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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 granulosal 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 granulosal cells and diameter of oocyte. Much of the variation in granulosal cell composition of type 1 follicles may occur at formation and this may account for the variability in granulosal 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 granulosal 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, $TGF\beta_{1,2 \text{ and } 3}$ 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' granulosal cells, granulosal cell), type 1a to transitory follicles (one layer of cells that are a mixture of 'flattened' and cuboidal granulosal cell), type 2 to primary follicles (one or two layers of cuboidal granulosal cells), type 3 to small preantral (two to four layers of granulosal cells), type 4 to large preantral (four to six layers of granulosal cells) and type 5 to small antral follicles (more than five layers of granulosal 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)	*n*	Layers of granulosal cells (shape)	Number of granulosal cells in largest cross-section	Total number of granulosal 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_B activin/ inhibin	FSHR	α-Inhibin	Follistatin	β_B activin/ inhibin	TGFβ 1 2/3	FGF_2*	LHR
Granulosal Follicle type	1		● ⊙					⊙			
	1a		● ⊙	● ⊙				⊙			
	2		● ⊙	● ○	● ⊙		⊙	⊙	⊙ ⊙	⊙	
	3		● ⊙	● ○	● ⊙	● ⊙	● ⊙	⊙	⊙ ⊙	⊙	
	4	● ⊙	● ⊙	● ○	● ⊙	● ⊙	● ⊙	⊙	⊙ ⊙	⊙	
	5	● ⊙	● ⊙	● ○	● ⊙	● ⊙	● ⊙	● ⊙	⊙ ⊙	⊙	
Oocyte		● ⊙ (1–5)	⊙ (1–5)	○			⊙	⊙	⊙ ⊙	⊙ (1–2)	
Theca interna									● ⊙ ● ⊙	⊙	● ⊙ (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

granulosal cells has a least one cuboidal granulosal cell (317 follicles examined; van Wezel and Rogers, 1996). In ewes a similar analysis revealed that 24.2% of follicles with one layer of granulosal 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 granulosal cells in the largest cross-section are similar to those for cows (Braw-Tal and Yossefi, 1997). The total number of granulosal cells around the oocyte in the ewe was determined using the nucleator and fractionator techniques of Gundersen (1988). The total population of granulosal cells in primordial follicles together with oocyte diameter are highly variable (Table 1). The variability in granulosal 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 granulosal cells relative to the diameter of the oocyte during the initiation of follicular growth (Braw-Tal and Yossefi, 1997; Lundy *et al.*, 1998). In

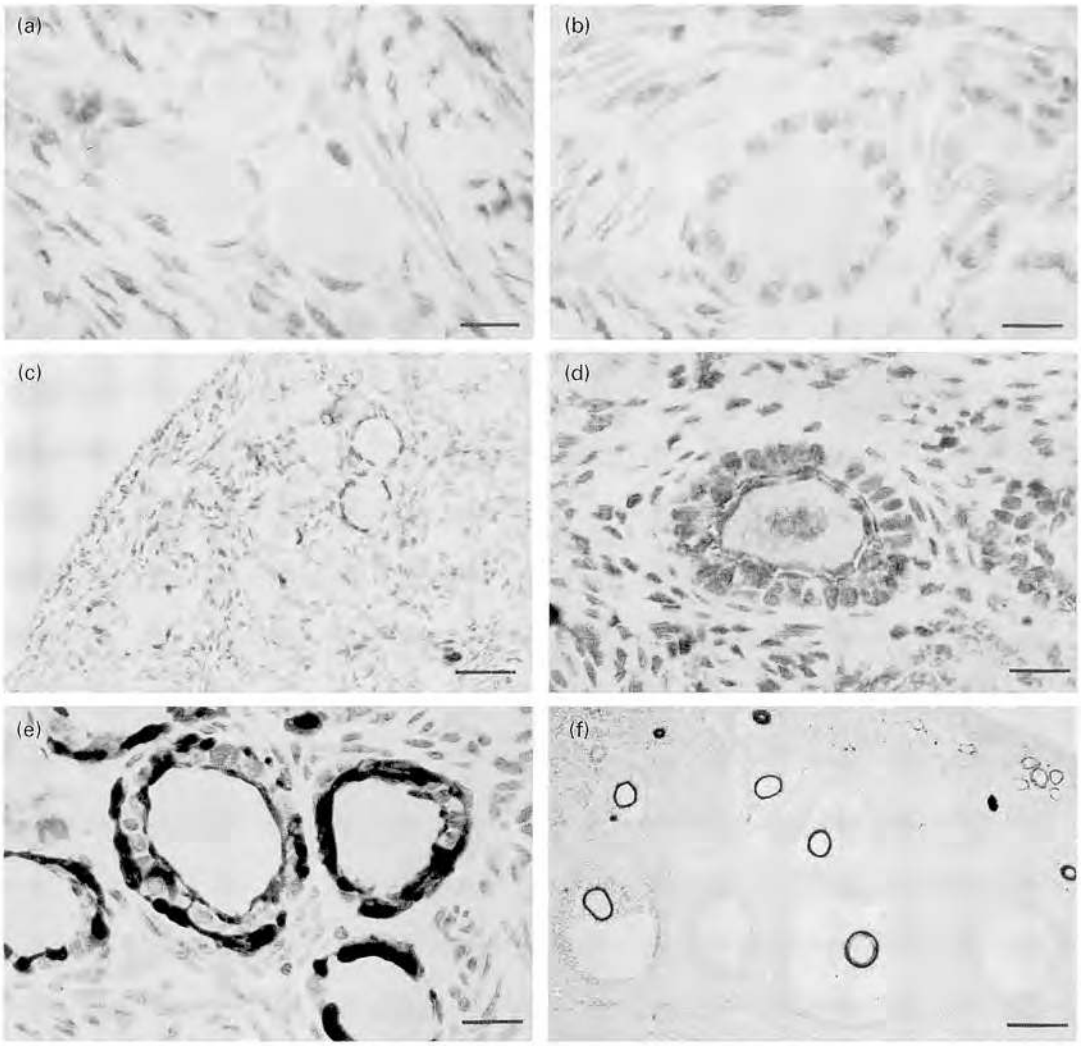

Fig. 1. Immunostaining for growth factor receptors or growth factors in ovarian follicles of the sheep ovary: (a) c-kit localized in oocytes of type 1 and 1a follicles and (b) in an oocyte of a type 2 follicle; (c) stem cell factor (SCF) localized to granulosal cells of type 1 follicles and (d) the oocyte and granulosal cells of a type 2 follicle; (e) inhibin β_B localized to granulosal 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 μm 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 μm, (c) 40 μm, (d) 16 μm, (e) 16 μm and (f) 160 μm.

cows and rats, very few granulosal 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 granulosal cells in type 1 follicles immunostain for PCNA (Lundy *et al.*, 1998). Therefore a key question that remains unanswered is

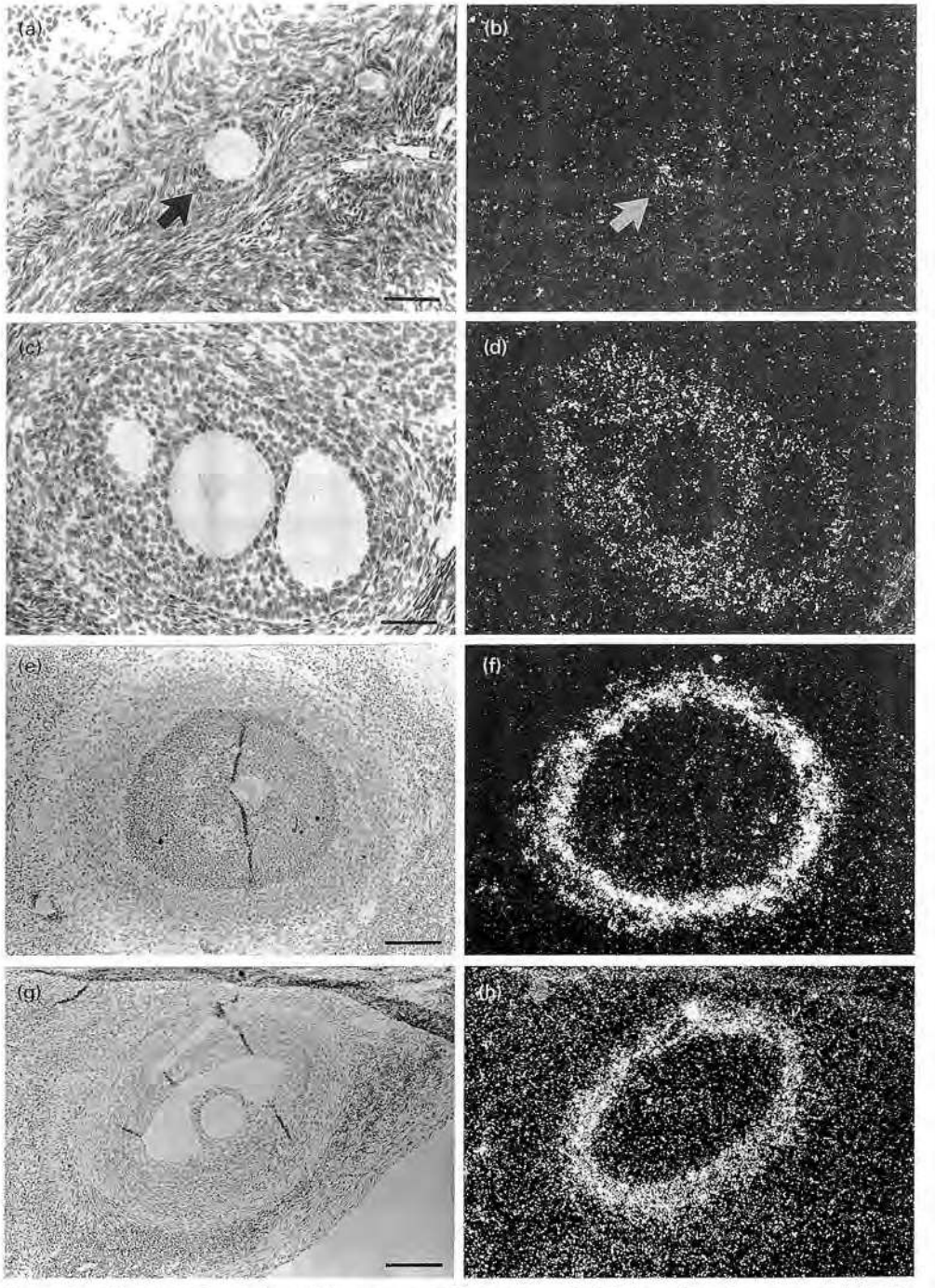

Fig. 2. Localization of gonadotrophin receptor mRNAs in small follicles of the sheep ovary: (a) light field and (b) dark field views of a type 1 and type 3 follicle with evidence of hybridization of ^{33}P-labelled FSH-receptor antisense riboprobe to granulosal cells of the type 3 (arrowed) but not type 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 granulosal 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 granulosal 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 granulosal 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 granulosal cells, respectively (Motro 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 granulosal 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 granulosal 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 granulosal 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 granulosal 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 ($FecX^I$) contain primordial and primary follicles with enlarging oocytes (expressing c-kit) but with no corresponding increase in the number of granulosal 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

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dark field views of ^{33}P-labelled p450scc 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 p450scc 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.

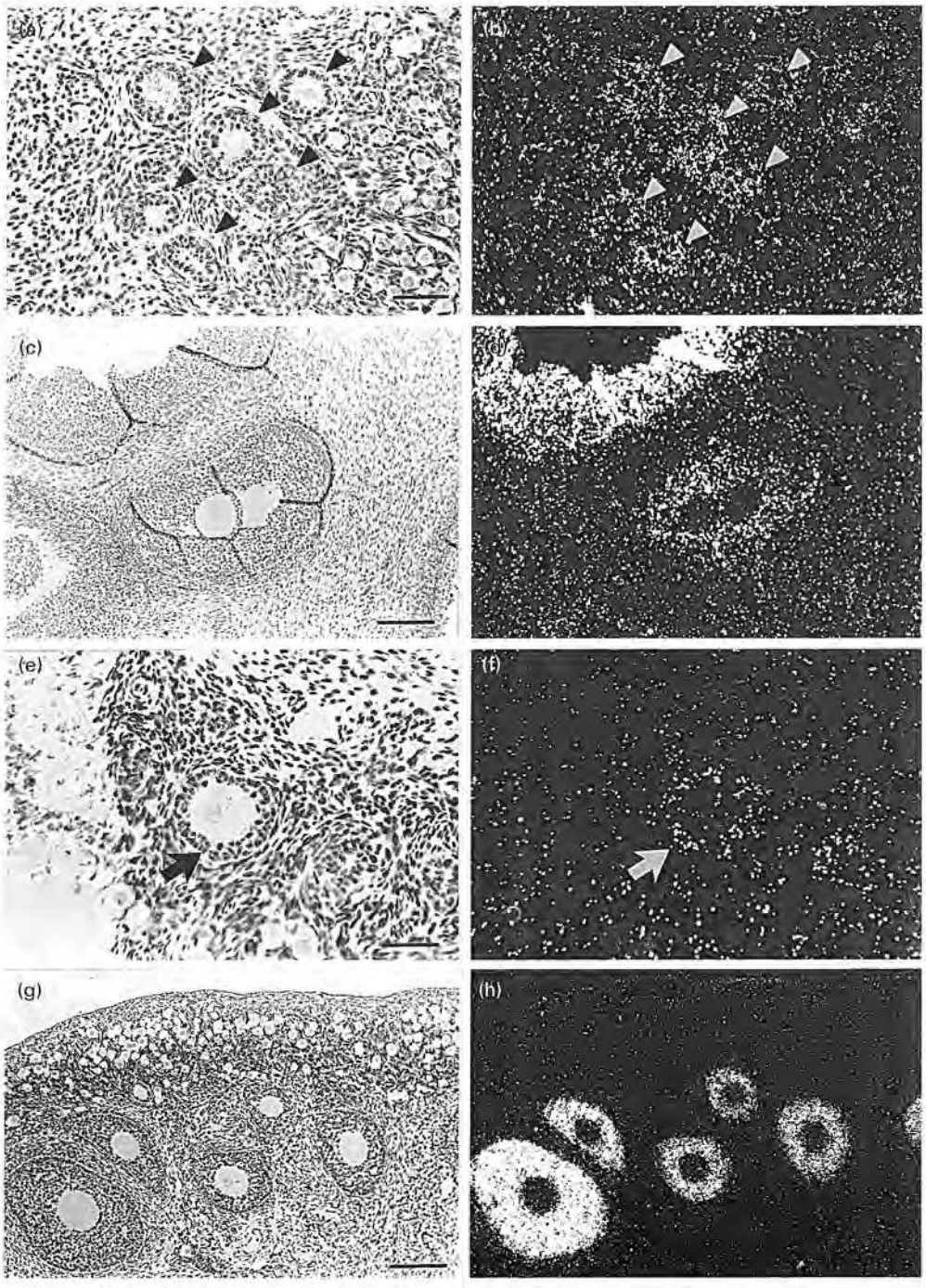

Fig. 3. Localization of inhibin and activin subunit mRNAs in small follicles of the sheep ovary: (a) light field and (b) dark field views of type 2 and 3 follicles (arrowheads) with evidence of hybridization of inhibin/activin β_B subunit antisense riboprobe to granulosal cells. Note there is no increase 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 granulosal 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 granulosal cells (Fig. 2). Autoradiographic analysis of ^{125}I-labelled FSH binding to preantral follicles is consistent with the presence of FSH-R in granulosal 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 granulosal cells or uptake of BrdU or thymidine by granulosal cells (Roy and Greenwald, 1989; Hulshof *et al.*, 1995; Wandji *et al.*, 1996a). Moreover, granulosal 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 granulosal cell proliferation and function in preantral follicles it is not an essential factor for proliferation of granulosal 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 $P450_{scc}$ and $P450_{17\text{-hydroxylase}}$ 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 granulosal 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 granulosal 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 granulosal 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 granulosal 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 granulosal 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 granulosal cells (Fig. 3). In contrast to the β_A

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riboprobe to granulosal 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 granulosal cells. For details on α-inhibin and β_A inhibin/activin *in situ* procedures and ^{35}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.

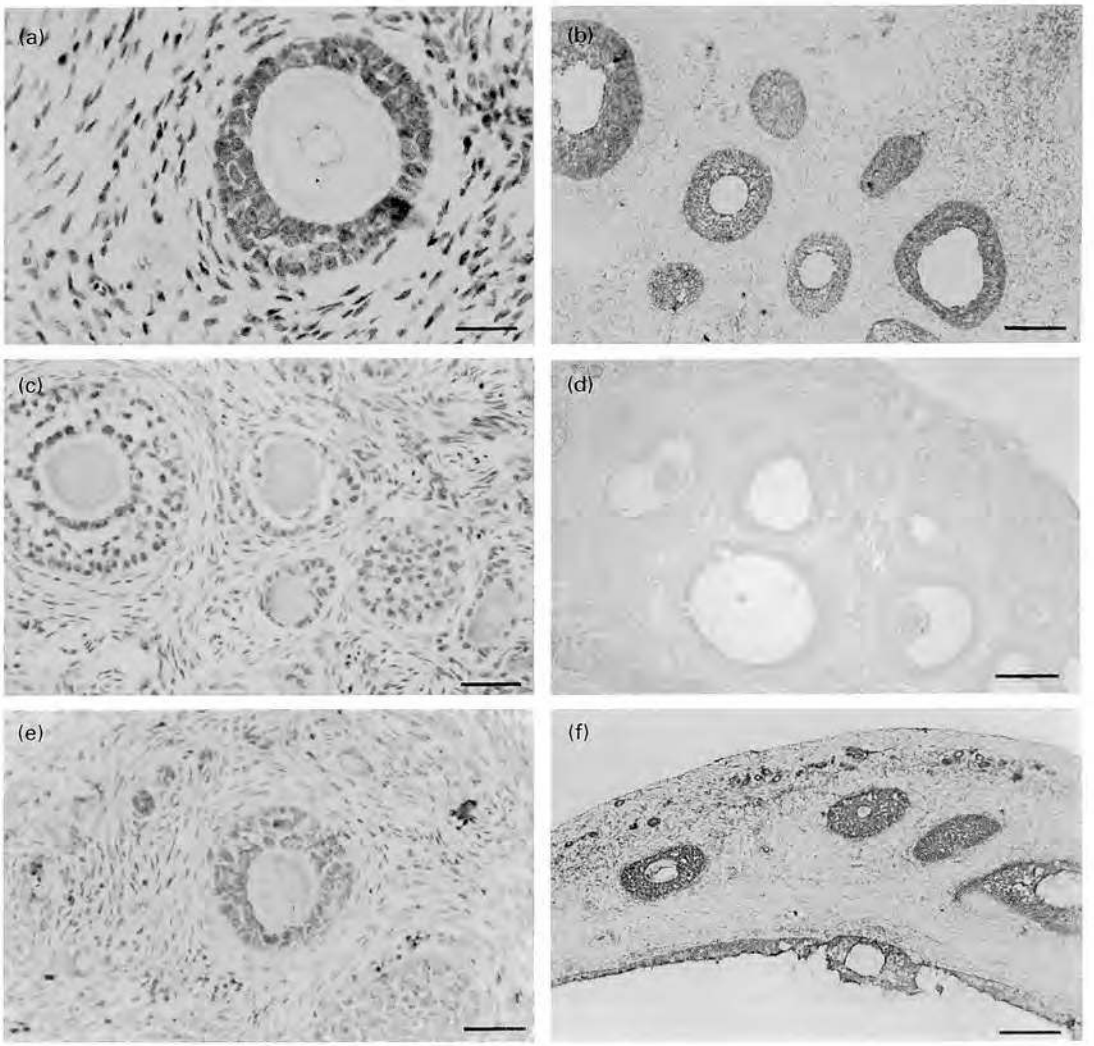

Fig. 4. Immunostaining for inhibin and/or activin subunits and follistatin in ovarian follicles of the sheep ovary: inhibin-α subunit immunostaining in type 3 follicle (a) and in type 4–5 follicles (b). Note intense immunostaining in granulosal cells and light immunostaining in some oocytes. (c,d) Inhibin/activin β_A subunit immunostaining in granulosal cells and oocytes of small (type 2–5) and both small and large antral follicles; (e,f) follistatin immunostaining in oocytes and granulosal 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 granulosal 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 granulosal cells of preantral follicles.

Inhibin α subunit mRNA was first observed in granulosal cells of developing ovine follicles with two or three layers of granulosal cells (Fig. 1). Thereafter the hybridization signal increased exclusively in granulosal 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 granulosal cells of follicles with two or three layers of granulosal 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 granulosal 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 granulosal cells. In normal animals the rate of proliferation of granulosal 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 (TGFβ) family (for example GDF-9).

In sheep, follistatin gene expression was first observed in granulosal 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 granulosal 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.

TGFβ and FGF

Transforming growth factor β is known to be produced by bovine thecal cells and to influence granulosal cell proliferation (Skinner *et al.*, 1987; Wandji *et al.*, 1996b). In the sheep ovary $TGF\beta_1$ mRNA was observed in stromal/interstitial tissues and first observed in theca interna in type 4 or 5 follicles, whereas $TGF\beta_3$ was noted mainly in smooth muscle cells around blood vessels in the theca

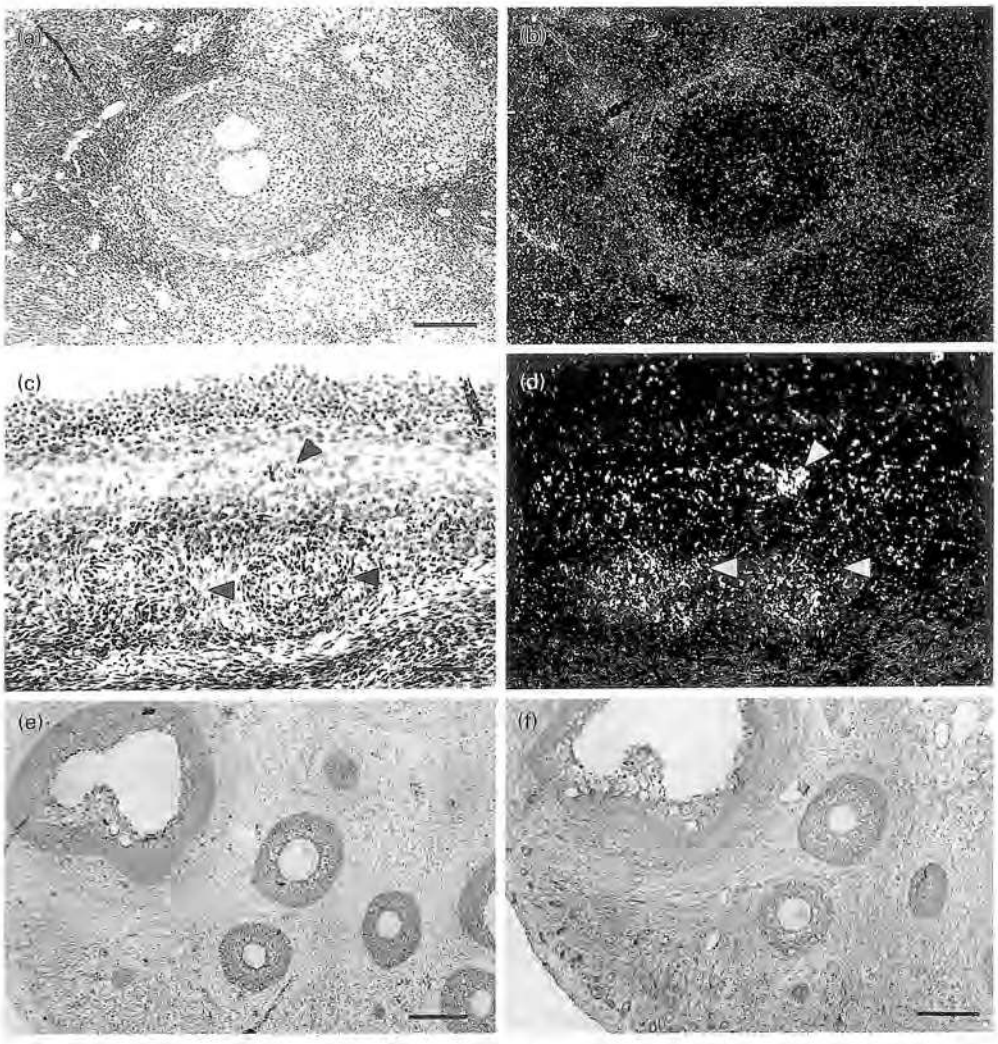

Fig. 5. Localisation of transforming growth factor β in the sheep ovary: (a) light field and (b) dark field views of a type 5 follicle with evidence of hybridization of the ^{33}P-labelled TGFβ$_1$ antisense riboprobe to theca interna and areas of the adjacent stroma/ interstitium but not granulosal cells; (c) light field and (d) dark field views of large antral follicle with evidence of hybridization of the ^{33}P-labelled TGFβ$_3$ antisense riboprobe to smooth muscle cells of blood vessels (arrowheads) and thecal interna but not granulosal cells; (e) immunostaining for TGFβ$_1$ in oocytes, granulosal cells of preantral and antral follicles and to a lesser extent in the theca interna and interstitium; (f) immunostaining for TGFβ$_{2,3}$ indicating widespread localization in the ovary. The TGFβ$_1$ riboprobe was from an RT-PCR derived 1172 bp ovine cDNA for the complete coding region. The TGFβ$_3$ riboprobe was from an RT–PCR derived 960 bp ovine cDNA. All sense probes showed no evidence of specific hybridization. The TGFβ$_1$ antibody (clone TB-21) was obtained from Anogen Inc, Mississauga, Ontario and the anti-TGFβ$_{2,3}$ (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 TGFβ$_2$ expression was similar to that of TGFβ$_1$ (D. Tisdall, unpublished). By contrast TGFβ$_1$ peptide was widespread throughout the ovary (Fig. 5): it was observed in oocytes, especially in primordial follicles and it was prominent in granulosal cells at the type 2 stage of development and to a lesser extent in the theca interna. Immunostaining with antibody that

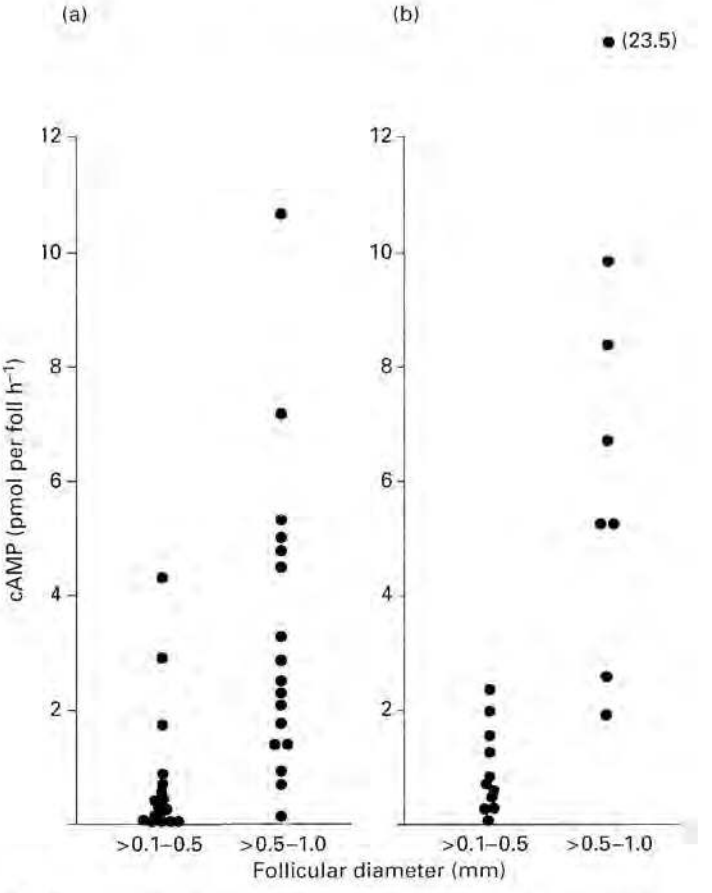

Fig. 6. The cyclic adenosine 3,5'-monophosphate (cAMP) responses of individual large preantral–early antral ovine follicles (follicle types 4 and 5) dissected from an individual (a) intact or (b) long-term (i.e. 60 days) hypophysectomized ewe. Each follicle from each ewe was incubated separately for 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 TGFβ$_2$ and TGFβ$_3$ but not TGFβ$_1$ indicated widespread distribution of these peptides throughout the ovary (see Fig. 5).

Granulosal 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, granulosal 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 granulosal 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 granulosal cells (i.e. stem cell factor, activin, FGF-2 and EGF), whereas others are likely to be inhibitory (follistatin, TGFβs) (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 granulosal 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 granulosal 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.

References

Boland NI, Humpherson PG, Leese HJ and Gosden RG (1993) Pattern of lactate production and steroidogenesis during growth and maturation of mouse ovarian follicles *in vitro*. *Biology of Reproduction* **48** 798–806

Braw-Tal R (1994) Expression of mRNA for follistatin and inhibin/activin subunits during follicular growth and atresia *Journal of Molecular Endocrinology* **13** 253–264

Braw-Tal R and Yossefi S (1997) Studies *in vivo* and *in vitro* on the initiation of follicle growth in the bovine ovary *Journal of Reproduction and Fertility* **109** 165–171

Braw-Tal R, Tisdall DJ, Hudson NL, Smith P and McNatty KP (1994) Follistatin but not α or β_A inhibin subunit mRNA is expressed in ovine fetal ovaries in late gestation *Journal of Molecular Endocrinology* **13** 1–9

Cameron VA, Nishimura E, Mathews LS, Lewis KA, Sawchenkko PE and Vale WW (1994) Hybridization histochemical localization of activin receptor subtypes in rat brain, pituitary, ovary and testis *Endocrinology* **134** 799–808

Clark DE, Tisdall DJ, Fidler AE and McNatty KP (1996) Localization of mRNA encoding c-kit during the initiation of folliculogenesis in ovine fetal ovaries *Journal of Reproduction and Fertility* **106** 329–335

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

Erickson GF, Kokka S and Rivier C (1995) Activin causes premature superovulation *Endocrinology* **136** 4804–4813

Findlay JR (1993) An update on the roles of inhibin, activin and follistatin as local regulators of folliculogenesis *Biology of Reproduction* **48** 15–23

Gospodarowicz D, Neufield G and Schweigener L (1986) Fibroblast growth factor *Molecular Cellular Endocrinology* **46** 187–204

Groome N and Lawrence M (1991) Preparation of monoclonal antibodies to the beta A subunit of ovarian inhibin using a synthetic peptide immunogen *Hybridoma* **10** 309–316

Groome N, Hancock J, Betteridge A, Lawrence M and Craven R (1990) Monoclonal and polyclonal antibodies reactive with

the 1–32 amino terminal sequence of the alpha subunit of human 32K inhibin *Hybridoma* 9 31–42

Groome NP, Illingworth PJ, O'Brien M, Pai R, Rodger FE, Mather JP and McNeilly AS (1996) Measurement of dimeric inhibin B throughout the human menstrual cycle *Journal of Clinical Endocrinology and Metabolism* **81** 1401–1405

Gundersen HJG (1988) The nucleator *Journal of Microscopy* **151** 3–21

Guo Q, Kumar T, Woodruff T, Hadswell L, De Mayo F and Matsuk M (1998) Overexpression of mouse follistatin causes reproductive defects in transgenic mice *Molecular Endocrinology* **12** 96–106

Hirshfield AN (1985) Comparison of granulosa cell proliferation in small follicles of hypophysectomized, prepubertal and mature rats *Biology of Reproduction* **32** 979–987

Hirshfield AN and De Santi AM (1995) Patterns of ovarian cell proliferation in rats during the embryonic period and the first three weeks post partum *Biology of Reproduction* **53** 1208–1221

Hsu SM, Raine L and Fanger H (1981) Use of a activin–biotin–peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabelled antibody (MAP) procedures *Journal of Histochemistry and Cytochemistry* **29** 577–580

Huang E, Manova K, Packer AI, Sanchez S, Bachvarova RF and Besmer P (1993) The murine steel panda mutation affects kit ligand expression and growth of early ovarian follicles *Developmental Biology* **157** 100–109

Hulshof SCJ, Figueiredo JR, Beckers JF, Bevers MM, van der Donk JA and van den Hurk R (1995) Effects of fetal bovine serum, FSH and 17β-estradiol on the culture of bovine preantral follicles *Theriogenology* **44** 217–226

Kumar T, Wang Y, Lu N and Matzuk M (1997) Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility *Nature Genetics* **15** 201–204

Li R, Phillips DM and Mather JP (1995) Activin promotes ovarian follicular development *in vitro*. *Endocrinology* **136** 849–856

Lundy T, Smith P, O'Connell A, Hudson NL and McNatty KP (1999) Populations of granulosa cells in small follicles of the sheep ovary *Journal of Reproduction and Fertility* **115** 251–262

McNatty KP (1978) Cyclic changes in antral fluid hormone concentrations in humans. In *Clinics in Endocrinology and Metabolism* Vol. 7 no 3 pp 577–600 Eds GT Ross and MB Lipsett. WB Saunders Co. Ltd, London

McNatty KP, Kieboom LE, McDiarmid J, Heath DA and Lun S (1986) Adenosine cyclic 3',5'-monophosphate and steroid production by small ovarian follicles from Booroola ewes with and without a fecundity gene *Journal of Reproduction and Fertility* **76** 471–480

McNatty KP, Heath DA, Hudson N and Clarke IJ (1990) Effect of long-term hypophysectomy on ovarian follicle populations and gonadotrophin-induced adenosine cyclic 3',5'-monophosphate output by follicles from Booroola ewes with or without the F gene *Journal of Reproduction and Fertility* **90** 515–522

McNatty KP, Smith P, Hudson NL, Heath DA, Lun S and O W-S (1992) Follicular development and steroidogenesis. In *Local Regulation of Ovarian Function* pp 21–38 Eds N-O Sjoberg, L Hamberger, PO Jansen, Ch Owman and HJT Coelingh-Bennink. Parthenon Publishing, Park Ridge, New Jersey

Magarelli P, Zachow R and Magoffin D (1996) Developmental and hormonal regulation of rat theca-cell differentiation factor secretion in ovarian follicles *Biology of Reproduction* **55** 416–420

Martens JW, de Winter JP, Timmerman MA, McLuskey A, van Schauk RH, Themmen AP and de Jong FH (1997) Inhibin interferes with activin signalling at the level of the activin receptor complex in Chinese hamster ovary cells *Endocrinology* **138** 2928–2936

Matzuk M, Kumar T, Shou W, Coerver K, Lau A, Behringer R and Finegold M (1996) Transgenic models to study the roles of inhibins and activins in reproduction, oncogenesis and development *Recent Progress in Hormone Research* **51** 123–137

Motro B and Bernstein A (1993) Dynamic changes in ovarian c-kit and steel expression during the estrous reproductive cycle *Developmental Dynamics* **197** 69–79

Neufield G, Ferrara N, Schweigerer L, Mitchell R and Gospodarowicz D (1987) Bovine granulosa cells produce basic fibroblast growth factor *Endocrinology* **121** 597–603

Oktay K, Schenken R and Nelson JT (1995) Proliferating cell nuclear antigen marks the initiation of follicular growth in the rat *Biology of Reproduction* **53** 295–301

Oktay K, Briggs D and Gosden RG (1997) Ontogeny of follicle-stimulating hormone receptor gene expression in isolated human follicles *Journal of Clinical Endocrinology and Metabolism* **82** 3748–3751

O'Shaughnessy PJ, McLelland D and McBride MW (1997) Regulation of luteinizing hormone-receptor and follicle-stimulating hormone-receptor messenger ribonucleic acid levels during development in the neonatal mouse ovary *Biology of Reproduction* **57** 602–608

Peters H, Byskov AG, Himelstein-Braw R and Faber M (1975) Follicle growth: the basic event in the mouse and human ovary *Journal of Reproduction and Fertility* **45** 559–566

Roberts VJ, Barth S, el-Roeiy and Yen SS (1993) Expression of inhibin/activin subunits and follistatin messenger ribonucleic acids and proteins in ovarian follicles and the corpus luteum during the human menstrual cycle *Journal of Clinical Endocrinology and Metabolism* **77** 1402–1410

Roy SK and Greenwald GS (1989) Hormonal requirements for the growth and differentiation of hamster preantral follicles in long-term culture *Journal of Reproduction and Fertility* **87** 103–114

Roy SK and Greenwald G (1990) Immunohistochemical localization of epidermal growth factor-like activity in the hamster ovary with a polyclonal antibody *Endocrinology* **126** 1309–1317

Singh B, Rutledge JM and Armstrong DT (1995) Epidermal growth factor and its receptor gene expression and peptide localisation in porcine ovarian follicles *Molecular Reproduction and Development* **40** 391–399

Skinner MK, Keshi-Oja J, Osteen K and Moses HL (1987) Ovarian theca cells produce transforming growth factor-beta which can regulate granulosa cell growth and differentiation *Endocrinology* **121** 786–792

Smith P, O W-S, Corrigan KA, Smith T, Lundy T, Davis GH and McNatty KP (1997) Ovarian morphology and endocrine characteristics of female sheep fetuses that are heterozygous or homozygous for the Inverdale prolificacy gene ($FecX^I$) *Biology of Reproduction* **57** 1183–1192

Sokka TA, Hamalainen TM, Kaipa A, Warren DW and Huhtaniemi IT (1996) Development of luteinizing hormone action in the perinatal rat ovary *Biology of Reproduction* **55** 663–670

Tisdall DJ, Hudson N, Smith P and McNatty KP (1994) Localization of ovine follistatin and α and β_A inhibin mRNA in the sheep ovary during the oestrous cycle *Journal of Molecular Endocrinology* **12** 181–193

Tisdall DJ, Watanabe K, Hudson NL, Smith P and McNatty KP (1995) FSH receptor gene expression during ovarian follicle development in sheep *Journal of Molecular Endocrinology* **15** 273–281

Tisdall DJ, Quirke LD, Smith P and McNatty KP (1997) Expression of the ovine stem cell factor during folliculogenesis in late fetal and adult ovaries *Journal of Molecular Endocrinology* **18** 127–135

Torney AH, Hodgson YM, Forage R and de Kretser DM (1989) Cellular localization of inhibin mRNA in the bovine ovary by *in situ* hybridization *Journal of Reproduction and Fertility* **86** 391–399

van Wezel IL and Rodgers RJ (1996) Morphological characterization of bovine primordial follicles and their environment *in vivo*. *Biology of Reproduction* **55** 1003–1011

van Wezel IL, Umapathysivam K, Tilley WD and Rodgers RJ (1995) Immunohistochemical localization of basic fibroblast growth factor in bovine ovarian follicles *Molecular and Cellular Endocrinology* **115** 133–140

Wandji S-A, Pelletier G and Sirard M-A (1992a) Ontogeny and cellular localization of ^{125}I-labelled insulin-like growth factor-I, ^{125}I-labelled follicle-stimulating hormone and ^{125}I-labelled human chorionic gonadotropin binding sites in ovaries from bovine fetuses and neonatal calves *Biology of Reproduction* **47** 814–822

Wandji S-A, Pelletier G and Sirad M-A (1992b) Ontogeny and cellular localization of ^{125}I-labelled basic fibroblast growth factor and ^{125}I-labelled epidermal growth factor binding sites in ovaries from bovine fetuses and neonatal calves *Biology of Reproduction* **47** 807–813

Wandji S-A, Sosen V, Voss AK, Eppig JJ and Fortune JE (1996a) Initiation *in vitro* of growth of bovine primordial follicles *Biology of Reproduction* **55** 942–948

Wandji S-A, Eppig JJ and Fortune JE (1996b) FSH and growth factors affect the growth and endocrine functions *in vitro* of granulosa cells of bovine preantral follicles *Theriogenology* **45** 817–832

Wang X-N and Greenwald GS (1993) Hypophysectomy of the cyclic mouse. 1. Effects on folliculogenesis, oocyte growth and follicle-stimulating hormone and human chorionic gonadotrophin receptors *Biology of Reproduction* **48** 585–594

Woodruff TK, Lyan RJ, Hansen SE, Rice GC and Mather JP (1990) Inhibin and activin locally regulate rat ovarian folliculogenesis *Endocrinology* **127** 3196–3205

Xu ZZ, Garverick HA, Smith GW, Smith MF, Hamilton SA and Young-Quist RS (1995) Expression of follicle-stimulating hormone and luteinizing hormone receptor messenger ribonucleic acids in bovine follicles during the first follicular wave *Biology of Reproduction* **53** 951–957

Yuan W, Lucy MC and Smith MF (1996) Messenger ribonucleic acid for insulin-like growth factors-1 and -11, insulin-like growth factor binding protein-2, gonadotropin receptors, and steroidogenic enzymes in porcine follicles *Biology of Reproduction* **55** 1045–1054

Yoshida H, Takakura N, Kataoka H, Kunisada T, Okamura H and Nishikawa S-I (1997) Stepwise requirement of c-kit tyrosine kinase in mouse ovarian follicle development *Developmental Biology* **184** 122–137