○	● ○	○	○	○	○
4	● ○	● ○	● ○	● ○	● ○	● ○	○	○	○	○
5	● ○	● ○	● ○	● ○	● ○	● ○	● ○	○	○	○
Oocyte	● ○	○	○			○	○	○	○	○
Theca interna	(1-5)	(1-5)						● ○ ● ○	○	● ○
										(4-5)

●, mRNA; ○, protein; ○, localized increasingly in oocyte plasma membrane and zona pellucida () = follicle type in which expression is observed, *Data from studies in cows, SCF = stem cell factor

granulosa cells has a least one cuboidal granulosa cell (317 follicles examined; van Wezel and Rogers, 1996). In ewes a similar analysis revealed that 24.2% of follicles with one layer of granulosa cells ($n = 215$ follicles) contained one or more cuboidal cells (Lundy *et al.*, 1998). A classification system for small ovarian follicles in the ewe is shown in Table 1.

The results for primordial follicles in ewes with respect to oocyte diameter and the number of granulosa cells in the largest cross-section are similar to those for cows (Braw-Tal and Yossefi, 1997). The total number of granulosa cells around the oocyte in the ewe was determined using the nucleator and fractionator techniques of Gundersen (1988). The total population of granulosa cells in primordial follicles together with oocyte diameter are highly variable (Table 1). The variability in granulosa cells in type 1 follicles may arise at the time when follicles are first forming (Hirshfield and De Santi, 1995). There appear to be important differences among species (for example between sheep and cattle) in both the number and activity of granulosa cells relative to the diameter of the oocyte during the initiation of follicular growth (Braw-Tal and Yossefi, 1997; Lundy *et al.*, 1998). In

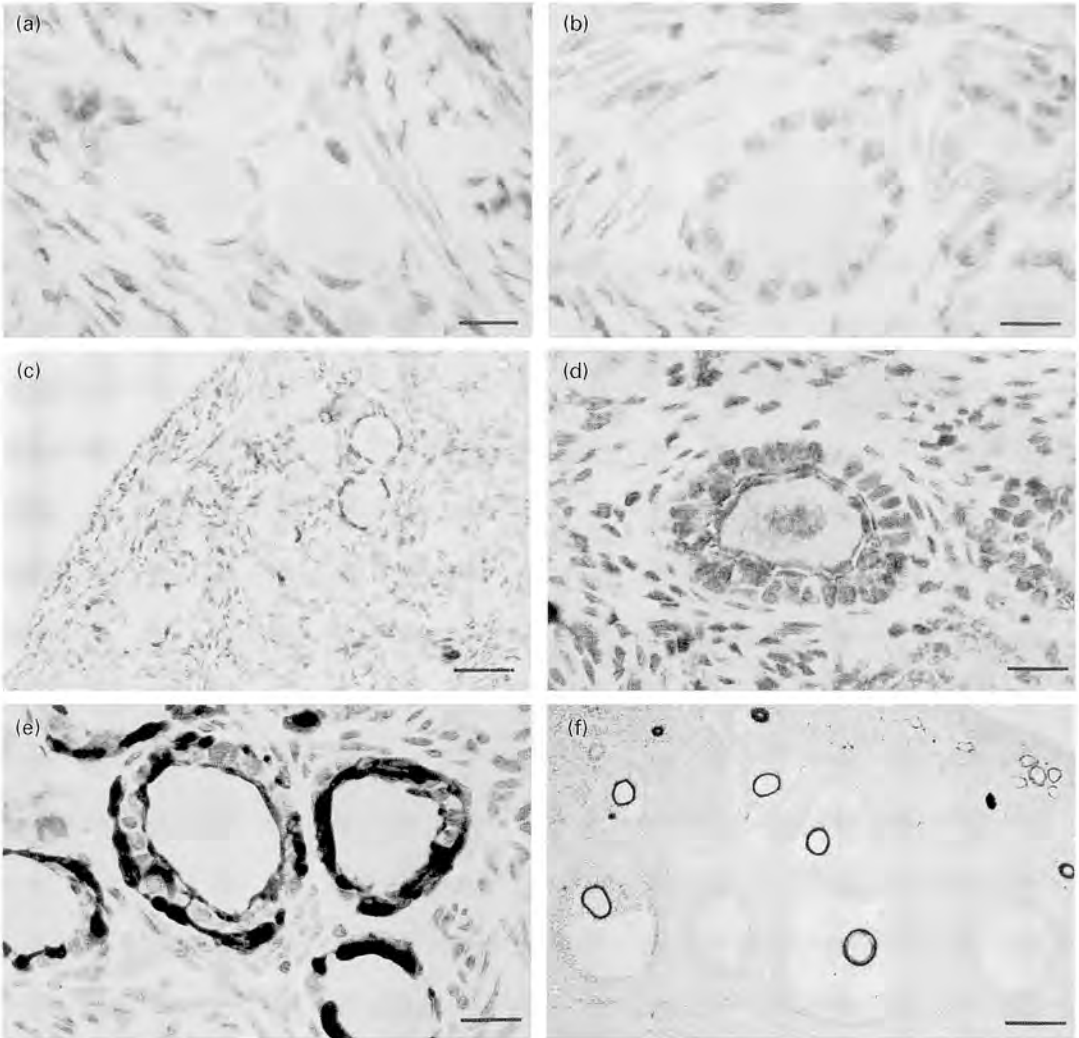


Fig. 1. Immunostaining for growth factor receptors or growth factors in ovarian follicles of the sheep ovary: (a) *c-kit* localized in oocytes of type 1 and 1a follicles and (b) in an oocyte of a type 2 follicle; (c) stem cell factor (SCF) localized to granulosa cells of type 1 follicles and (d) the oocyte and granulosa cells of a type 2 follicle; (e) inhibin β_B localized to granulosa cells of type 1a and 2 follicles and (f) zona pellucida of larger preantral and antral follicles. All immunostaining was performed with paraformaldehyde fixed paraffin wax embedded 5 μ m sections. All immunostaining was blocked when the antibodies were either preincubated with their respective antigens or replaced by non-immune serum. The immunohistochemical procedures used were those described by Hsu *et al.* (1981). The *c-kit* antibody (C19) was supplied by Santa Cruz Biotechnology Inc, Santa Cruz (CA). The stem cell factor antibody (RGAS005) and recombinant ovine stem cell factor (+1 to +206 amino acid sequence RGAS006) were generated at the Wallaceville Animal Research Centre. Inhibin β_B antibody was clone C5 described in Groome *et al.* (1996). Scale bars represent (a) and (b) 13.3 μ m, (c) 40 μ m, (d) 16 μ m, (e) 16 μ m and (f) 160 μ m.

cows and rats, very few granulosa cells in type 1 follicles immunostain for proliferating cell nuclear antigen (PCNA), an essential nuclear protein involved in cell proliferation (Oktay *et al.*, 1995; Wandji *et al.*, 1996a). By contrast in ewes, about 30% or more of the granulosa cells in type 1 follicles immunostain for PCNA (Lundy *et al.*, 1998). Therefore a key question that remains unanswered is

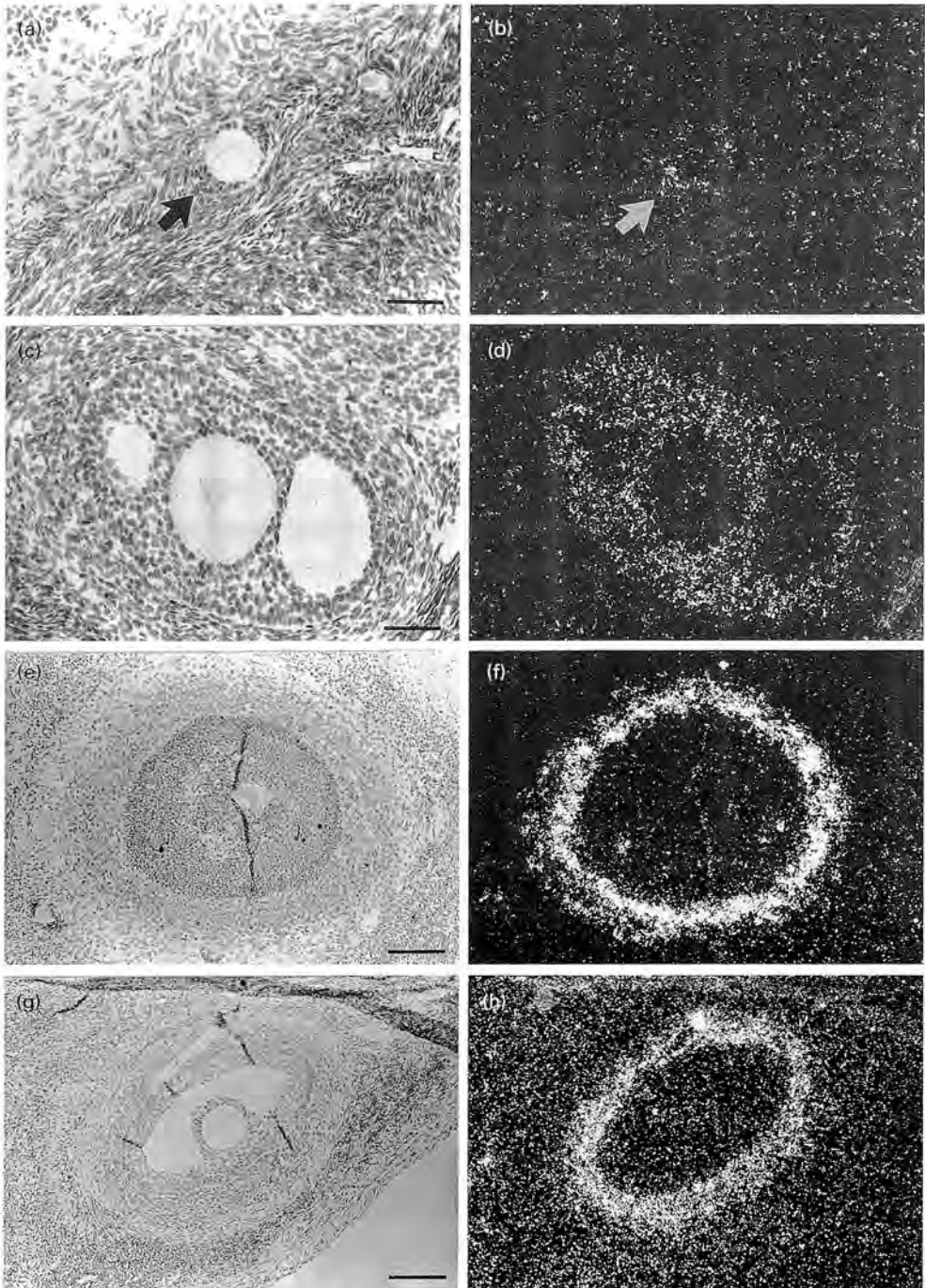


Fig. 2. Localization of gonadotrophin receptor mRNAs in small follicles of the sheep ovary: (a) light field and (b) dark field views of a type 1 and type 3 follicle with evidence of hybridization of ^{33}P -labelled FSH-receptor antisense riboprobe to granulosal cells of the type 3 (arrowed) but not type 1 follicle; (c) light field and (d) dark field views of a type 5 follicle showing hybridization of the FSH-receptor antisense riboprobe to granulosal cells; (e) light field and (f) dark field views of ^{33}P -labelled LH receptor antisense riboprobe hybridized to theca interna of a type 4-5 follicle; (g) light field and (h)

whether type 1 follicles are a pool of quiescent follicles. Compared with the uncertainty about the growth status of type 1 follicles, the collective evidence from several species is that type 1a follicles have entered the growth phase. In cows, ewes and rats, cuboidal granulosa cells in type 1a follicles may immunostain for PCNA or incorporate tritiated thymidine (Oktay *et al.*, 1995; Wandji *et al.*, 1996a; Braw-Tal and Yossefi, 1997; Lundy *et al.*, 1998). Moreover in ewes, type 1a follicles contain significantly more granulosa cells and the mean oocyte diameters are larger compared with type 1 follicles (Table 1). As ovine follicles transit through the types 1 to 3 growth phases, the mean total number of granulosa cells undergo at least six doublings before entering the type 4 phase which in turn may include two doubling cycles. Thus when considering factors that might regulate early follicular growth it may be important to consider: (i) the classification system being used; (ii) the animal model being studied; (iii) whether type 1 follicles are all quiescent follicles and; (iv) the likelihood that types 2, 3 and 4 follicles each represent more than one cell-doubling cycle with the possibility that each cycle is under some form of regulatory control. It is suggested that future studies need to focus more closely on the characteristics of the types of follicle being studied. Some of the growth regulatory factors associated with these early phases of growth are discussed below (see also Table 2).

Growth Factors and Receptors

C-kit/Stem cell factor

The tyrosine kinase receptor *c-kit* and its ligand, stem cell factor (SCF), have been localized to oocytes and granulosa cells, respectively (Motto and Bernstein, 1993). In the mouse, inhibition of the interaction between SCF and *c-kit* prevents the transformation of primordial follicles to primary follicles without blocking the formation of primordial follicles (Huang *et al.*, 1993; Yoshida *et al.*, 1997). In the sheep ovary, SCF mRNA has been detected in granulosa cell and *c-kit* mRNA in the oocyte at all stages of follicular growth from the primordial phase (Clark *et al.*, 1996; Tisdall *et al.*, 1997). Furthermore, in sheep, *c-kit* protein can be localized to oocytes of primordial and growing follicles and SCF protein to granulosa cells and oocytes of both primordial and primary follicles (Fig. 1). These findings are consistent with the view that activation of the *c-kit* tyrosine kinase system by SCF is an important factor in the growth of primordial follicles. One interesting finding from the study of Yoshida *et al.* (1997) was that the granulosa cell ceased to proliferate after the administration of *c-kit* antibody to mice. Thus a very early signal from oocytes might be necessary to promote proliferation of granulosa cells. Potential candidates might be growth differentiating factor 9 (GDF-9) (Dong *et al.*, 1996), epidermal growth factor or its receptor (Singh *et al.*, 1995) or a product from the X chromosome, since sheep that are homozygous for an X-linked mutation (*FecX¹*) contain primordial and primary follicles with enlarging oocytes (expressing *c-kit*) but with no corresponding increase in the number of granulosa cells (P. Smith, T. Lundy and K. P. McNatty, unpublished).

Gonadotrophin receptors

Follicle stimulating hormone (FSH) is unlikely to be a critical factor for initiating the growth of primordial follicles. There is convincing evidence from sheep, humans, cows and pigs that the gene for the FSH receptor (FSH-R) is not expressed until the follicle has reached the type 2-3 stage of development (Tisdall *et al.*, 1995; Xu *et al.*, 1995; Yuan *et al.*, 1996; Oktay *et al.*, 1997). At this and all

dark field views of ³³P-labelled p450sc antisense riboprobe hybridized to theca interna of a type 5 follicle. All sense probes showed no evidence of specific hybridization (see Tisdall *et al.*, 1995 for methodology). The ovine LH-R cDNA was obtained from G. Niswender, Colorado State University, Fort Collins, CO and the bovine p450sc from M. Waterman, Vanderbilt University School of Medicine, Nashville, TN. Scale bars represent (a,b,c,d) 80 µm; (e,f,g,h) 160 µm.

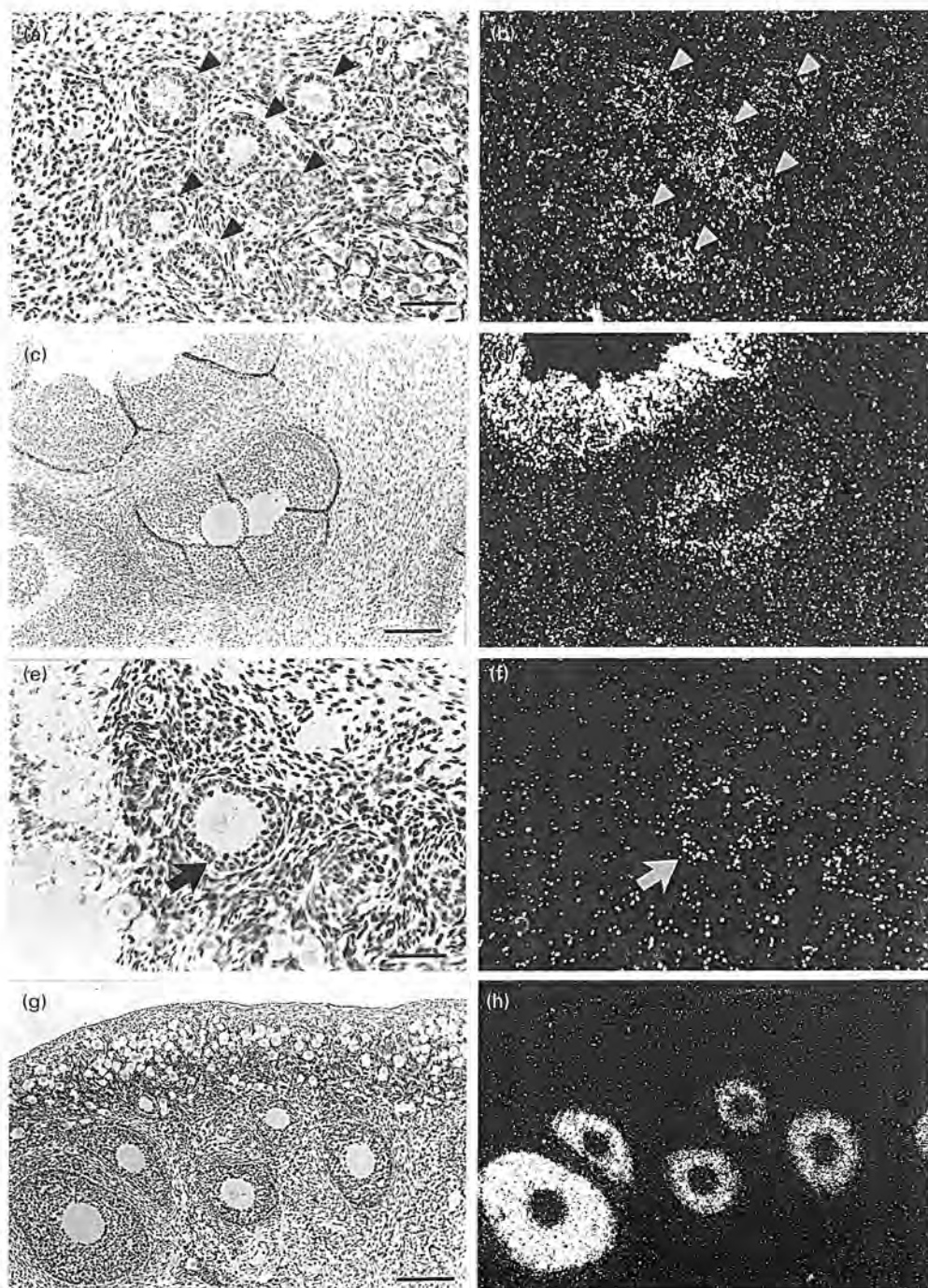


Fig. 3. Localization of inhibin and activin subunit mRNAs in small follicles of the sheep ovary: (a) light field and (b) dark field views of type 2 and 3 follicles (arrowheads) with evidence of hybridization of inhibin/activin β_{H} subunit antisense riboprobe to granulosa cells. Note there is no increase above background in type 1 follicles (b); (c) light field and (d) dark field views of type 5 and larger follicles with hybridization of inhibin/activin β_{A} subunit antisense riboprobe to granulosa cells; (e) light field and (f) dark field views of a type 3 follicle (arrows) with hybridization of inhibin α subunit antisense

subsequent stages of development FSH-R mRNA is localized exclusively to granulosa cells (Fig. 2). Autoradiographic analysis of ^{125}I -labelled FSH binding to preantral follicles is consistent with the presence of FSH-R in granulosa cells of type 2–4 but not type 1 or 1a follicles (Wandji *et al.*, 1992a). It is likely that the FSH-R is functionally active during preantral development because culture of preantral bovine, hamster and human follicles in serum-free media supplemented with FSH leads to significant increases in the number of granulosa cells or uptake of BrdU or thymidine by granulosa cells (Roy and Greenwald, 1989; Hulshof *et al.*, 1995; Wandji *et al.*, 1996a). Moreover, granulosa cells in large preantral–early antral follicles can synthesize cAMP or lactate in response to FSH *in vitro* (McNatty *et al.*, 1992; Boland *et al.*, 1993).

Although it can be demonstrated that FSH has stimulatory effects on granulosa cell proliferation and function in preantral follicles it is not an essential factor for proliferation of granulosa cells (Hirshfield, 1985) or the formation of a theca interna (Magarelli *et al.*, 1996). FSH-deficient mice produced by gene targeting of embryo stem cells (Kumar *et al.*, 1997) or hypohysectomized hamsters, rats or sheep all contain normally developing small follicles (Hirshfield, 1985; McNatty *et al.*, 1990; Wang and Greenwald, 1993). Furthermore, slices of ovarian cortex from the cow cultured in media devoid of gonadotrophins led to an increase in the number of primary and small preantral follicles and a corresponding decrease in the number of primordial follicles (Wandji *et al.*, 1996a; Braw-Tal and Yossefi, 1997) indicating that the initiation of follicular growth is likely to involve paracrine or autocrine rather than endocrine factors.

In sheep, the theca interna develops in type 3 follicles and in cattle in type 4 follicles (Braw-Tal and Yossefi, 1997; Lundy *et al.*, 1998). Luteinizing hormone receptor (LH-R) mRNA is demonstrable in theca interna of type 4–5 preantral follicles of pigs, cows and sheep (Xu *et al.*, 1995; Yuan *et al.*, 1996; Fig. 2). In rodents, the mRNAs encoding the intra- and extracellular domains of the LH-R appear concomitantly with the appearance of differentiated thecal cells but increased expression of LH-R during follicular growth is a gonadotrophin-dependent event (Sokka *et al.*, 1996; O'Shaughnessy *et al.*, 1997). In cattle, pigs and sheep the mRNAs for P450_{sc} and P450_{17-hydroxylase} enzymes are present in theca interna of type 4–5 follicles, which also synthesize androgens *in vitro* (Fig. 2; McNatty *et al.*, 1986; Yuan *et al.*, 1996). In domestic ruminants there is evidence that type 4–5 follicles have functional FSH-R and LH-R in granulosa cells and thecal cells, respectively, and that in ewes these follicles are capable of synthesizing progestins, androgens and oestrogens *in vitro* (McNatty *et al.*, 1986; Yuan *et al.*, 1996; Wandji *et al.*, 1996b).

Inhibin/activin subunits

Inhibins and activins are dimeric growth factors of the transforming growth factor β superfamily. The subunits inhibin α , inhibin/activin β_A and inhibin/activin β_B together with the activin receptors type I, IIA and IIB are expressed in ovarian cells during follicular development (Roberts *et al.*, 1993; Cameron *et al.*, 1994). In sheep ovaries the mRNA and peptide for β_B inhibin/activin subunit is first detected in granulosa cells of type 1a–3 follicles (Figs 1 and 3). The smallest follicles containing the β_B inhibin/activin peptide (using antisera clone C5; Groome *et al.*, 1996) were those containing one layer of granulosa cells but with at least one cuboidal cell (Fig. 1). At later stages of growth the β_B peptide was found increasingly in the zona pellucida although gene expression continued in granulosa cells (Fig. 1). However, no hybridization of the antisense β_B probe was noted in the oocyte, theca interna or interstitial cells. Unlike the early appearance of β_B mRNA in granulosa cells that for inhibin/activin β_A was not found until follicles reached the type 5 stage of growth (Torney *et al.*, 1989; Braw-Tal, 1994; Tisdall *et al.*, 1994). At this stage and thereafter β_A inhibin/activin mRNA was localized exclusively to granulosa cells (Fig. 3). In contrast to the β_A

riboprobe to granulosa cells; (g) light field and (h) dark field views of types 3–5 but not type 1–2 follicles with hybridization of follistatin subunit antisense riboprobe to granulosa cells. For details on α -inhibin and β_A inhibin/activin *in situ* procedures and ^{33}P -labelled riboprobes see Braw-Tal *et al.* (1994) and Tisdall *et al.* (1994). The β_B cDNA was supplied by R. Rodgers (Flinders University of South Australia, SA, Australia). Scale bars represent (a, b, e, f) 80 μm ; (c, d, g, h) 160 μm .

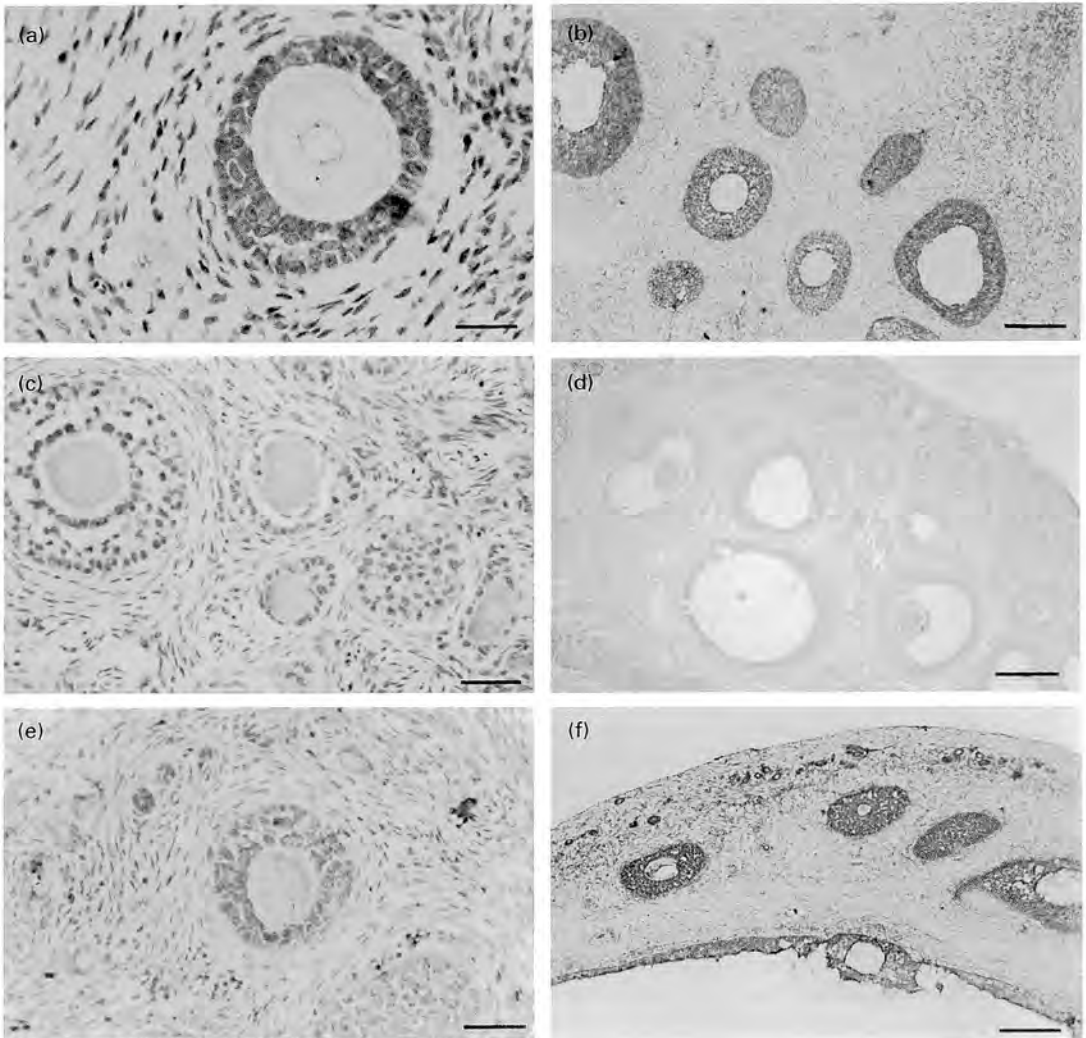


Fig. 4. Immunostaining for inhibin and/or activin subunits and follistatin in ovarian follicles of the sheep ovary: inhibin- α subunit immunostaining in type 3 follicle (a) and in type 4–5 follicles (b). Note intense immunostaining in granulosa cells and light immunostaining in some oocytes. (c,d) Inhibin/activin β_A subunit immunostaining in granulosa cells and oocytes of small (type 2–5) and both small and large antral follicles; (e,f) follistatin immunostaining in oocytes and granulosa cells of both preantral and antral follicles. For details on histological and immunohistochemical procedures see legend to Fig. 1. For details on the inhibin- α subunit antisera (clone R1) and β_A inhibin/activin antisera (clone E4) see Groome *et al.* (1990) and Groome and Lawrence (1991). The follistatin antisera (RGAS005) was generated against a 315 amino acid recombinant ovine follistatin (RGAS006) and both products were produced at the Wallaceville Animal Research Centre. Scale bars represent (a) 26.7 μm ; (b, d, f) 160 μm ; (c, e) 40 μm .

inhibin/activin mRNA results, immunohistochemical studies (using antisera Clone E4; Groome and Lawrence, 1991) localized the β_A inhibin/activin peptide to oocytes as well as to granulosa cell at all stages of follicular growth (for example primordial, primary, preantral and antral growth) (Fig. 4). One interpretation of this finding is that the monoclonal antibody to β_A peptide binds β_A inhibin/activin subunit which originated from an extragonadal source via the blood supply.

Collectively these results provide evidence for the presence of β inhibin/activin subunit peptides in oocytes or granulosa cells of preantral follicles.

Inhibin α subunit mRNA was first observed in granulosa cells of developing ovine follicles with two or three layers of granulosa cells (Fig. 1). Thereafter the hybridization signal increased exclusively in granulosa cells of both preantral and antral follicles (Braw-Tal, 1994). Similarly immunohistochemical studies with an inhibin α -subunit antibody (Clone R1; Groome *et al.*, 1990) demonstrated specific binding to granulosa cells of follicles with two or three layers of granulosa cells with no binding to theca interna, although a low level of binding was associated with the oocyte (Fig. 4).

The presence of β activin/inhibin subunits at several sites may be indicative of the presence of activin receptors in both oocytes and granulosa cell or other factors that bind the β activin/inhibin subunits (for example follistatin). Of interest is the finding that type 1a–2 follicles represent the only stages of development in which β_B activin/inhibin expression occurs in the absence of the α inhibin subunit. Mice homozygous for a deletion of the inhibin α subunit gene secrete large amounts of activin A and B and develop sex cord–stromal tumours (Matzuk *et al.*, 1996); these tumours in the female may arise from uncontrolled proliferation of granulosa cells. In normal animals the rate of proliferation of granulosa cells may be influenced in part by the endogenous production of inhibin in type 3 and larger follicles thereby blocking the action of activin via the type II activin receptor (Martens *et al.*, 1997). In domestic ruminants it remains to be determined when the activin receptors first appear and whether activin B has an important role in either upregulating FSH receptors (see Findlay, 1993 for review) or in promoting the development of a type 3 follicle.

Follistatin

Overexpression of follistatin in transgenic mice inhibits follicular growth at the primary and subsequent stages of development (Guo *et al.*, 1998). Thus follistatin may regulate the actions of activin or other members of the transforming growth factor β (TGF β) family (for example GDF-9).

In sheep, follistatin gene expression was first observed in granulosa cells of type 3 follicles (Braw-Tal, 1994; Braw-Tal *et al.*, 1994) (Fig. 3). Thereafter it was observed in almost all preantral, and non-atretic antral follicles throughout their different growth phases. Follistatin protein was first observed in granulosa cells of type 2 follicles and at subsequent phases of growth. The earlier detection of protein over mRNA for follistatin may be the result of differences in sensitivities in the methods used or to the protein originating from an extracellular source. In addition, follistatin peptide can be localized to oocytes in types 1–5 follicles as well as large preantral follicles (Dr R. Braw-Tal, personal communication; Fig. 4).

Collectively the immunohistochemical data infer that follistatin and activin and perhaps to a lesser extent inhibin are associated with the oocyte throughout follicular growth. In this context, exposure of oocytes to excess activin A stimulates meiotic maturation in follicle-enclosed oocytes and oocyte degeneration in immature follicles (Woodruff *et al.*, 1990; Erickson *et al.*, 1995). During preantral and early antral follicular growth, follistatin may act as a binding protein to prevent premature oocyte maturation. It is worth noting for most follicular growth phases that the β_A activin/inhibin peptide and follistatin are consistently co-localized where the two peptides have been studied together. In contrast the β_B activin/inhibin peptide does not co-localize with follistatin. Instead the β_B subunit is strongly associated with both the oocyte plasma membrane and zona pellucida inferring a different mechanism of action.

TGF β and FGF

Transforming growth factor β is known to be produced by bovine thecal cells and to influence granulosa cell proliferation (Skinner *et al.*, 1987; Wandji *et al.*, 1996b). In the sheep ovary TGF β_1 mRNA was observed in stromal/interstitial tissues and first observed in theca interna in type 4 or 5 follicles, whereas TGF β_3 was noted mainly in smooth muscle cells around blood vessels in the theca

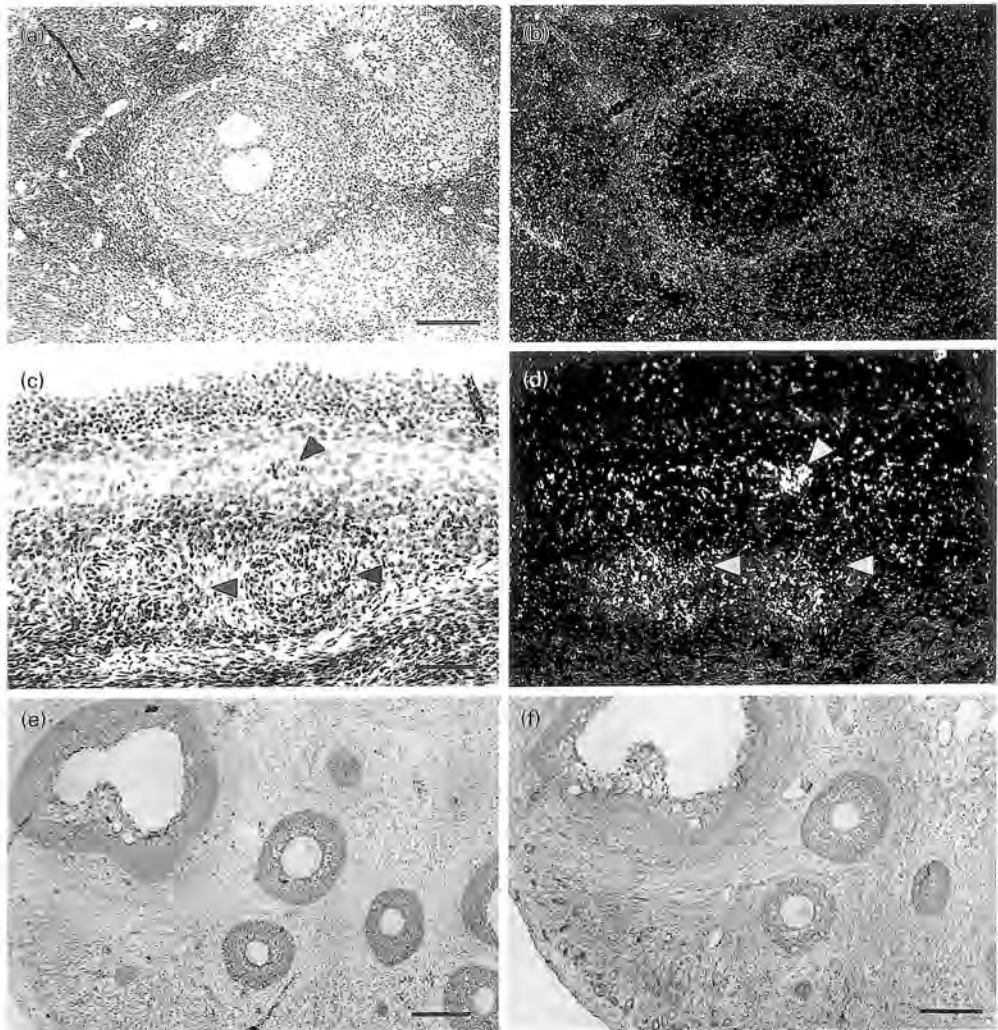


Fig. 5. Localisation of transforming growth factor β in the sheep ovary: (a) light field and (b) dark field views of a type 5 follicle with evidence of hybridization of the ^{33}P -labelled $\text{TGF}\beta_1$ antisense riboprobe to theca interna and areas of the adjacent stroma/ interstitium but not granulosa cells; (c) light field and (d) dark field views of large antral follicle with evidence of hybridization of the ^{33}P -labelled $\text{TGF}\beta_3$ antisense riboprobe to smooth muscle cells of blood vessels (arrowheads) and theca interna but not granulosa cells; (e) immunostaining for $\text{TGF}\beta_1$ in oocytes, granulosa cells of preantral and antral follicles and to a lesser extent in the theca interna and interstitium; (f) immunostaining for $\text{TGF}\beta_{2,3}$ indicating widespread localization in the ovary. The $\text{TGF}\beta_1$ riboprobe was from an RT-PCR derived 1172 bp ovine cDNA for the complete coding region. The $\text{TGF}\beta_3$ riboprobe was from an RT-PCR derived 960 bp ovine cDNA. All sense probes showed no evidence of specific hybridization. The $\text{TGF}\beta_1$ antibody (clone TB-21) was obtained from Anogen Inc, Mississauga, Ontario and the anti- $\text{TGF}\beta_{2,3}$ (clone AB-1) from Oncogene Science Inc, Uniondale, NY. For further details on immunohistochemical procedures see Fig. 1. Scale bars represent (a, b) 80 μm ; (c, d) 73 μm ; (e, f) 160 μm .

layer (Fig. 5). The pattern of $\text{TGF}\beta_2$ expression was similar to that of $\text{TGF}\beta_1$ (D. Tisdall, unpublished). By contrast $\text{TGF}\beta_1$ peptide was widespread throughout the ovary (Fig. 5): it was observed in oocytes, especially in primordial follicles and it was prominent in granulosa cells at the type 2 stage of development and to a lesser extent in the theca interna. Immunostaining with antibody that

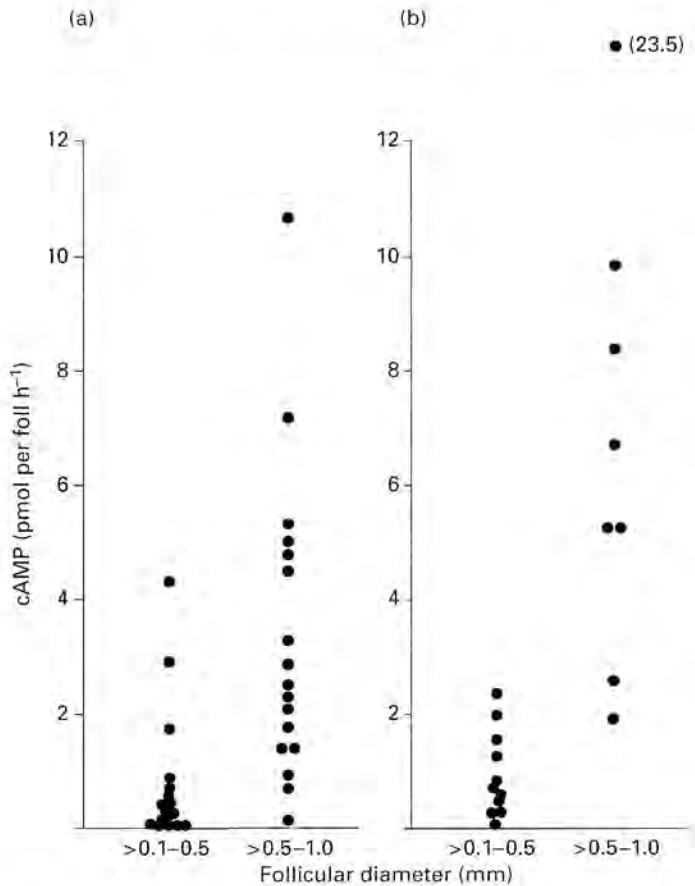


Fig. 6. The cyclic adenosine 3,5'-monophosphate (cAMP) responses of individual large preantral-early antral ovine follicles (follicle types 4 and 5) dissected from an individual (a) intact or (b) long-term (i.e. 60 days) hypophysectomized ewe. Each follicle from each ewe was incubated separately for 1 h in 1 ml Dulbecco's phosphate-buffered saline + 0.1% w v⁻¹ bovine serum albumin + ovine LH (NIH-LH-S23, 1 µg ml⁻¹) + and FSH (NIH-FSH-S11, 1 µg ml⁻¹) (see McNatty *et al.*, 1986 for experimental details).

recognizes TGFβ₂ and TGFβ₃ but not TGFβ₁, indicated widespread distribution of these peptides throughout the ovary (see Fig. 5).

Granulosa cells in culture express the gene encoding fibroblast growth factor 2 (FGF-2) (Neufield *et al.*, 1987). However, the ontogeny of FGF-2 gene expression in developing follicles and other ovarian cellular sources is not well understood. Immunocytochemical studies reveal that FGF-2 peptide is widespread throughout the bovine ovary being present in oocytes of primordial and primary follicles, granulosa cells of large preantral and antral follicles, theca interna, ovarian surface epithelium and smooth muscle cells surrounding blood vessels (Van Wezel *et al.*, 1995). Moreover FGF receptors are found in granulosa cells of type 2 follicles (Wandji *et al.*, 1992b).

In addition to the localization of TGFβs and FGF-2 to oocytes of primordial and primary follicles, epidermal growth factor (EGF) has been localized to oocytes of primordial follicles of hamsters and pigs and TGFα to oocytes of primary follicles (Roy and Greenwald, 1990; Singh *et al.*, 1995). Collectively, therefore, oocytes in primordial follicles appear to be bathed in growth factors, some of

which are likely to have stimulatory effects on granulosa cells (i.e. stem cell factor, activin, FGF-2 and EGF), whereas others are likely to be inhibitory (follistatin, TGF β s) (Gospodarowicz *et al.*, 1986; Li *et al.*, 1995; Wandji *et al.*, 1996b; Guo *et al.*, 1998). The relative importance of these growth factors or their receptors in the initiation of follicular growth remains to be determined.

Hierarchical Development

In domestic ruminants, initiation of follicular growth begins in late fetal life and continues without interruption during infancy, pregnancy, lactation and the oestrous cycle. From studies in rodents it has been proposed that follicles grow sequentially and continue to grow until they either become atretic or ovulate (Peters *et al.*, 1975). In studies of granulosa cell populations and steroid concentrations in follicular fluid of antral follicles from sheep, cows and humans it is evident that, at any time, no two follicles share an identical cell composition or steroid microenvironment (for example McNatty, 1978). These data are consistent with the notion that follicles develop in a hierarchical manner. Further evidence to support this notion was obtained from examining the gonadotrophin-induced cyclic AMP responses of late preantral–early antral follicles isolated from either hypophysectomized or control ewes (Fig. 6). These data also infer that preantral follicles may develop in a hierarchical manner and that this pattern of development is, at least in part, independent of gonadotrophins.

Conclusions

The growth of a primordial follicle to the early antral phase involves eight doublings of the population of granulosa cells and a 3–4-fold enlargement of the oocyte. In domestic ruminants most, if not all, of these growth phases can occur in the absence of gonadotrophins. However, based upon evidence from animal mutants, many of these growth phases are dependent on locally produced growth factors or receptors including c-kit, stem cell factor, members of the transforming growth factor superfamily (for example β_A inhibin/activin, α -inhibin, GDF-9) and follistatin. The evidence from studies in domestic ruminants shows that these growth factors together with receptors for both growth factors and gonadotrophins are expressed during early follicular growth in a stage- and cell-specific manner.

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