Gene expression and protein localisation for activin-A, follistatin and activin receptors in goat ovaries

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Abstract

We studied the protein and mRNA expression of activin-A, follistatin and activin receptors in goat ovaries to find evidence of their possible role in ovarian activity, particularly in the various stages of follicle development. Ovaries of cyclic goats were collected and then either fixed in paraformaldehyde for immunohistochemical localisation of activin-A, follistatin, activin receptors IIA/B (ActR-IIA/B) and IA (ActR-IA) proteins or used to obtain samples to demonstrate mRNA expression of activin-A (β A subunit), follistatin, ActR-IIA, -IIB, -IA and -IB, using RT-PCR. For this latter goal, primordial, primary and secondary follicles were isolated mechanically, washed to remove the stromal cells and then used for RT-PCR. In addition, oocytes, cumulus, mural granulosa and theca cells from small (<3 mm) and large (3–6 mm) antral follicles, luteal cells and surface epithelium were collected to study mRNA expression. Activin-A and follistatin proteins were found in oocytes of all follicle classes, granulosa cells from the primary follicle stage

onwards, theca cells of antral follicles, corpora lutea and ovarian surface epithelium. In antral follicles, these proteins were detected both in cumulus and mural granulosa cells. ActR-IIA/B protein was found at the same follicular sites, and also in granulosa cells of primordial follicles onward. The localisation of ActR-IA corresponded with that of ActR-IIA/B, but the former protein was absent in the theca of large antral follicles. The mRNAs for activin-A (β A subunit), follistatin, and ActR-IIA, -IIB, -IA and -IB were detected at all follicular and cellular types studied, except that ActR-IIB was not found in follicles that had not developed an antrum yet. In conclusion, in goat ovaries, transcripts of activin-A (β A subunit), its receptors and its binding protein follistatin are expressed and their proteins formed at all follicular stages and in corpora lutea. These findings indicate a role of activin-A in the local regulatory system during the entire follicular development and during luteal activity.

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Introduction

Mammalian folliculogenesis involves the developmental progression from a primordial follicle, containing a single layer of granulosa cells around the oocyte, to a large preovulatory follicle consisting of multiple layers of mural granulosa cells enclosing a cumulus–oocyte complex. During this process, the oocyte and granulosa cells grow and differentiate, while theca cells are recruited from stromal tissue. After ovulation, granulosa and theca cells differentiate into luteal cells. This entire process is regulated and coordinated by endocrine hormones such as the gonadotropins, and by local growth factors in an autocrine or paracrine manner (Richards *et al.* 2002).

Activin was originally identified as a peptide growth factor from the ovarian follicular fluid that stimulates FSH secretion in cultured anterior pituitary cells (Vale

et al. 1986). It is a homodimer or heterodimer of two similar but distinct β subunits (β A and β B). The dimerisation of activin β subunits gives rise to three forms of activin, that is, activin A (β A β A), activin B (β B β B) and activin AB (β A β B). Both β A and β B subunits can also combine with the α subunit and form inhibin-A (α β A) and -B (α β B) respectively. In the ovary, activin-A is the most studied type (Tisdall *et al.* 1994, Yokota *et al.* 1997, Zhao *et al.* 2001, Thomas *et al.* 2003) and plays an important role in ovarian follicle development (Ohshima *et al.* 2002, Lovell *et al.* 2003), but activin-B has also been described (Bristol & Woodruff 2004). The activities of activin are modulated by its binding protein follistatin, which was originally purified from mammalian follicular fluid as a FSH inhibitor (Phillips & de Kretser 1998). Follistatin binds activin with high affinity, and its binding effectively neutralises the bioactivities of activin in a

variety of target tissues (Knight & Glister 2001, Fisher *et al.* 2003).

Like most of the TGF β superfamily members, activin signals through two types of closely related receptors designated type I and type II, each represented by two isoforms, that is, activin receptor types IA (ActR-IA), IB (ActR-IB), IIA (ActR-IIA) and IIB (ActR-IIB). Activin first binds to a type II receptor, which in turn recruits and activates a type I receptor by phosphorylation. The activated complex of activin and its receptors then stimulates the downstream intracellular signalling molecules that are translocated to the nucleus to regulate target gene transcription (Pangas & Woodruff 2000). ActR-IB is the predominant type I receptor for activin, while ActR-IA might have specificity for either activin or bone morphogenetic protein signals (Massagué & Chen 2000). *In vitro* studies have demonstrated that activin-A stimulates pre-antral follicle development in bovine (Hulshof *et al.* 1997) and rodent isolated follicles (Liu *et al.* 1998, Smits *et al.* 1998, Zhao *et al.* 2001), increases FSH receptor and FSH-induced luteinising hormone (LH) receptor production in rat granulosa cells (Minegishi *et al.* 1999, Tsuchiya *et al.* 1999) and suppresses androgen production in human theca cells (Hillier *et al.* 1991). Activin also regulates ovarian steroidogenesis in primates (Alak *et al.* 1998) and oocyte maturation and developmental competence in cattle (Silva & Knight 1998). In contrast, there are also reports that activin-A has no effect on bovine primary follicle development (Fortune 2003) and oocyte maturation in the rat (Tsafiri *et al.* 1989) and pig (Coskun & Lin 1994).

Expression of protein and mRNA for activin-A and activin receptors in the ovary has been localised in both oocyte and granulosa cells of follicles at various developmental stages (rodents: Wu *et al.* 1994, Zhao *et al.* 2001; primates: Roberts *et al.* 1993, Sidis *et al.* 1998; pigs: van den Hurk & van de Pavert 2001; and ruminants: Tisdall *et al.* 1994, Hulshof *et al.* 1997, Izadyar *et al.* 1998). In addition, activin-A and activin receptors have been detected in human theca cells (Pangas *et al.* 2002) and porcine corpora lutea (van den Hurk & van de Pavert 2001). Interestingly, follistatin has also been localised in granulosa cells of rat (Nakatani *et al.* 1991), sheep (Tisdall *et al.* 1994) and man (Roberts *et al.* 1993), and in oocytes, granulosa cells and corpora lutea of cattle (Izadyar *et al.* 1998, Singh & Adams 1998). Although there is convincing evidence that activin signalling is important for ovarian function, information on its localisation and function is mainly obtained from rodents and cows. Furthermore, most of these studies emphasise only antral follicles, and some of them show conflicting results. To improve our knowledge about the factors that control ovarian follicle development in mammals and to explore possible physiological differences, it is very important to conduct studies with other species, such as goats. Goats are present on all continents and are commercially seen as highly attractive

livestock, since they constitute an important source of products such as meat, milk, fibre and skin.

The present study was carried out to examine the possible existence of an intrafollicular activin-follistatin-activin receptor system and its distribution in ovaries of cyclic goats to find evidence of a possible role of this system in ovarian activity, folliculogenesis especially. Therefore, mRNA expression of activin-A (β A subunit), its type I and type II receptors and its binding protein follistatin was investigated by reverse-transcription polymerase chain reaction (RT-PCR), while the presence and cellular localisation of the corresponding proteins were analysed by immunohistochemistry.

Materials and Methods

Ovaries

During the breeding season, ovaries ($n=56$) were recovered from slaughtered cyclic adult mixed-breed goats and transported to the laboratory in a thermos flask within 1 h. Sixteen of those ovaries were fixed overnight at room temperature in 4% (w/v) paraformaldehyde in PBS (pH 7.4), and subsequently dehydrated and embedded in paraffin wax (Histoplast; Shandon Scientific Ltd, Pittsburgh, PA, USA) in preparation for immunohistochemical studies. The remaining 40 ovaries were used to recover cells and tissues for RT-PCR.

Immunohistochemistry

Localisation of activin-A, follistatin and ActR-IIA/B and -IA was performed on serial 5- μ m sections cut from ovaries of eight different goats. These sections were mounted on poly-L-lysine-coated slides, dried overnight at 37 °C, deparaffinised in xylene and rehydrated in a graded ethanol series. Endogenous peroxidase was blocked by incubating the deparaffinised sections in 3% hydrogen peroxide in methanol for 10 min. The sections were then washed with PBS, and the epitopes were activated by microwaving the sections for 7 min at 900 W in 0.01 M citrate buffer (pH 6.0). After microwave treatment, the sections were washed in PBS/0.05% Tween (PBS-T; Merck, Darmstadt, Germany) before being incubated for 30 min with 5% normal goat serum in PBS to minimise non-specific binding. The primary antibodies were as follows: 1) rabbit antiactivin-A (Innogenetics, Ghent, Belgium); 2) mouse antifollistatin (R&D System Europe, Abingdon, UK); 3) rabbit anti-ActR-IIA that cross-reacts with ActR-IIB (Celgen, Leuven, Belgium) and mouse anti-ActR-IA (R&D System Europe). All antibodies were diluted 1:50. Unfortunately, the available anti-ActR-IB is not suitable for use in goat tissue. The sections were incubated overnight at 4 °C in appropriate dilutions of the antibodies. All other incubations and washes were

performed at room temperature. After incubation with an antibody, sections were washed three times with PBS-T and incubated for 45 min with biotinylated secondary antibody (goat antirabbit or goat antimouse IgG from Vector Laboratories, Burlingame, CA, USA), diluted 1:200 in PBS containing 5% normal goat serum. Next, the sections were washed three times in PBS-T before being incubated for 45 min with an avidin-biotin complex (1:600) (Vectastain Elite ABC kits; Vector Laboratories). The sections were then washed three times in PBS and stained with diaminobenzidine (DAB) (0.05% DAB in Tris/HCl, pH 7.6, and 0.03% H₂O₂ – Sigma tablets) until a precipitate formed or for a maximum of 20 min. The stained sections were rinsed in PBS and water, and counterstained for 10 s in Mayer's haematoxylin. Finally, the sections were washed for 10 min in running tap water, and subsequently dehydrated in a graded ethanol series followed by xylene treatment and mounting in Pertex (Cellpath Ltd, Hemel Hempstead, UK). The staining intensity was scored as follows: absent (–), weak (+), moderate (++), or strong (+++). Sections were analysed by two independent researchers. Controls for non-specific staining were performed by (1) replacing the primary antibody with IgGs from the same species in which the specific antibody was raised, at the same concentration, and (2) incubation with diaminobenzidine reagent alone to exclude the possibility of non-suppressed endogenous peroxidase activity.

Classification and measurement of follicles

Ovarian follicles were classified as 1) primordial (one layer of flattened/cuboidal granulosa cells), 2) primary (a single layer of cuboidal granulosa cells), 3) secondary (two or more layers of cuboidal granulosa cells), 4) small antral follicles (<3 mm in diameter, with multiple granulosa cells enclosing an antrum), and 5) large antral follicles (3–6 mm). The diameter of follicles was calculated by the method described by Van den Hurk *et al.* (1994).

Collection of cells and tissues for RT-PCR

The ovaries were rinsed in saline (0.9% NaCl) containing antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin), after which 10 of them were used for isolation of primordial, primary and secondary follicles. The remaining ovaries were used for collection of oocytes, cumulus cells, mural granulosa cells and thecal cells from small and large antral follicles; and samples of corpora lutea and ovarian surface epithelium.

Early-stage follicles, that is, primordial, primary and secondary, were isolated by the mechanical procedure described previously (Lucci *et al.* 1999). After isolation, these follicles were washed several times to remove the stromal cells completely, and then placed by category into separate Eppendorf tubes in groups of 15. This procedure

was completed within 2 h, and all samples were stored at –80 °C until the RNA was extracted. In previous work from our group, histological analysis was performed to confirm goat preantral follicle classification after isolation (Lucci *et al.* 1999).

From a second group of ovaries ($n=20$), cumulus-oocyte complexes (COCs) were aspirated from small (1–3 mm) and large (3–6 mm) antral follicles with an 18-gauge needle attached to a tube in line with a vacuum pump. From the follicle content so collected, compact COCs were selected as described by Van Tol & Bevers (1998). Thereafter, the cumulus was separated from the oocyte by a combination of vortexing and aspiration via a narrow-bore Pasteur pipette. Denuded oocytes, and cumulus and mural granulosa cells were separated, washed four times in PBS, packed in tubes in groups of either 10 denuded oocytes, cumulus cells from 10 COCs or samples of mural granulosa, and stored at –80 °C until RNA extraction.

To collect theca cells, small ($n=10$) and large antral follicles ($n=10$) were isolated from ovaries ($n=5$) and dissected free of stromal tissue with forceps, as described previously (Van Tol & Bevers 1998). The follicles were then bisected, and the granulosa cells were scraped off with a scalpel. Next, the theca cell layers were vortexed for 1 min in 1 ml HEPES-buffered M199 (Gibco BRL, Paisley, UK) supplemented with penicillin/streptomycin, transferred to a fresh 1 ml of buffer, vortexed again for 1 min, washed twice in 2 ml HEPES-buffered M199, collected and stored at –80 °C. From another group of ovaries ($n=5$), small pieces of corpus luteum and surface epithelium were collected and stored at –80 °C until RNA extraction. Three samples of each tissue sample were analysed.

Extraction of total RNA and reverse transcription

Isolation of total RNA combined with on-column DNase digestion was performed with the RNeasy mini kit and the RNase-free DNase set (Qiagen). According to the manufacturer's instructions, 350 µl lysis buffer were added to each frozen sample, and the lysate was aspirated through a 20-gauge needle before being centrifuged at 10 000 *g* for 3 min at room temperature. The lysates of theca cells, corpus luteum and ovarian surface samples were then subjected to proteinase K treatment (6.7 mAU/ml, Qiagen) at 55 °C for 10 min. Thereafter, all lysates were diluted 1:1 with 70% ethanol and introduced to a mini-column. After binding of the RNA to the column, DNA digestion was performed with RNase-free DNase (340 Kunitz units/ml) for 15 min at room temperature. After washing of the column three times, the RNA was eluted with 30 µl RNase-free water.

Prior to the reverse-transcription reaction, the eluted RNA samples were incubated for 5 min at 70 °C, and chilled on ice. Reverse transcription was then performed

Table 1 Oligonucleotide primers used for PCR analysis of goat cells and tissues

	Primer sequence (5' → 3')	Sense	Position	Genbank accession number
Target gene				
Activin-A	ACCTCGGAGATCATCACGTT	s	357–376	GI: 563747 (2002)
	CAGTCATTCCAGCCAATGTC	as (2)	990–1009	<i>Bos taurus</i> Act-βA
Follistatin	CACGACTTGAGGTTGGCGAA	as (1)	1146–1165	
	TGTGAGAACGTGGACTGTGG	s	289–308	GI: 404023 (1994)
	ACAGGCTCCTCAGACTTACT	as	835–854	<i>Bos taurus</i> follistatin
ActR-IIA	AACGAGGCACCAGTGTGAT	s	760–769	GI: 393113 (1995)
	CTGCATGTCTTCAAGAGAGG	as	1277–1296	<i>Bos taurus</i> ActR-IIA
ActR-IIB	CAACTTCCAGAGAGACGCCT	s	1280–1299	GI: 31341841 (2003)
	CTTCTTGTGCCACCACT	as (2)	1444–1463	<i>Bos taurus</i> ActR-IIB
	ACACTCGTCTCTCCACACAG	as (1)	1574–1593	
ActR-IA	AGATGAGAAGCCCAAGGTAAA	s	193–213	GI: 31341405 (1996)
	GTGCCATATTCACATCTCTGG	as	603–624	<i>Bos taurus</i> ActR-IA
ActR-IB	AAGATGCAGTCACTGACACC	s	14–33	GI: 28193915 (2003)
	GCCTCATAACTCTGCCACCA	as	310–329	<i>Ovis aries</i> ActR-IB
GAPDH	AGGCCATCACCATCTTCCAG	s	179–198	GI: 2285902 (1997)
	GGCGTTGGACAGTGGTCATAA	as	485–504	<i>Bos taurus</i> GAPDH

s: sense; as: anti-sense, anti-sense primer used in the first (1) and second round (2).

in a total volume of 20 µl made up of 10 µl sample RNA, 4 µl 5X reverse transcriptase buffer (Gibco BRL, Breda, The Netherlands), 8 units RNAsin, 150 units Superscript II reverse transcriptase (Gibco BRL), 0.036 U random primers (Life Technologies BV, Leiden, The Netherlands) and 10 mM DTT and 0.5 mM of each dNTP. The mixture was incubated for 1 h at 42 °C and for 5 min at 80 °C, and then stored at – 20 °C. Minus RT blanks were prepared under the same conditions, but without inclusion of reverse transcriptase.

Amplification of cDNA

PCR reactions were carried out in 200 µl tubes (Biozym, Landgraaf, The Netherlands), using 1 µl cDNA as template in 25 µl of a mixture of 2 mM MgCl₂, 200 µM of each dNTP and 0.5 µM each of primers, and 0.625 units Taq DNA polymerase (HotStarTaq; Qiagen). The primers used for amplification of the inhibin/activin βA subunit, follistatin, Act-RIIA, -RIIB, -RIA, and -RIB, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), are presented in Table 1.

The thermal cycling profile for the first round of PCR was as follows: initial denaturation and activation of the polymerase for 15 min at 94 °C, followed by 40 cycles of 15 s at 94 °C, 30 s at 55 °C and 45 s at 72 °C. Final extension was for 10 min at 72 °C. During the amplification of inhibin/activin βA subunit and ActR-IIB cDNA, hemi-nesting was used to increase the specificity and sensitivity. For hemi-nesting, 1 µl first-round product was transferred to another 200 µl tube with 24 µl PCR amplification mixture containing the second antisense primer, and amplified for 25 cycles with the same thermal cycling profile. All reactions were performed in a 24-well thermocycler (Perkin-Elmer, Gouda, The Netherlands).

Finally, 10 µl product was resolved by electrophoresis in 1% agarose gels containing ethidium bromide. A 100 base pair (bp) DNA ladder (Gibco BRL) was included as a reference for fragment size, and images of each gel were recorded with a digital camera (Olympus C-4040, New York, NY, USA).

A standard sequencing procedure (ABI PRISM 310 Genetic analyzer, Applied Biosystems) was used to verify the specificity of the PCR products.

Results

Protein localisation for activin-A, follistatin and activin receptors

Activin-A and follistatin proteins were detected in oocytes of primordial follicle stage onward (Fig. 1A and J), and in granulosa cells of primary (Fig. 1B and K) and secondary follicles (Fig. 1C and L). Occasionally, activin-A was found in granulosa cells of primordial follicles (Fig. 1A), but no reaction to either activin-A or follistatin was found in theca cells from secondary follicles (Fig. 1C and L). ActR-IIA/B and ActR-IA proteins were found in oocyte and granulosa cells of primordial (Fig. 2A and J), primary (Fig. 2B and K) and secondary follicles (Fig. 2C and L), but not in theca cells of these follicles.

In antral stages, all follicular compartments, that is, the oocyte, cumulus cells, mural granulosa cells and thecal cells, generally had a moderate to strong reaction to both activin-A (Fig. 1D–F) and follistatin (Fig. 1M–O), except for small antral follicles that had a weak reaction to activin-A (Fig. 1D, Table 2). Additionally, these same sites had a positive reaction to both ActR-IIA/B (Fig. 2D–F) and ActR-IA (Fig. 2M–O), except the theca cells of large antral follicles, which did not react to ActR-IA (Fig. 2O).

Activin-A

Follistatin

Figure 1 Activin-A and follistatin immunoreactivity in the different structures found within goat ovaries. (A and J) Primordial follicle, (B and K) primary follicle, (C and L) secondary follicle, (D and M) small antral follicle, (E and N) COC of a large antral follicle, (F and O) mural granulosa and theca cells from a large antral follicle, (G and P) corpus luteum, (H and Q) ovarian surface epithelium and (I and R) negative control reaction. O: oocyte; G: granulosa cells; MGC: mural granulosa cells; CC: cumulus cells; T: theca cells; CL: corpus luteum; S: ovarian surface epithelium. Scale bars represent 25 μm.

ActR-IIA/B **ActR-IA**

Figure 2 ActR-IIA/B, and ActR-IA immunoreactivity in the different structures found within goat ovaries. (A and J) Primordial follicle, (B and K) primary follicle, (C and L) secondary follicle, (D and M) small antral follicle, (E and N) COC of a large antral follicle, (F and O) mural granulosa and theca cells from a large antral follicle, (G and P) corpus luteum, (H and Q) ovarian surface epithelium and (I and R) negative control reaction. O: oocyte; G: granulosa cells; MGC: mural granulosa cells; CC: cumulus cells; T: theca cells; CL: corpus luteum; S: ovarian surface epithelium. Scale bars represent 25 µm.

Table 2 Relative intensity of immunohistochemical staining for activin-A, follistatin, ActR-IIA/B and ActR-IA, and localisation of mRNA for activin-A, follistatin, ActR-IIA, ActR-IIB, ActR-IA and ActR-IB in the ovaries of goats

	Activin-A		Follistatin		ActR-IIA/B	ActR-IIA	ActR-IIB	ActR-IA	ActR-IA	ActR-IB
	Protein	mRNA	Protein	mRNA	Protein	mRNA	mRNA	Protein	mRNA	mRNA
Structure										
Primordial follicle										
Oocyte	++	+	+	+	++	+	—*	++	+	+
Granulosa	+/-	+	—	+	++	+	—	+	+	+
Primary follicle										
Oocyte	+	+	+	+	++	+	—*	+	+	+
Granulosa	++	+	+/-	+	++	+	—	+	+	+
Secondary follicle										
Oocyte	++	+	+	+	++	+	—*	+	+	+
Granulosa	++	+	+	+	++	+	—*	+	+	+
Theca	—	—	—	—	—	—	—	—	—	—
Antral follicle <3 mm										
Oocyte	+	+	+++	+	++	+	+	++	+	+
Cumulus	+	+	+++	+	++	+	+	+	+	+
Mural granulosa	+	+	+++	+	++	+	+	+	+	+
Theca	+	+	+	+	+/-	+	+	+	+	+
Antral follicle <3–6 mm										
Oocyte	++	+	++	+	+++	+	+	++	+	+
Cumulus	++	+	+	+	+++	+	+	++	+	+
Mural granulosa	+++	+	+++	+	+++	+	+	++	+	+
Theca	++	+	++	+	+	+	+	—	+	+
Corpus luteum	+++	+	++	+	++	+	+	+++	+	+
Ovarian surface	+++	+	++	+	++	+	+	+++	+	+

*Whole follicles.
(-/+) occasionally found; (—) absent; (+) weak; (++) moderate; (+++) strong immunoreaction.

Apart from follicles, immunoreactions to activin-A, follistatin, ActR-IIA/B and ActR-IA were observed in corpora lutea (Fig. 1G and P) and ovarian surface epithelium (Fig. 1H and Q). The relative intensity of immunohistochemical staining for activin-A, follistatin, ActR-IIA/B and ActR-IA in all cell types studied is illustrated in Table 2. For all antibodies tested, control reactions (Figs 1I and R and 2I and R) confirmed the absence of non-specific staining.

mRNA expression for activin β A subunit, follistatin and activin receptors

Amplification of cDNA from primordial, primary and secondary follicles resulted in specific products for inhibin/activin β A subunit, follistatin, ActR-IIA, -IA and -IB (Fig. 3). Transcripts for both the β A subunit of activin (653 bp) and follistatin (566 bp), and all activin receptors (IIA (527 bp), IIB (184 bp), IA (431 bp) and IB (315 bp)) were also detected in cDNA from oocytes, cumulus, mural granulosa and theca cells collected from small and large antral follicles as well as from corpus luteum and ovarian surface epithelium (Fig. 3). The expression of the house-keeping gene (GAPDH) is also illustrated in Fig. 3. Sequence analysis of the amplified β A subunit of activin, follistatin, and ActR-IIA, -IIB, -IA and -IB products confirmed their specificity. Amplification of -RT (with-

out reverse transcriptase) or water blanks (cDNA replaced by water) controls yielded no specific products in any of the reactions.

Discussion

The current study has demonstrated the presence of protein for both the inhibin/activin β A subunit and follistatin in primordial, primary and secondary goat follicles. Both proteins are synthesised in these follicles, since the present RT-PCR studies additionally showed their mRNA expression. Activin-A protein was previously detected in oocyte and granulosa cells from early follicles in sheep (McNatty *et al.* 1999), cow (Hulshof *et al.* 1997), man (Yamoto *et al.* 1992), pig (van den Hurk & van de Pavert 2001), rat (Zhao *et al.* 2001) and cat (Bristol & Woodruff 2004). However, Pangas *et al.* (2002) could not detect activin-A protein in mouse early follicles. For this follicular category, activin-A mRNA was demonstrated in pig by *in situ* hybridisation (van den Hurk & van de Pavert 2001), but not in sheep (Braw-Tal 1994, Tisdall *et al.* 1994). The follistatin protein was previously demonstrated in sheep oocytes of primordial follicles and in oocytes and granulosa cells of primary and secondary follicles (McNatty *et al.* 1999), but its mRNA was detected only in granulosa cells of secondary follicles (Braw-Tal 1994, Tisdall *et al.*

Figure 3 Expression of activin-A, follistatin, ActR-IIA, ActR-IIB, ActR-IA and ActR-IB mRNA in different follicle and cell types in goat ovaries. Follicle and cell types are indicated at the top. One hundred base pair ladders are included as markers for fragment size. Tests to ensure sample purity were performed in previous research (J R V Silva, R van den Hurk, H T A van Tol, B A J Roelen & J R Figueiredo, unpublished observations).

1994). Because follistatin binds and neutralises activin-A (de Winter *et al.* 1996, Sidis *et al.* 2001, 2002, Fisher *et al.* 2003), co-expression of both compounds in goat primordial, primary and secondary follicles suggests that follistatin may control the biological action of activin-A during early follicle growth. To support possible autocrine and/or paracrine actions of activin-A in such goat follicles, we demonstrated the proteins for ActR-IIA/B and ActR-IA, as well as the mRNA for ActR-IIA, -IA and -IB. The only exception was ActR-IIB, since no cDNA amplification from primordial, primary and secondary follicles was detected. Previous studies have shown ActR-IIA/B protein in early follicles from cow (Hulshof *et al.* 1997), while those of cat and rat have ActR-IIA, -IIB, -IA and -IB (Drummond *et al.* 2002, Bristol & Woodruff 2004). In contrast, ActR-IIA protein was not found in mouse early follicles (Pangas *et al.* 2002). In pig, both protein and mRNA for ActR-IIA were detected in these follicle types (van den Hurk & van de Pavert 2001). *In vitro* studies have shown that activin-A stimulates early

follicle development in cows (Hulshof *et al.* 1997), sheep (Thomas *et al.* 2003) and rodents (Liu *et al.* 1998, Smitz *et al.* 1998, Zhao *et al.* 2001).

In caprine antral follicles, both activin-A and follistatin proteins were present in the oocyte, cumulus cells, mural granulosa cells and theca cells. These compounds apparently are formed in these cells, since their mRNA was also detected at the same locations. Transcription of βA subunit can also form inhibin-A, but this will occur only when there is an excess of βA subunit production (Findlay 1993, Knight & Glistler 2001). The role of inhibin on folliculogenesis has been recently reviewed by Knight & Glistler 2001. Activin-A protein has been detected in oocytes and granulosa cells in rat (Ogawa *et al.* 1991), human (Yamoto *et al.* 1992, Roberts *et al.* 1993, Wada *et al.* 1996), bovine (Izadyar *et al.* 1998, Silva *et al.* 2003) and porcine (van den Hurk & van de Pavert 2001) antral follicles. In man (Roberts *et al.* 1993) and pig (van den Hurk & van de Pavert 2001), theca cells of antral follicles also immunoreacted to the activin-A protein. Thus far, expression of

activin-A mRNA in antral follicles has appeared to be restricted to the granulosa cells in rat (Meunier *et al.* 1988), primate (Roberts *et al.* 1993), sheep (Braw-Tal 1994, Tisdall *et al.* 1994) and pig (van den Hurk & van de Pavert 2001), while in cows it was also detected in oocytes (Izadyar *et al.* 1998). Follistatin protein and mRNA have been demonstrated in oocyte and granulosa cells from bovine antral follicles (Izadyar *et al.* 1998, Silva *et al.* 2003). Expression of follistatin mRNA in antral follicles has also been reported in rat (Nakatani *et al.* 1991), man (Roberts *et al.* 1993, Sidis *et al.* 1998) and sheep (Braw-Tal 1994, Tisdall *et al.* 1994), and it appeared to be confined to granulosa cells.

We furthermore demonstrated ActR-IIA/B and ActR-IA proteins and the mRNAs for ActR-IIA, -IIB, -IA and -IB in oocytes, cumulus cells, mural granulosa cells and theca cells in the goat. Previously, our group reported the expression of ActR-IIA mRNA and protein in oocytes and granulosa cells of pig (van den Hurk & van de Pavert 2001) and cow (Izadyar *et al.* 1998) antral follicles, while in those of the human, ActR-IIA and -IIB were detected in granulosa cells and theca cells (Pangas *et al.* 2002). In mouse and human antral follicles, mRNAs for all four activin receptor subtypes were expressed in both oocytes and granulosa cells (Sidis *et al.* 1998). *In vitro*, activin-A stimulated antrum formation (rat: Zhao *et al.* 2001), FSH receptor and FSH-induced LH receptor production (rat: Minegishi *et al.* 1999, Tsuchiya *et al.* 1999), cytochrome P-450 aromatase activity, and thus oestrogen synthesis, in granulosa cells (human: Mukasa *et al.* 2003). Activin also suppressed androgen production in theca cells from different species (human: Hillier *et al.* 1991; bovine: Wrathall & Knight 1995; and ovine: Campbell & Baird 2001). Consequently, activin is thought to play a crucial role in basal growth, recruitment and selection of antral follicles through stimulation of proliferation and FSH receptor expression in granulosa cells and modulation of steroidogenesis in granulosa and theca cells, its actions being time and concentration dependent and regulated by follistatin (reviewed by Findlay *et al.* 1993, 2002, Driancourt 2001, Knight & Glister 2001). Activin-A is also involved in the regulation of oocyte maturation in several species (bovine: Silva & Knight 1998; human: Alak *et al.* 1998; and mouse: Sidis *et al.* 1998). The distribution pattern of activin-A, its binding protein follistatin and activin receptors in goat antral follicles points to an important role of these proteins in antral follicle development in this species.

Apart from follicles, we found evidence for the presence of an activin–follistatin–activin receptor system in corpora lutea and ovarian surface epithelium, because of the presence of both mRNA and protein for activin-A, follistatin and all four types of activin receptors at these sites. With regard to corpora lutea, expression of protein and mRNA for activin-A and follistatin has previously been demonstrated in man (Roberts *et al.* 1993, Wada

et al. 1996) and cow (Singh & Adams 1998). Both Act-RIIA protein and mRNA were detected in pig luteal tissue (van den Hurk & van de Pavert 2001), and, *in vitro*, activin-A suppressed progesterone production by primate luteal cells (Brannian *et al.* 1992). Expression of activin-A protein and mRNA in ovarian surface epithelium has previously been demonstrated in pig (van den Hurk & van de Pavert 2001) and cow (Hulshof *et al.* 1997). Choi *et al.* (2001) demonstrated that activin inhibits growth and induces apoptosis in cultured ovarian surface epithelial cells. As mentioned above, the activin–follistatin system has been demonstrated to control growth and differentiation of antral follicles by affecting gonadotrophin receptor synthesis and steroidogenesis. The activin–follistatin system presence in goat corpora lutea and surface epithelium may therefore point to a regulatory function in or fine tuning of their activity.

In conclusion, activin-A, its binding protein follistatin and its receptors are formed in all types of goat follicles and in antral follicles in all compartments. In addition, they are generated in corpora lutea and ovarian surface epithelium. The widespread distribution pattern of the follistatin–activin–activin receptor system in goat ovaries points to a crucial role of this system in various reproductive processes, including follicle growth and differentiation, and luteal activity.

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