The activin-follistatin system and in vitro early follicle development in goats

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Abstract

The aim of the present study was to investigate the effects of activin-A and follistatin on in vitro primordial and primary follicle development in goats. To study primordial follicle development (experiment 1), pieces of ovarian cortex were cultured in vitro for 5 days in minimal essential medium (MEM) supplemented with activin-A (0, 10 or 100 ng/ml), follistatin (0, 10 or 100 ng/ml) or combinations of the two. After culture, the numbers of primordial follicles and more advanced follicle stages were calculated and compared with those in non-cultured tissue. Protein and mRNA expression of activin-A, follistatin, Kit ligand (KL), growth differentiation factor-9 (GDF-9) and bone morphogenetic protein-15 (BMP-15) in non-cultured and cultured follicles were studied by immunohistochemistry and PCR. To evaluate primary follicle growth (experiment 2), freshly isolated follicles were cultured for 6 days in MEM plus 100 ng/ml activin-A, 100 ng/ml follistatin or 100 ng/ml activin-A plus 200 ng/ml follistatin. Morphology, follicle and oocyte diameters in cultured tissue and isolated follicles before and after culture were assessed. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) reactions were performed to study DNA fragmentation in follicles. In experiment 1, it was found that goat primordial follicles were activated to develop into more advanced stages, i.e. intermediate and primary follicles, during in vitro culture, but neither activin-A nor follistatin affected the number of primordial follicles that entered the growth phase. Activin-A treatment enhanced the number of morphologically normal follicles and stimulated their growth during cortical tissue culture. The effects were, however, not counteracted by follistatin. The follicles in cultured goat tissue maintained their expression of proteins and mRNA for activin-A, follistatin, KL, GDF-9 and BMP-15. Fewer than 30% of the atretic follicles in cultured cortical tissue had TUNEL-positive (oocyte or granulosa) cells. Activin-A did not affect the occurrence of TUNEL-positive cells in follicles within cortical tissue. In experiment 2, addition of activin-A to cultured isolated primary follicles significantly stimulated their growth, the effect being counteracted by follistatin. Absence of such a neutralizing effect of follistatin in the cultures with ovarian cortical tissue can be due to lower dose of follistatin used and incomplete blockage of activin in these experiments.

Introduction

Ovarian folliculogenesis is a complex process whereby oocytes and their surrounding somatic cells develop through primordial, primary, secondary and antral stages. During the last decade, many attempts have been made to activate primordial follicles in vitro and enable their growth up to maturation stages (for reviews see van den Hurk et al. 2000, Driancourt 2001, Eppig 2001, Matzuk et al. 2002, Fortune 2003, van den Hurk & Zhao 2005).
Several studies with farm animals and primates have successfully shown the activation and transition of primordial follicles to primary stages (caprine, Silva et al. 2004a; bovine, Wandji et al. 1996, Cushman et al. 2002; baboon, Fortune et al. 1998; human, Hovatta et al. 1997, Hreinsson et al. 2002) during in vitro culture of ovarian cortical slices. However, using these mammalian models primary follicles do not grow to secondary stages, not even when tissues were cultured in vitro for 20 days (Wandji et al. 1997, Fortune et al. 1998). Only when mouse ovarian tissue was used could primordial follicles be activated and further grown to secondary and antral stages, whereby oocytes were competent to undergo maturation, fertilization and embryo development (O’Brien et al. 2003). This growth was brought about in a two-step culture system, in which the first step consisted of whole ovary culture to obtain primordial to primary follicle transition, and the second step was isolation and culture of primary and secondary follicles. Probably, this strategy is also required to promote primary follicle growth in domestic ruminants and primates. Although the mechanisms regulating the activation of primordial follicles and growth of primary and secondary follicles are not completely understood, accumulating evidence indicates that these processes are locally regulated by various paracrine and autocrine factors, among which are activins, growth differentiation factor-9 (GDF-9), bone morphogenetic protein-15 (BMP-15) and Kit ligand (KL; for reviews, see van den Hurk et al. 2000, Findlay et al. 2002, Fortune 2003, van den Hurk & Zhao 2005).

Activins are heterodimers (βAβB, activin AB) or two homodimers (βAβA, activin-A; βBβB, activin B) of the β subunits of inhibin. Like inhibins, they were originally described as being gonadally produced regulators of pituitary hormone release, but they are currently known to have a broader range of effects (Phillips 2005). With over 500 hits at PubMed’s website, including the five studies from our group (Izadayar et al. 1996, 1998, Hulshof et al. 1997, van de Pavert & van den Hurk 2001, Zhao et al. 2001), the function of activin-A in the ovary has been most extensively studied. Follistatin is structurally unrelated to the activins, but binds with high affinity to the β subunits and so is able to neutralize the activities of activin in a variety of target tissues, including the ovary (Phillips & de Krester 1998).

Activins signal through two types of closely related receptor, designated type I and type II, each represented by two isoforms, i.e. activin receptors type IA (ActR-IA), IB (ActR-IB), II A (ActR-IIA) and II B (ActR-IIIB). Activin first binds to a type II receptor, which in turn recruits and activates a type I receptor by phosphorylation (Pangas & Woodruff 2000), with ActR-IB being the predominant type I receptor for activin (Masagué & Chen 2000). Protein and mRNA for activin-A and activin receptors have been localized in both the oocyte and the granulosa cells of goat early follicles, as have those for follistatin (Silva et al. 2004b). Besides the demonstration of proteins and mRNA for activin and their receptors in bovine and rodent preantral follicles (i.e. primordial, primary and secondary follicles), in vitro studies with isolated primary and secondary follicles have demonstrated that, in these species, activin-A is involved in their development (bovine: Hulshof et al. 1997, rodents: Smits et al. 1998, Zhao et al. 2001). There are, however, also reports that showed an age-dependent effect of activin-A on medium-sized preantral follicles isolated from mice (Mizunuma et al. 1999) and no effect on primary to secondary follicle transition has been reported in bovine follicles (Fortune 2003). Except for a possible signaling role of activin-A in rodent and bovine ovaries, information on its involvement in early folliculogenesis in other mammalian species, like the goat, is lacking. To improve our knowledge on the biological activity of activin-A and follistatin on early follicle development in mammals and to explore possible physiological differences, it is very important to conduct studies with other species, like goats. Goats are present on all continents and are commercially viewed as highly attractive livestock animals, since they have been used for many purposes such as milk, meat and skin production.

In the present study, experiments were carried out to examine the possible effects of the activin-follistatin system on primordial follicle activation during culture of goat ovarian cortical slices, and on growth of primary follicles after their isolation from goat ovaries. For the study of primordial follicle activation, ovarian cortical tissue was cultured for 5 days in the absence and presence of activin-A, follistatin or a combination of activin-A and follistatin and the effects were studied on (1) the rate at which primordial follicles were transformed into developing follicles, (2) oocyte and follicle diameter, (3) atresia and DNA fragmentation (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling; TUNEL) and (4) on protein and mRNA expression of activin-A, follistatin, GDF-9, BMP-15 and KL, using immunohistochemistry and PCR. Furthermore, primary follicle growth was investigated by culturing freshly isolated primary follicles for 6 days in the presence or absence of activin-A and/or follistatin and with subsequent evaluation of the effects of these compounds on follicle diameter and DNA fragmentation.

**Materials and Methods**

**Experiment 1: culture of cortical slices**

**Ovaries and experimental design** Ovaries (n = 10) from cyclic adult mixed-breed goats were collected at a local slaughterhouse, washed in saline solution containing antibiotics (100 µg/ml penicillin and 100 µg/ml streptomycin) and transported to the laboratory. Cortical tissue from
one pair of ovaries was used in each of five different replicates. Each replicate was performed on a different occasion.

In the laboratory, the ovaries (n=10) were stripped of surrounding fat and fibrous tissue and the ovarian cortex was recovered and divided into pieces of approximately 3 mm × 3 mm (1 mm thick). For each animal, one slice was selected randomly and fixed immediately in 4% paraformaldehyde (non-cultured control). The remaining slices of ovarian cortex were cultured individually in 1 ml culture medium in 24-well culture dishes at 39 °C in an atmosphere of 5% CO₂ in air. Control culture medium was Eagle’s α-minimal essential medium (MEM) supplemented with SPIT (5 ng/ml sodium, 110 µg/ml pyruvate, 10 µg/ml insulin and 5·5 µg/ml transferrin), 1·25 mg/ml BSA, 100 µg/ml penicillin and 100 µg/ml streptomycin (all from Sigma Chemicals). For the experimental conditions, the medium was supplemented with either human recombinant activin-A (10 or 100 ng/ml; R&D Systems Europe, Abingdon, Oxon, UK), follistatin (10 or 100 ng/ml; R&D Systems Europe) or a combination of both, i.e. 10 or 100 ng/ml activin-A plus either 10 or 100 ng/ml follistatin. The ovarian slices from each animal were cultured for 5 days and, on days 2 and 4, the culture medium was replaced with fresh medium.

Protein localization for activin-A, follistatin, GDF-9, BMP-15 and KL in cortical slices Either immediately after recovery or after 5 days of culture, slices of ovarian tissue were fixed by immersion in buffered 4% formaldehyde for 18 h, dehydrated and embedded in paraffin wax. Thereafter, cortical slices were serially sectioned (5 µm thick) and mounted on glass microscope slides. Immunohistochemistry was performed as described by Silva et al. (2004b). In brief, the epitopes were activated by microwave heating the sections for 7 min at 900 W in 0·01 M citrate buffer (pH 6·0) and non-specific binding was prevented by incubation for 30 min with 5% normal goat serum in PBS. The primary antibodies were (1) rabbit anti-activin-A (1:50; Innogenetics, Ghent, Belgium), (2) mouse anti-follistatin (1:50; R&D Systems Europe), (3) mouse anti-KL (1:20; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-GDF-9 (1:100) and mouse anti-BMP-15 (1:20; both from Wyeth Research, Cambridge, MA, USA). The sections were incubated overnight at 4 °C in appropriate dilutions of the antibodies. The sections were then incubated for 45 min with biotinylated secondary antibody (goat anti-rabbit or goat anti-mouse IgG from Vector Laboratories, Burlingame, CA, USA), diluted 1:200 in PBS containing 5% normal goat serum. Next, the sections were incubated for 45 min with avidin–biotin complex (1:600; Vectastain Elite ABC kits; Vector Laboratories). Protein localization was determined with diaminobenzidine (DAB; 0·05% DAB in Tris/HCl, pH 7·6/0·03% H₂O₂; Sigma Chemicals). The sections were counterstained with hematoxylin, dehydrated and mounted in Pertex (Cellpath, Hemel Hempstead, Herts, UK). 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 DAB reagent alone to exclude the possibility of non-suppressed endogenous peroxidase activity. Specificity of the antibodies was tested previously (Silva et al. 2004b, 2004c).

The early-stage follicles were classified as primordial (one layer of flattened granulosa cells around the oocyte) or developing follicles, i.e. intermediate (both flattened and cuboidal granulosa cells around the oocyte), primary (a single layer of cuboidal granulosa cells around the oocyte) or secondary (two or more layers of cuboidal granulosa cells) follicles. Follicles were further classified as intact when, in 5 µm thick haematoxylin/eosin-stained histological sections, a morphologically normal oocyte was surrounded by organized granulosa cells, or atretic (degenerative) when a shrunken oocyte containing a pyknotic nucleus was surrounded by disorganized granulosa cells, which were detached from the basement membrane. When evaluating follicle activation and growth, the number of primordial or developing follicles per fragment was calculated before (day 0) or after 5 days of culture in the various media. To avoid counting a follicle more than once in serial sections, follicles were counted and evaluated only in the section where the oocyte nucleus was visible. Oocyte and follicle diameters were measured at 40 × magnification using a light microscope (Zeiss, Cologne, Germany) fitted with an eyepiece micrometer.

Gene expression for activin-A, follistatin, GDF-9, BMP-15 and KL in cortical slices To evaluate gene expression, three slices were collected from uncultured cortical tissue (day 0) and from tissues that were cultured in the respective media for 5 days, and then stored at −80 °C until RNA extraction. Isolation of total RNA combined with on-column DNase digestion was performed using the RNaseasy mini kit and the RNase-free DNase set (Qiagen) according to the manufacturer’s instructions as described previously (Silva et al. 2004b). Prior to the reverse transcription reaction, the eluted RNA samples were incubated for 5 min at 70 °C. Reverse transcription was then performed in a total volume of 20 µl, made up of 10 µl sample RNA, 4 µl 5 × reverse transcriptase buffer (Gibco BRL), 8 U RNasin (Promega), 150 U Superscript II reverse transcriptase (Gibco BRL) and 0·036 U random primers (Life Technologies BV), and containing 10 mM dithiothreitol and 0·5 mM of each dNTP. The mixture was incubated for 1 h at 42 °C and 5 min at 80 °C, and then stored at −20 °C. Reverse transcriptase blanks were prepared under the same conditions, but without inclusion of reverse transcriptase. PCR reactions were carried out in 200 µl tubes (Biozym, Landgraaf, The Netherlands), using 1 µl cDNA
The sections were washed in PBS, covered with DAB (described above) and then counterstained with haematoxylin, dehydrated and mounted.

Experiment 2: culture of isolated primary follicles

Ovaries and follicle isolation To evaluate the effect of activin and follistatin on isolated early follicles, ovaries (n=10) from five adult goats were collected in a slaughterhouse and transported to the laboratory. In each of five different replicates performed on different occasions, primary follicles were isolated from one pair of ovaries from the same animal, using the mechanical procedure that was previously described by Lucci et al. (1999). After isolation, these follicles were washed repeated times to completely remove the stromal cells. The removal of stromal cells was verified using an inverted microscope and then the follicles were used for in vitro culture.

Primary follicle culture For the control group, the follicles (n=60) were cultured in groups of 3–5 in 5-well culture dishes (Bibby Sterilin, Stone, Staffs, UK) containing 250 μl culture medium (MEM) supplemented with SPIT, 1·25 mg/ml BSA, 100 μg/ml penicillin and 100 μg/ml streptomycin. Experimental media consisted of the above-described control media to which recombinant human activin-A (n=66) or follistatin (n=63) was added at 100 ng/ml, or both activin A and follistatin (n=62) were added at 100 and 200 ng/ml, respectively. Isolated follicles were randomly assigned to the treatment groups. Plates were incubated for 6 days in a sterile humidified atmosphere with 5% CO₂ at 39 °C. Each set of cultures (n=5) took place under identical conditions and half of
the medium was replaced every second day. The morphology of each individual follicle before, during and immediately after culture was studied using an inverted microscope. The diameter of normal follicles, i.e. follicles that did not show signs of oocyte retraction or those of granulosa cell disorganization, were measured using a crossed micrometer under a microscope on days 0 and 6.

Assessment of DNA fragmentation of isolated follicles The occurrence of DNA fragmentation in follicles before and after culture was detected using the TUNEL technique. In each treatment, 10 follicles were randomly collected from five different replicates and evaluated. First, 4% paraformaldehyde-fixed follicles were incubated twice for 15 min in PBS containing 150 mM glycine and 1 mg/ml polyvinylalcohol (PVA) to reduce free aldehydes and to block nonspecific reactions. Next, they were permeabilized by immersion for 15 min at 4 °C in 0·1% (v/v) Triton X-100 in PBS. The permeabilized follicles were then washed twice in PBS containing 1 mg/ml PVA (PBS-PVA; pH 7·4) before being incubated in 20 µl drops of fluorescein-conjugated dUTP and TdT (Roche) for 1 h at 37 °C in a dark, moist chamber. Finally, to enable DNA visualization, the follicles were washed twice in PBS-PVA and incubated with 0·1 µg/ml 4,6-diamino-2-phenylindole (DAPI) in PBS for 10 min.

Stained follicles were mounted on glass slides with an antifading medium (Vectashield) to prevent photobleaching and examined using a confocal laser scanning microscope (Bio-Rad Radiance 2100 MP) mounted on a Nikon TE300 inverted microscope (Nikon, Badhoevedorp, The Netherlands) to detect TUNEL-positive cells. The number of granulosa cells per follicle, as well as the number of cells with fragmented DNA, were determined using a conventional immunofluorescence microscope equipped with an eyepiece counting grid.

Statistical analysis

In the first experiment, one-way analysis of variance and Tukey’s HSD test were used to compare the numbers of primordial and developing follicles, the follicle and oocyte diameters, the number of granulosa cells in normal follicles and the numbers of degenerated follicles before and after culture of cortical slices. Chi-square tests (Instat for Windows) were applied for statistical analysis of the obtained percentages of atretic and morphologically normal follicles with fragmented DNA within cortical tissue, respectively. In the second experiment, analysis of variance and Tukey’s HSD test were also used to compare the diameter of isolated primary follicles, the number of granulosa cells and the numbers of TUNEL-positive cells before and after culture in the different experimental media. The percentages of atretic follicles were compared by chi-square test. Differences were considered significant when P<0·05.

Results

Experiment 1

Primordial follicle activation and growth during in vitro culture of cortical slices The total numbers of follicles, i.e. normal plus atretic follicles, that were evaluated in each of these treatments did not differ significantly from each other (range from 82·9 ± 1·2·4 to 101·4 ± 16·1; P>0·05). The numbers of normal primordial and developing follicles in non-cultured tissue or in tissue cultured for 5 days under different treatment conditions are shown in Fig. 1. Non-cultured ovarian tissue contained predominantly primordial follicles and only a few developing follicles. Based on granulosa cell morphology, in all cultures tested the number of primordial follicles was reduced significantly (P<0·05) after 5 days of culture, concomitant with a significant increase in the number of morphologically normal developing follicles (Fig. 1). Compared with culturing in MEM (control), no significant effect of added activin-A, follistatin or combinations of activin and follistatin on the number of developing follicles was observed (P>0·05). Overall, the distribution of developing follicles after culture was 26·9% intermediate, 69·0% primary and 4·1% secondary follicles.

The follicle and oocyte diameters as well as the number of follicular granulosa cells per section were evaluated and are shown in Table 2. Since follicle diameter and number of granulosa cells in secondary (multilaminar) follicles are more variable, the data from these follicles are shown separately from those of the (unilaminar) follicles that have only one layer of granulosa cells, i.e. primordial, intermediate and primary follicles. Compared with uncultured cortical slices, significant increases (P<0·05) in unilaminar follicle and their oocyte diameters were observed when tissues were cultured for 5 days in medium containing 100 ng/ml activin-A. Unilaminar follicle diameters but not their oocyte diameters had also increased when ovarian slices were cultured in 100 ng/ml activin-A plus 10 ng/ml follistatin. When compared with those cultured in control medium, the diameters of unilaminar follicles and their oocytes had increased (P<0·05) after 5 days culture in activin-A (100 ng/ml), but the effect had not disappeared (P>0·05) after addition of follistatin (100 ng/ ml) to the medium with activin-A. When compared with uncultured tissues, all cultured ovarian slices except those cultured in MEM (control), MEM+10 ng/ml activin-A and MEM+10 ng/ml activin-A+100 ng/ml follistatin showed a significant increase (P<0·05) in the number of follicular granulosa cells. For multilaminar follicles, no significant differences (P>0·05) in follicle and oocyte diameters or in numbers of granulosa cells were found when cultured tissues were compared mutually or with uncultured tissues.

Atresia and DNA fragmentation in follicles within cortical slices Figure 2 shows the numbers of atretic
primordial and developing follicles before and after 5 days of culture. When compared with uncultured cortical slices, those that were cultured for 5 days all contained significantly increased numbers of atretic developing follicles. After 5 days of culture, among the tested media, the one containing 100 ng/ml activin-A significantly reduced the number of atretic developing follicles ($P < 0.05$); the effect was not counteracted when either 10 or 100 ng/ml follistatin was added to activin-A. In none of the normal follicles within uncultured cortical slices could DNA fragmentation be demonstrated by TUNEL labelling. After culture, the great majority of the nuclei of morphologically normal follicles still had TUNEL-negative nuclei (Fig. 3F); only 4.4% (5/113) of normal follicles contained oocytes and/or granulosa cells with TUNEL-labelled nuclei. From the atretic follicles, 28% (25/90) showed a TUNEL reaction in one or more nuclei. For the latter data follicles were pooled, since no significant differences among treatments ($P > 0.05$; data not presented) were observed. Figure 3 shows primary follicles with an absence

**Table 2** Follicle/oocyte diameters and number of granulosa cells per section for follicle with one (unilaminar follicles) or two or more (multilaminar follicles) layers of granulosa cells (multilaminar follicles) in the uncultured controls and after 5-day culture in different treatments

<table>
<thead>
<tr>
<th>Treatment after uncultured</th>
<th>Follicle (µm; mean ± S.D.)</th>
<th>Oocyte</th>
<th>No GC</th>
<th>Follicle (µm; mean ± S.D.)</th>
<th>Oocyte</th>
<th>No GC</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 days of culture</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEM</td>
<td>30.6 ± 4.7</td>
<td>26.9 ± 2.9</td>
<td>8.2 ± 4.5</td>
<td>108.4 ± 47.1</td>
<td>54.0 ± 19.5</td>
<td>69.2 ± 26.4</td>
</tr>
<tr>
<td>A10</td>
<td>31.7 ± 3.7*</td>
<td>26.3 ± 3.1a</td>
<td>11.9 ± 4.1ab</td>
<td>99.0 ± 45.6</td>
<td>46.7 ± 16.0</td>
<td>63.2 ± 25.3</td>
</tr>
<tr>
<td>A100</td>
<td>33.4 ± 7.9abc</td>
<td>27.9 ± 4.4abc</td>
<td>13.0 ± 7.2ab</td>
<td>104.7 ± 27.3</td>
<td>51.7 ± 22.4</td>
<td>86.4 ± 46.3</td>
</tr>
<tr>
<td>F10</td>
<td>35.5 ± 6.9abc</td>
<td>29.6 ± 4.9ab</td>
<td>12.4 ± 3.7ab</td>
<td>110.0 ± 25.7</td>
<td>58.5 ± 14.6</td>
<td>92.8 ± 42.5</td>
</tr>
<tr>
<td>F100</td>
<td>32.8 ± 4.6abc</td>
<td>27.2 ± 2.8abc</td>
<td>12.0 ± 3.1ab</td>
<td>105.0 ± 21.5</td>
<td>46.2 ± 10.9</td>
<td>81.2 ± 21.3</td>
</tr>
<tr>
<td>A10 + F10</td>
<td>32.5 ± 5.4abc</td>
<td>27.0 ± 3.2abc</td>
<td>12.2 ± 5.6ab</td>
<td>87.5 ± 33.7</td>
<td>46.2 ± 14.3</td>
<td>69.8 ± 28.7</td>
</tr>
<tr>
<td>A100 + F10</td>
<td>31.4 ± 3.8abc</td>
<td>26.2 ± 2.1abc</td>
<td>11.8 ± 5.5ab</td>
<td>81.5 ± 52.4</td>
<td>46.5 ± 16.0</td>
<td>57.4 ± 37.4</td>
</tr>
<tr>
<td>A100 + F100</td>
<td>30.8 ± 4.2abc</td>
<td>25.9 ± 2.6abc</td>
<td>10.9 ± 4.7a</td>
<td>94.2 ± 47.0</td>
<td>48.7 ± 18.3</td>
<td>76.0 ± 35.1</td>
</tr>
<tr>
<td>A100 + F100</td>
<td>35.5 ± 5.5abc</td>
<td>29.3 ± 4.2a</td>
<td>15.2 ± 5.3ab</td>
<td>95.9 ± 34.0</td>
<td>55.5 ± 17.1</td>
<td>97.4 ± 56.3</td>
</tr>
<tr>
<td>A100 + F100</td>
<td>32.2 ± 3.6abc</td>
<td>26.8 ± 2.6abc</td>
<td>13.2 ± 4.2ab</td>
<td>91.0 ± 44.9</td>
<td>49.7 ± 19.4</td>
<td>84.0 ± 46.4</td>
</tr>
</tbody>
</table>

In each treatment, 30 unilaminar and 5 multilaminar follicles were measured. No GC, number of granulosa cells; A, activin-A; F, follistatin; 10 or 100, concentrations in ng/ml.

*Denotes values that differ significantly from uncultured control (day 0). a–dValues with different letters denote significant differences among culture media within a given column ($P < 0.05$).
Expression of activin-A, follistatin, GDF-9, BMP-15 and KL. Both before and after 5 days culture and independent of the in vitro treatment applied, all or the great majority of morphologically normal primordial or growing follicles (98–100%) expressed the proteins for activin-A, follistatin, GDF-9, BMP-15 and KL (Fig. 3A–E). Oocytes showed strong activin-A, follistatin, GDF-9 and BMP-15 immunoreactions, but no KL reaction. Granulosa cells also intensively immunostained for activin-A, follistatin and GDF-9, but weakly for BMP-15 and moderately to strongly for KL, the stronger reaction being especially present at the apical side of the granulosa cells where they border the oocyte (Fig. 3E). Using specific primers for activin-A, follistatin, GDF-9, BMP-15 and KL, amplification of cDNA from uncultured and all cultured tissues resulted in specific products, demonstrating that the mRNAs for those factors were synthesized during the in vitro culture period. Sequence analysis of the amplified activin-A, follistatin, GDF-9, BMP-15 and KL products confirmed their specificity. Amplification of reverse transcriptase blanks or water controls yielded no specific products in any of the reactions (Fig. 4).

Experiment 2

In vitro growth of isolated primary follicles. A total of 251 isolated primary follicles were cultured in vitro (pool of 60–66 follicles per treatment that were obtained from five replicates performed on different occasions). As illustrated in Table 3, isolated primary follicles that were cultured for 6 days in medium containing 100 pg/ml activin-A showed a significant increase ($P<0.05$) in follicle diameter when compared with control medium (MEM), MEM + 100 ng/ml follistatin or MEM + 100 ng/ml activin-A + 200 ng/ml follistatin. Follicles showing signs of atresia were not included in these diameter calculations. No significant differences in the number of granulosa cells per follicle were observed among the treatments, but (normal) follicles cultured in activin-A containing medium had higher numbers of granulosa cells when compared with uncultured follicles ($P<0.05$; Table 4). From the follicles that were cultured for 6 days, 30.0% (18/60) showed signs of atresia after culture in control medium (MEM). This percentage was not significantly different from those cultured in media containing activin-A (24.6%; 16/66), follistatin (31.8%; 20/63) or both (33.9%; 21/62). Figure 5 shows a morphologically normal primary follicle after 6 days of culture, which contains a clear oocyte surrounded by granulosa cells, and an atretic follicle with a dark retracted oocyte and disorganized granulosa cells.

DNA fragmentation in isolated follicles. Uncultured follicles that were considered morphologically normal did not show a TUNEL reaction (Table 4, Fig. 6A). After 6 days of culture, normal follicles generally had few (1–3) granulosa cells with a TUNEL-positive nucleus (Fig. 6B). The presence of follistatin in the culture medium significantly increased the percentage of TUNEL-positive follicles (Fig. 6F) and presence of TUNEL reactivity in granulosa cell (Fig. 3G) and oocyte (Fig. 3H) nuclei.
granulosa cells per follicle when compared with uncultured follicles and follicles cultured in medium containing activin-A (Table 4). After culture, all atretic follicles contained fragmented DNA in their oocyte nucleus and in a high percentage of their granulosa cells (32.4 ± 6.2). Figure 5C shows confocal images of primary follicles with TUNEL fluorescence in both granulosa and oocyte nuclei.

**Discussion**

This study demonstrates a decrease in the number of primordial follicles and concomitant increase in the number of developing follicles during in vitro culture of goat ovarian cortical tissue for 5 days in a defined culture medium, wherein activin-A and follistatin did not have an additional beneficial effect. The finding is in accordance with those from earlier in vitro studies with cattle (Wandji et al. 1996, Braw-Tal & Yossefi 1997) and primate (Hovatta et al. 1997, Fortune et al. 1998) follicles. These studies described that activation of primordial follicles as occurring ‘spontaneously’, i.e. without the addition of growth factors or hormones. The in vitro conditions thus have facilitated follicle development in cultured ovarian slices, possibly through release of stimulatory factors or cessation of production of inhibitory factors by oocytes or stromal, granulosa or pre-thecal cells within the cultured ovarian cortical tissue. Such an endogenous process may have masked an effect of the tested compounds that have been added to the culture media. For example, it cannot be excluded that absence of a positive effect of added activin-A on the transition of primordial follicles into more advanced stages is due to high production of its blocking factor follistatin within the cultured ovarian slices, nor that in cultured control tissues endogenous
activin-A already reaches a sufficient level to activate primordial follicles. With use of real-time PCR, we have tried to quantify the expression of mRNAs for activin-A and follistatin as well as for KL, GDF-9 and BMP-15 in cultured and non-cultured cortical slices, but in all cases this appeared impossible, because of the very low expression levels (JRV Silva & R. van den Hurk, unpublished results). However, activin-A, follistatin, KL, GDF-9 and BMP-15 were apparently synthesized continuously in follicles during in vitro culture of ovarian slices, since with use of nested PCR we detected the expression of their mRNAs in these tissues both before and after (5 days) culture, while the respective proteins were immunocyto-chemically demonstrated in the enclosed early ovarian follicles. Activin-A was found to be involved in germ cell proliferation in human (Martins da Silva et al. 2004), secondary follicle development in cattle (Hulshof et al. 1997), mouse (Smitz et al. 1998) and rat (Zhao et al. 2001), and antrum formation in rat follicles (Zhao et al. 2001), but there is no evidence for a role of activin-A and

![Figure 4](image)

**Figure 4** mRNA expression for activin-A, follistatin, GDF-9, BMP-15, KL and GAPDH in goat ovarian cortical tissue after 5 days culture.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Follicle diameter (μm; mean ± s.d.)</th>
<th>Follicle diameter increase at day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM (n=60)</td>
<td>46.7 ± 8.5</td>
<td>50.0 ± 9.9</td>
</tr>
<tr>
<td>MEM+100 ng Act.-A (n=66)</td>
<td>45.5 ± 7.9</td>
<td>53.9 ± 9.8</td>
</tr>
<tr>
<td>MEM+100 ng follistatin (n=63)</td>
<td>50.2 ± 10.4</td>
<td>53.4 ± 11.7</td>
</tr>
<tr>
<td>MEM+100 ng Act.-A+200 ng follistatin (n=62)</td>
<td>51.1 ± 9.4</td>
<td>53.0 ± 9.1</td>
</tr>
</tbody>
</table>

Only non-atretic follicles are included; their number are given in brackets.
*Values in the same column with different letters denote significant difference among culture media (P<0.05).
follistatin in primordial follicle activation. KL promotes the transition from primordial to primary follicle in mice and recruitment of theca cells from the stromal tissue surrounding primordial follicles (Parrott & Skinner 1997, 2000). Also, earlier data from studies with rodents (Vitt et al. 2000, Otsuka et al. 2000, Nilsson & Skinner 2002) suggest that GDF-9 and BMP-15 are important regulators of granulosa cell mitosis and early follicular development. The importance of these factors for early folliculogenesis is confirmed by the findings of

### Table 4

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of granulosa cells per follicle</th>
<th>% of TUNEL-positive granulosa cells per follicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control — day 0</td>
<td>61.6 ± 6.0ᵃ</td>
<td>0</td>
</tr>
<tr>
<td>MEM — day 6</td>
<td>65.0 ± 13.7ᵃᵇ</td>
<td>3.3 ± 1.5ᵃᵇ</td>
</tr>
<tr>
<td>MEM + Act.-A (100 ng/ml) — day 6</td>
<td>78.4 ± 10.1ᵇ</td>
<td>1.6 ± 0.6ᵃ</td>
</tr>
<tr>
<td>MEM + follistatin (100 ng/ml) — day 6</td>
<td>66.6 ± 11.3ᵃᵇ</td>
<td>3.5 ± 1.7ᵇ</td>
</tr>
<tr>
<td>MEM + Act.-A (100 ng/ml) + follistatin (200 ng/ml) — day 6</td>
<td>69.8 ± 13.6ᵃᵇ</td>
<td>2.0 ± 1.8ᵇ</td>
</tr>
</tbody>
</table>

ⁿ=10 follicles per treatment. ᵃᵇValues in the same column with different letters denote significant difference among culture media (P<0.05).

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Figure 5 A morphologically normal (A) and a degenerated (B) isolated preantral follicle after 6-day culture. Scale bars: 25 μm. *Shrunken oocyte.

Figure 6 Confocal images of morphologically normal follicles before (A) and after 6 days of culture (B), and of a follicle that became atretic during *in vitro* culture (C). Note the grey TUNEL-positive fluorescence in granulosa cell nuclei in (B) and (C) and the oocyte nucleus in (C). Normal nuclei appear white (DAPI staining).
Dong et al. (1996) and Galloway et al. (2000), which showed that GDF-9-deficient mice and BMP-15-deficient sheep are infertile because follicle development does not proceed beyond the primary stage. Several other factors, such as fibroblastic growth factor-2 (Nilsson et al. 2001), leukemia inhibitory factor (Nilsson et al. 2002) and BMP-7 (Lee et al. 2004) have been previously demonstrated to induce primordial to primary follicle transition in rats and mice and are probably produced within the ovarian cortex.

When compared with control medium, the diameter of unilaminar follicles had increased in the current studies in which ovarian tissue was cultured in the presence of activin-A (100 ng/ml). This effect may be due to an increase in both oocyte diameter and number of larger cuboidal granulosa cells. Correspondingly, activin-A stimulated the growth of cultured goat isolated primary follicles. In contrast with the cultured cortical tissue enclosed follicles, follistatin (200 ng/ml) blocked the effect of activin-A on the growth of isolated primary follicles. This difference in effects of follistatin between the cultures of slices and isolated follicles could be due to an incomplete neutralization of activin activity within cortical tissue, since lower dose of follistatin was used. The fact the follistatin needs to penetrate the tissue slice to access the follicle can also account for the lack of effect. Possibly, enough activin-A is left for stimulation of the growth of the very early unilaminar stages, i.e. primordial and intermediate follicles, but not enough to activate the growth of primary follicles. On the other hand, it is known that follistatin binds activin with high affinity, whereby its binding effectively neutralizes the bioactivity of activin (Knight & Glister 2001, Fisher et al. 2003). Differences in activin-A neutralization could also be due to differences in endogenous expression of activin-A between cultured isolated follicles and cultured cortical tissue enclosed follicles. Nevertheless, the presence of activin-A and its receptors in early follicles (Silva et al. 2004b) is indicative for a functional activin–activin receptor complex and explains the effect of activin-A on oocyte and follicle growth in cultured unilaminar follicles. A concentration of activin-A comparable with that used in the current study also directly influenced sheep early oocyte and preantral follicle development in vitro (Thomas et al. 2003). Furthermore, activin-A appeared to stimulate the in vitro growth of bovine (Hulshof et al. 1997), rat (Zhao et al. 2001) and mouse (Smits et al. 1998) primary and/or secondary follicles. Based on these data, activin-A seems to be an intrafollicular factor that controls primary follicle development, its effect being dependent on the availability of follistatin. Yet there is a single report that showed that activin-A has no effect on primary to secondary follicle transition in cows (Fortune 2003).

The current data furthermore showed that addition of 100 ng/ml activin-A significantly reduced the number of atretic developing follicles in cultured cortical tissue, the effect not being counteracted by the addition of either 10 or 100 ng/ml follistatin. Activin-A acted as a follicular survival factor only when follicles are enclosed within ovarian cortical tissue, since we did not find a similar effect when isolated primary follicles were cultured. Previously, Hulshof et al. (1997) and Zhao et al. (2001) did not find an in vitro effect of activin-A on follicle survival of isolated bovine and rat small-sized preantral follicles, respectively. Less than 30% of the atretic follicles in our culture experiments with cortical slices showed DNA fragmentation, while this phenomenon was found in all atretic follicles that arose in isolated follicle cultures, especially in high percentages of their granulosa cells. Probably, cultured cortical tissue-enclosed follicles have less access to oxygen and nutrients than cultured isolated follicles, which may favour the occurrence of necrosis as a possible way of degeneration. Jennings et al. (1975) showed that hypoxia induced changes in the cellular membrane permeability, which cause changes in the levels of Na+, K+ and Cl− and are followed by changes in the distribution of intracellular Ca2+, which in turn may lead to changes in the cellular volume and necrosis. In contrast, isolated follicles have better access to oxygen and nutrients and are dying via apoptosis. This apoptotic process is triggered by diverse signals that lead to activation of intracellular caspases in a pathway completely different from necrosis (Tilly 1996).

In conclusion, this study has demonstrated that, after 5 days of culture of ovarian cortical slices, large numbers of goat primordial follicles have been transformed into more advanced stages, especially primary follicles, and that addition of activin-A and follistatin to the culture medium had no effect on this process. Activin-A (100 ng/ml), however, did promote the in vitro survival and growth of activated follicles in cortical tissue as well as the growth but not the survival of isolated primary follicles. Follistatin did counteract the activin-A effects on isolated primary follicles but not those on cortical tissue-enclosed follicles, probably because of differences in activin-A amounts that are needed for activation of the respective follicle stages. The lower dose of follistatin associated with difficulties to access the follicles within cortical tissue could account for the lack of effect. These results provide a basis for future studies with the aim of growing caprine oocytes and follicles from the earliest (primordial) follicle stage up to the preovulatory stage at which oocytes can be matured and fertilized in vitro.

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