Gene expression for LH receptor, 17 α -hydroxylase and StAR in the theca interna of preantral and early antral follicles in the bovine ovary

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Abstract

The onset of gene expression for three proteins that play pivotal roles in theca interna function, namely the LH receptor (LH-R), cytochrome P450 17 α -hydroxylase (17 α OH) and the steroidogenic acute regulatory protein (StAR), was determined. Ovaries were obtained on day 9 of the oestrus cycle from mature synchronized dairy cows ($n = 5$) and gene expression in preantral and antral follicles up to 4 mm in diameter was evaluated by *in situ* hybridization. LH-R and 17 α OH mRNAs were observed first, in the theca interna of large preantral follicles (type 4), concurrent with its morphological differentiation. StAR mRNA appeared later during follicular growth, in follicles >1 mm in diameter (type 6). LH-R and 17 α OH mRNAs were found exclusively in the thecal cells, whereas StAR mRNA appeared in thecal cells, granulosa cells of late atretic follicles and oocytes. In early atresia, thecal cells expressed all three mRNAs, and their expression decreased gradually as atresia progressed. Atresia in granulosa cells was characterized by massive apoptosis of periantral, but not peribasal cells, that differentiated into luteal-like cells expressing StAR.

In summary, our study suggests that in spite of the presence of 17 α OH, a key enzyme in steroidogenesis, the ability to produce steroids by bovine follicles smaller than 1 mm in diameter must be very limited due to the absence of StAR protein. During the early stages of atresia, thecal cells remain morphologically and functionally healthy, and continue to express all three studied mRNAs.

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Introduction

The development of ovarian follicles is a dynamic process involving highly coordinated proliferation and differentiation of the follicular compartments (theca interna, granulosa cells and an oocyte). The theca interna is known to play a crucial role during this process, providing structural integrity for the follicle and an endocrine milieu through its extensive blood supply. A critical steroid-mediated interaction between thecal and granulosa cells involves the ability of the thecal cells to synthesize androgens, which are used by the granulosa cells for aromatization to oestradiol. Thecal cells have also been shown to produce several non-steroidal paracrine factors which can influence the growth and differentiation of both granulosa and thecal cells (Roberts & Skinner 1991, Parrot & Skinner 1997).

Differentiation of theca interna cells from steroidogenically inactive to androgenic involves expression of the biosynthetic enzymes of the steroid pathway that catalyze cholesterol to progestins and ultimately to androgens.

The first step in steroid biosynthesis is the transport of cholesterol from the outer to the inner mitochondrial membrane by the steroidogenic acute regulatory protein (StAR) (reviewed by Stocco 2001). Conversion of pregnenolone to dehydroepiandrosterone (DHEA) via the Δ^5 steroidogenic pathway, which is preferred in the bovine ovary (Fortune 1986), is controlled by cytochrome P450 17 α -hydroxylase/17–20 lyase (17 α OH, CYP17). Expression of 17 α OH is regulated by luteinizing hormone (LH), which is considered to play a major role in determining thecal cell differentiation (Erickson & Magoffin 1983, McNatty *et al.* 1984a, Fortune 1994). Expression of thecal 17 α OH mRNA precedes that of aromatase in bovine granulosa cells of the growing follicles (Xu *et al.* 1995b, Bao *et al.* 1997).

Over the last decade, the localization and activities of several key proteins in the bovine theca interna during follicle recruitment and selection have been intensively studied (reviewed by Bao & Garverick 1998). Much less attention, however, has been paid to the onset of gene

expression during the early stages of bovine follicle growth. Moreover, the available data are contradictory: Xu *et al.* (1995a, 1995b) reported that the expression of LH-R and 17 α OH mRNAs is first observed shortly after antrum formation. Bao *et al.* (1997) claimed that LH-R and 17 α OH mRNAs are first detected in the preantral follicles. Moreover, information about the onset of StAR expression in bovine follicles is very limited. StAR mRNA was reported to first appear in follicles 'after antrum formation' (Bao *et al.* 1998), but the precise stage of follicle growth was not defined.

The aims of the present study were: (1) to determine the onset of mRNA expression for LH-R, 17 α OH and StAR in bovine follicles, (2) to relate this to the stage of follicle growth, and (3) to determine the effect of atresia on LH-R, 17 α OH and StAR mRNA expression during the early stages of follicle development.

Materials and Methods

Tissue collection and preparation

Mature, cyclic Holstein dairy cows ($n = 5$) were used in this study. Cows were synchronized for oestrus with an invaginal progesterone insert (CIDR; Eazi Breed, Hamilton, NZ) for 9 days, and 500 μ g of cloprostenol, a PGF2 α analogue (Estrumate; Coopers, Berkhamsted, UK) was injected i.m. 7 days after insertion of the progesterone implant. Cows expressed oestrus behavior within 48 h after removal of the CIDR. Ovulation (Day 1) was confirmed by ultrasonography (Aloka, Tokyo, Japan, 7.5 MHz transducer). On Day 7 of the subsequent cycle, PGF2 α analogue (500 μ g) was injected and 40 h later, ovaries were collected at the local abattoir after the cows had been sacrificed. Large (dominant and subordinate) and medium-sized follicles were collected and used in an experiment described elsewhere (Roth *et al.* 2001). To confirm the stage of the cycle, the concentration of oestradiol and progesterone in the follicular fluid of large follicles was measured. The oestradiol:progesterone ratio in the follicular fluid of the dominant follicles was 22.3 ± 4.0 (mean $\pm$ S.E.M.), indicating that the ovaries had been collected before LH surge.

Blocks of ovarian tissue containing cortex with follicles up to 4 mm in diameter (2–3 pieces from each ovary) were collected and fixed immediately in 4% (w/v) phosphate-buffered paraformaldehyde for 24 h, dehydrated and embedded in paraffin wax. Tissue sections were cut (5 μ m thick) and placed onto microscope slides that had been coated with 3-aminopropyltriethoxysilane (Aldrich Chemical Co, Milwaukee, WI, USA), and used for *in situ* hybridization.

This study was reviewed and approved by the National Committee for Animal Experimentation Ethics, and was conducted in accordance with the Israeli Law on Animal Welfare and Experimentation 1994.

Follicle classification

Follicles were classified as described previously (Braw-Tal & Yossefi 1997), with some modifications: (a) primordial follicles (type 1) – oocyte surrounded by flattened granulosa cells; (b) primary follicles (type 2) – oocyte surrounded by one layer of cuboidal cells; (c) small preantral follicles (type 3) – two or three layers of granulosa cells; (d) large preantral follicles (type 4) – four or more layers of granulosa cells; (e) early antral follicles were divided into three groups according to their diameters – 0.4–1.0 mm (type 5), 1.1–2.0 mm (type 6), and 2.1–4.0 mm (type 7). Primary and preantral follicles ($n = 54$) were evaluated in the section through the oocyte nucleus. The diameters of antral non-atretic ($n = 20$) and atretic ($n = 16$) follicles were measured at right angles in the section in which the oocyte or cumulus cells were present, and the mean diameters were calculated. In follicles where such a section could not be found, diameters were measured in the largest estimated cross-section. Antral follicles were examined for the presence of pycnotic and/or apoptotic cells and classified as non-atretic, early atretic (stage I), advanced (stage II) and late atretic (stage III) according to Braw-Tal (1994). Briefly, follicles designated as non-atretic were those with occasional mitotic figs and none or up to five pycnotic/apoptotic nuclei among the granulosa cells. Early atretic follicles (stage I) were those with more than five pycnotic/apoptotic nuclei and none or only occasional mitotic figs among the granulosa cells. Advanced atretic follicles (stage II) were those with no mitotically dividing granulosa cells, more than 10% pycnotic/apoptotic nuclei and atretic bodies in the follicular fluid. Late atretic follicles (stage III) were those in which 1–2 layers of granulosa cells remained or were not present at all, and the oocyte was often necrotic or fragmented. Follistatin gene expression in granulosa cells is a constitutive event throughout most stages of follicle growth and it decreases in atretic follicles (Braw-Tal 1994). We therefore used follistatin gene expression as an additional indicator of follicular status. The theca interna in non-atretic follicles was examined, the cell layers were counted and its morphological appearance was characterized.

Probe preparation

Bovine cDNA probes for StAR, LH-R and 17 α OH were products of RT-PCR reaction using oligonucleotide primers as previously described (Mamluk *et al.* 1998, 1999). PCR products were cloned into pGEM-T vector and individual clones were isolated and sequenced by the Biological Services Unit at the Weizmann Institute of Sciences, Rehovot, Israel. cDNA probes were used as templates for the *in vitro* synthesis of RNA. Ovine follistatin cDNA probe was a generous gift from Dr D Tisdall (Wallaceville Animal Research Center, Upper Hutt, New Zealand). Antisense and sense [α -³⁵S]-UTP-labelled single-stranded RNA was transcribed using SP6/T7

transcription kit (Boehringer Mannheim, Mannheim, Germany) and DNA template removed by incubation with DNase I (1 U/mg DNA; Boehringer Mannheim) for 15 min at 37°C. The RNA was purified by phenol/chloroform extraction and precipitated with 3 M sodium acetate and ethanol, resuspended in 10 mM dithiothreitol (DTT) and stored at -70°C (Braw-Tal *et al.* 1994).

In situ hybridization

Sections were dewaxed in xylene, rehydrated in a graded series of alcohols and rinsed in diethyl pyrocarbonate-treated water. Sections were used for *in situ* hybridization as previously described (Braw-Tal *et al.* 1994). All the sections were sequentially incubated with the following (a) 0.2 M HCl for 10 min at RT, (b) 2 × SSC (1 × SSC is 150 mM sodium chloride, 15 mM sodium citrate) for 30 min at RT, (c) proteinase K (Boehringer Mannheim) 2 µg/ml in 0.2 M Tris-HCl (pH 7.2), 50 mM EDTA for 30 min at 37°C, (d) 2 × SSC for 5 min, at RT. The slides were dehydrated in a graded series of alcohols and air-dried.

Hybridization buffer contained 50% (w/v) formamide, 0.3 M NaCl, 10 mM Tris-HCl (pH 6.8), 10 mM sodium phosphate (pH 6.8), 5 mM EDTA (pH 8.0), 1 × Denhardt's (0.02% w/v) each of BSA, Ficoll and polyvinylpyrrolidone, 10% (w/v) dextran sulphate, 50 mM DTT (dithiothreitol), 1 mg/ml tRNA and 30 000 c.p.m./µl of α-³⁵S-UTP-labelled riboprobe. The hybridization mixture was denatured at 65°C for 5 min and applied to the pre-treated and dried tissue sections and incubated overnight at 48–50°C. After hybridization, the sections were washed stringently in the following solutions: (a) 5 × SSC, 1% (v/v) β-mercaptoethanol (β-ME) for 30 min at 50°C, (b) 2 × 5 SSC, 50% formamide, 1% β-ME for 30 min at 65°C, (c) 2 × SSC three times for 5 min each at 37°C. Tissue sections were treated with RNase A 2 µg/ml (Boehringer Mannheim) in 0.1 M Tris-HCl (pH 7.5), 0.4 M NaCl, 5 mM EDTA for 30 min at 37°C. Slides were then washed in (a) 50% formamide, 2 × SSC at 65°C for 30 min, (b) 2 × SSC at 37°C for 15 min, (c) 0.2 × SSC at 37°C for 15 min. The sections were dehydrated by passing through a graded series of alcohols containing 0.3 M ammonium acetate, air-dried, and coated with liquid emulsion (LM-1; Amersham International plc, Amersham, Bucks, UK). After 2–3 weeks of exposure at 4°C, the slides were developed (D19; Kodak, Rochester, NY, USA), and counterstained with haematoxylin. For each probe, hybridization was repeated three times with sections from different parts of the tissue. The first randomly run sections were hybridized with both sense and antisense [α-³⁵S]-labelled probe's. As the signal for the sense probe was comparable to that of the background (data not shown), all sections were hybridized with antisense probe only. Sections from 54 preantral, 20 antral non-atretic and 16 antral atretic follicles were hybridized with the aforementioned probes and examined.

Image analysis

Hybridization intensity (silver-grain density) was quantified in antral follicles as the number of pixels within a marked area, measured with the Image ProPlus analysis system (Media Cybernetics Inc, Silver Spring, MD, USA). For each antral follicle (12 non-atretic and 8 stage II atretic follicles), three fields at roughly 120° to each other were measured, and the measurement was repeated in two different sections. Background silver grain density was measured in the follicular cavity of the same follicle. The specific hybridization intensity was calculated as the average hybridization intensity for the three different sections of the same follicle minus the average background, and was expressed as the number of pixels per unit area.

TUNEL assay

Apoptosis was assessed by *in situ* 3' end labelling of DNA fragments using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labelling (TUNEL) assay. TUNEL was performed with the ApopTag apoptosis detection kit (Intergen, Norcross, GA, USA) according to the manufacturer's instructions. Briefly, ovarian sections were deparaffinized, rehydrated and incubated with 20 µg/ml Proteinase K (Sigma, St Louis, MO, USA) for 15 min at 37°C. Endogenous peroxidases were neutralized by incubation with 3% H₂O₂. The sections were incubated with TdT for 1 h at 37°C, followed by incubation with anti-digoxigenin-peroxidase antibodies. Sections were stained with DAB solution (3,3'-diaminobenzidine, Sigma) and counterstained with haematoxylin or light green (methyl green).

Statistical analysis

Mean, S.E.M. values and analysis of variance (ANOVA) were computed according to standard procedure. A probability of $P < 0.05$ was considered significant.

Results

Theca interna in non-atretic small bovine follicles

Formation of theca interna

Primordial and primary follicles (types 1 and 2) did not have a morphologically defined theca interna; cells adjacent to the basement membrane were not morphologically different from other stromal cells. Thecal cells were first detected in small preantral follicles (type 3) as a few elongated cells attached to the basement membrane (Fig. 1). In large preantral follicles (type 4), the theca interna was composed of more than one layer of darkly stained cells, arranged parallel to the basement membrane and clearly distinguishable from the stromal cells. As follicular growth progressed, the number of thecal layers increased and in type 7 follicles (2–4 mm in diameter), the theca interna consisted of 14.5 ± 0.6 layers of thecal

Figure 1 Correlation between follicle type and number of thecal cell layers in bovine ovary ($n = 5$). Type 2 are primary follicles; types 3 and 4 are small and large preantral follicles, respectively. Types 5, 6 and 7 are small antral follicles with mean diameters of 0.4–1.0, 1.1–2.0 and 2.1–4.0 mm respectively. Values are mean $\pm$ S.E., $n = 6$ –8 follicles for each type, gc – granulosa cells, th – theca interna.

cells (Fig. 1). The theca interna could be distinguished from the surrounding stroma by richer vascularization, less abundant extracellular matrix, and cells arranged parallel to the basement membrane.

Expression of LH-R, 17 α OH and StAR mRNA

Expression of LH-R mRNA was observed exclusively in the thecal cells. Expression was first detectable in large preantral follicles (type 4) with morphologically defined theca interna cells (Fig. 2B). The hybridization signal appeared mainly in the cells adjacent to the basement membrane. As growth of the follicle progressed, the number of thecal cells expressing LH-R increased. In non-atretic follicles, larger than 2 mm in diameter (type 7), LH-R was strongly expressed in all theca interna cells (Fig. 3B).

Expression of 17 α OH mRNA was found exclusively in theca interna cells with a pattern of expression very similar to that of LH-R. It appeared first in the thecal cells of large preantral follicles (type 4, Fig. 2C) adjacent to the basement membrane. The hybridization signal increased as follicular growth progressed and the number of morphologically differentiated thecal cells increased. The strongest expression of 17 α OH was found in all thecal cells of follicles larger than 2 mm (type 7, Fig. 3C).

Expression of StAR mRNA was not detected in follicles smaller than 1 mm in diameter (Figs 2D and 4B). In larger follicles (type 7), the hybridization signal was strong but was not evenly distributed throughout the thecal layers (Fig. 3D). StAR mRNA expression was most abundant in cells adjacent to the basement membrane and it decreased gradually along the theca layers. In the thecal cells most distant from the basement membrane, no StAR mRNA was detected. There were also some cells near the basement membrane that did not express StAR mRNA.

Figure 2 Gene expression in large preantral bovine follicle (type 4). Bright-field (A) and dark-field (B, C and D) views are shown. Ovarian sections of the same follicle were hybridized with 35 S-labelled LH-R (B), 17 α OH (C) or StAR (D) antisense probe, processed for liquid emulsion autoradiography and counterstained with haematoxylin for visualization. Expression for LH-R and 17 α OH mRNAs in thecal cells was detected. StAR expression was seen in the oocyte but not in thecal cells. o – oocyte, gc–granulosa cells, th–thecal cells. Scale bar = 80 μ m.

Besides thecal cells, expression of StAR was found in the oocytes at all studied stages of follicular development (Figs 2D and 4B).

Atresia in small bovine follicles

Morphological changes

No atretic preantral follicles were found. The first sign of atresia (pycnotic or apoptotic granulosa cells) coincided with the beginning of antrum formation. All advanced and late atretic follicles (stages II and III, respectively) were larger than 2 mm in diameter (type 7).

Early atretic follicles (stage I) had very few (<1%) pycnotic or apoptotic granulosa cells, occasionally, there were some dividing granulosa cells. The theca interna of these follicles did not differ morphologically from the theca of non-atretic follicles.

Advanced atretic follicles (stage II) were characterized by a reduced number of granulosa layers, and many (>10%) pycnotic and apoptotic (TUNEL-positive) granulosa cells within the layers close to and in the antrum. Not all pycnotic cells were TUNEL-positive (Fig. 5A). Peribasal granulosa cells looked morphologically healthy, even in the advanced atretic follicle. The theca interna was hypertrophied, and without its characteristic arrangement of layers parallel to the basement

Figure 3 Gene expression in non-atretic antral follicle, 2500 μm in diameter (type 7). Bright field (A) and darkfield (B, C and D) views are shown. Ovarian sections of the same follicle were hybridized with ^{35}S -labelled LH-R (B), $17\alpha\text{OH}$ (C) or StAR (D) antisense probe, processed for liquid emulsion autoradiography and counterstained with haematoxylin for visualization. Strong expression for LH-R, $17\alpha\text{OH}$ and StAR mRNAs in thecal cells was detected. Note that the hybridization signal for StAR was not evenly distributed throughout the thecal layers: it was most abundant in cells adjacent to the basement membrane (peribasal) and was weak or absent in the most distant cells. gc-granulosa cells, th-theca cells, s-stromal cells, ff-follicular fluid. Scale bar = 10 μm .

membrane. Apoptotic thecal cells were occasionally found in these follicles (Fig. 5A).

Late atretic follicles (stage III) had very few or no pycnotic or apoptotic granulosa cells in the follicular antrum. Granulosa cells attached to the basement membrane were often hypertrophied, showing a luteinized-like appearance. The theca interna was hypertrophied and disorganized.

Expression of LH-R, $17\alpha\text{OH}$ and StAR mRNA

Theca interna in early atretic follicles (stage I) did not show a significant decrease in hybridization signal as compared with non-atretic follicles, in response to the probes used in the present study. As atresia progressed, the hybridization signal for all probes gradually decreased. In theca interna of advanced atretic follicles (stage II) the labelling was significantly lower as compared with non-atretic follicles ($P < 0.05$, Fig. 7). Peribasal granulosa cells of late atretic follicles (stage III) expressed StAR mRNA. StAR expression in the theca interna of these follicles was absent (Fig. 6B).

Expression of follistatin mRNA

Follistatin mRNA was strongly expressed in granulosa cells of all non-atretic (Fig. 5C) and in early atretic (stage I)

follicles. In advanced atretic (stage II) follicles follistatin expression was either weak or absent (Fig. 5C).

Discussion

Our study defines the precise stage during follicular development of the onset of gene expression for three proteins that play a pivotal role in theca interna function, namely LH-R, $17\alpha\text{OH}$ and StAR. We show that mRNAs for LH-R and $17\alpha\text{OH}$ appear very early during follicle growth, when the follicle reaches the large preantral stage (type 4), whereas StAR mRNA is expressed later, in follicles which are at least 1 mm in diameter (type 6). Moreover, our study shows that LH-R and $17\alpha\text{OH}$ expression appears concurrently with the morphological differentiation of the theca interna and remains there during the early stages of follicle atresia. Supporting our findings, Rodgers *et al.* (1986) showed that $17\alpha\text{OH}$ protein expression is concomitant with theca interna differentiation in the bovine ovary. By providing the exact stage of follicular growth at which LH-R and $17\alpha\text{OH}$ mRNAs appear, our study supports and extends the previous findings of Bao *et al.* (1997), but not those of Xu *et al.* (1995a, b), who reported the presence for LH-R and $17\alpha\text{OH}$ mRNAs shortly after antrum formation, and thus later during follicle growth. Neither of the two latter studies related gene expression for these two proteins to

Figure 4 StAR gene expression in non-atretic antral follicle, 500 μm in diameter (type 5). Bright-field (A) and dark-field (B) views are shown. Ovarian section was hybridized with ^{35}S -labelled StAR anti-sense probe (B), processed for liquid-emulsion autoradiography and counterstained with haematoxylin for visualization. StAR expression was seen in the oocyte but not in thecal cells. Oocyte is marked with a broken line, gc-granulosa cells, th-the cal cells, s-stromal cells, ff-follicular fluid. Scale bar = 10 μm .

thecal development. The ontogeny of the expression pattern for LH-R and $17\alpha\text{OH}$ reported herein is similar to that shown by Logan *et al.* (2002) for sheep ovary.

It is well accepted that StAR protein controls the rate-limiting step in steroidogenesis (Stocco 2001). Our study shows that the onset of StAR mRNA expression occurs in antral follicles of 1 mm in diameter. Thus the expression of StAR follows that of LH-R and $17\alpha\text{OH}$. Bao *et al.* (1998) also reported that the mRNAs of LH-R and steroidogenic enzymes occur earlier than that of StAR. It would therefore be reasonable to assume that, in spite of the presence of two key steroidogenic enzymes: $17\alpha\text{OH}$ (Xu *et al.* 1995b, Bao *et al.* 1997, present study) and cytochrome P450 side-chain cleavage (Xu *et al.* 1995b), the ability of thecal cells from bovine follicles smaller than 1 mm in diameter to produce androgens must be very limited, because of the absence of StAR. As shown in our study, granulosa cells of bovine preantral and early antral follicles do not express StAR. Also in more advanced stages of follicle development StAR mRNA was not detected in granulosa cells (Bao *et al.* 1998). In contrast, in sheep

ovary, both thecal and granulosa cells of large follicles express StAR mRNA (Logan *et al.* 2002). This could imply that oestradiol- 17β production by the dominant follicle in cattle, but not in sheep, is completely dependant on the substrate supplied by the thecal cells. This supports the observation of Tian *et al.* (1995), who showed that the increase in oestradiol production by bovine preovulatory follicles is primarily due to the greater availability of substrate secreted by thecal cells rather than an increase in the aromatizing ability of granulosa cells. Thus in the bovine ovary, thecal cells play a critical role in the maintenance of steroidogenesis in the preovulatory follicle; any impairment in thecal cell function will correspondingly affect the function of the preovulatory follicle. As a case in point, we have recently shown that exposure of cows to heat stress results in reduced steroid production by thecal cells and impaired function of the preovulatory follicle (Roth *et al.* 2001).

In our study, we observed that the mRNAs of studied proteins appear first in theca interna cells adjacent to the basement membrane. This suggests that the granulosa cells and/or basement membrane are involved in thecal cell differentiation. The kit ligand (KL), secreted by granulosa cells, has been shown to stimulate cell growth and androgen synthesis in the bovine theca interna *in vitro* (Parrot & Skinner 1997). Moreover, it has been recently implied that the interaction between granulosa and thecal cells depends not only on the paracrine communication between them, but also on their contact with the basal lamina (Rodgers & Irving-Rodgers 2002, Allegrucci *et al.* 2003).

The expression of LH-R, $17\alpha\text{OH}$ and StAR mRNAs increased with increasing follicular size: the hybridization signal became more intense as more cells differentiated and expressed the mRNAs. Follicles larger than 2 mm in diameter strongly expressed LH-R and $17\alpha\text{OH}$ mRNAs in most thecal cells, though some of the cells still remained unlabelled, which implies that theca cells are not a homogeneous population of cells, and that some might be steroidogenically more active than others. Similarly, Conley *et al.* (1995) reported that not all thecal cells express $17\alpha\text{OH}$ protein in sheep or bovine follicles.

Atresia of small antral follicles in cows, as described in the present study, was first evident when pycnotic and apoptotic cells appeared in the granulosa cells adjacent to the antrum. Though apoptosis is the main path of granulosa cell death, not all pycnotic nuclei in the examined follicles were TUNEL positive, suggesting that some granulosa cells died by other death pathways, as reported by Van Wezel *et al.* (1999). Atresia was associated with a gradual decrease in follistatin expression by granulosa cells, as during atresia of the ovine follicle (Braw-Tal 1994). Theca interna cells remained morphologically and functionally healthy during the early stages of atresia, expressing all three of the mRNAs studied here, and continued to produce steroids (McNatty *et al.* 1984a, Henderson *et al.* 1987, Jolly *et al.* 1994). As atresia progressed, there was a decrease in all three mRNAs expression in

Figure 5 Gene expression in advanced atretic (stage II) antral follicle (type 7). Ovarian sections of the same follicle were stained for detection of apoptosis by TUNEL assay (A) or hybridized with ^{35}S -labelled StAR (B), or follistatin (C) antisense probe, processed for liquid-emulsion autoradiography and counterstained with haematoxylin for visualization. Most granulosa cells were TUNEL positive (apoptotic), some pycnotic cells were TUNEL negative. No apoptotic cells were detected in cells adjacent to the basement membrane (closed arrows). Theca interna cells were hypertrophied, with occasional apoptotic cells (open arrows) and no StAR expression. Peribasal granulosa cells expressed StAR mRNA (closed arrows). Strong expression of follistatin was seen in granulosa cells of the non-atretic follicle, but not the atretic one. hf- non-atretic follicle, af- atretic (stage II) follicle, gc-granulosa cells, th-theca cells, s-stromal cells, ff-follicular fluid. Scale bar = 10 μm .

Figure 6 StAR gene expression in late atretic (stage III) follicle (type 7). Only peribasal granulosa cells remained (A). Strong expression for StAR mRNA in peribasal granulosa cells was detected (B). No StAR expression in theca cells. gc-granulosa cells, th-theca cells, s-stromal cells. Scale bar = 10 μm .

theca cells, and apoptotic cells were occasionally detected. In the bovine ovary, theca cells are able to produce androgens long before granulosa cells are able to convert them to oestradiol (follicles > 4 mm in diameter; McNatty *et al.* 1984a, Xu *et al.* 1995b) and they continue to produce steroids long after granulosa cells die (Jolly *et al.* 1994), confirming the central role of the theca in follicular steroidogenesis.

It has been recognized in many species that granulosa cells are not a homogeneous population of cells. Three types of granulosa cells are distinguishable in antral follicles by their spatial location, namely, the periantral, peribasal (cells that are in close proximity to the antrum or the basement membrane respectively) and cumulus cells. They differ, in part, in their morphology (Rodgers *et al.* 2001), onset of gene expression (Braw-Tal 1994), responsiveness to gonadotrophin (Rouillier *et al.* 1996), steroid production (Roberts & Echternkamp 1994), and susceptibility to apoptosis (Hay & Cran 1978, Manabe *et al.* 1996, Van Wezel *et al.* 1999). Our present study provides additional evidence of diversity in the population of granulosa cells in small bovine follicles, namely, the fate of these cells during the atretic process. We observed that periantral cells die by pycnosis or apoptosis and are phagocytosed and removed by neighbouring granulosa cells, as demonstrated previously (Bykov 1979, Van Wezel *et al.* 1999, Rodgers *et al.* 2001). Peribasal cells, on the other hand, did not become apoptotic, but rather differentiated morphologically and functionally into

Figure 7 Hybridization intensity of ^{35}S -labelled LH-R, 17 α OH and StAR antisense probe to theca interna cells of non-atretic (shaded bars, $n = 12$) and advanced atretic (stage II) follicles (light bars, $n = 8$). Note the significant decrease in hybridization intensity in atretic follicles ($P < 0.05$).

luteal-like cells, which lost the ability to express follistatin, but instead expressed StAR (present study) and P450 side-chain cleavage (Clark *et al.* 2004). It has been hypothesized that there are two types of atresia in small bovine follicles: basal and antral (Irving-Rodgers *et al.* 2001, Clark *et al.* 2004). Alternatively, we suggest that these are consecutive stages of the atretic process: in the early stage of atresia, periantral granulosa cells die by apoptosis or pyknosis and are removed from the follicle. As atresia progresses, peribasal cells differentiate into luteal-like cells that express StAR and P450 side-chain cleavage and most likely are able to secrete progesterone. Supporting this concept, it has been shown that as bovine follicles undergo atresia, the progesterone content of the follicular fluid increases significantly (McNatty *et al.* 1984b, Jolly *et al.* 1994). However, the lack of apoptosis in peribasal granulosa cells is not clear. It has recently been suggested that thecal cells secrete factors that suppress apoptosis of granulosa cells in bovine follicles (Tajima *et al.* 2002). This may also be the case here, but further examination is required.

The detection of StAR mRNA in the oocyte was both surprising and unexpected. Similar findings have been reported recently by Logan *et al.* (2002) for sheep and by Pollack *et al.* (1997) for human foetal oocytes. To date, the function of StAR has been associated with steroidogenesis in adrenal glands and gonads. However, the oocyte is not the only non-steroidal cell type where StAR mRNA expression has been found: it has been reported in other non-steroidal tissues, such as human kidney (Pollack *et al.* 1997), trout brain, spleen and ova (Kusakabe *et al.* 2002) and mouse brain (King *et al.* 2002). Whether StAR has roles in metabolic processes other than the transport of cholesterol to the mitochondria remains to be elucidated.

In summary, our study shows that the onset of gene expression for LH-R, 17 α OH and StAR is stage-specific during follicular development. LH-R and 17 α OH mRNAs appear in large preantral follicles (type 4), concomitantly with theca interna differentiation. It is followed by StAR expression in 1 mm antral follicles (type 6). Thus steroidogenesis in bovine follicles potentially starts in follicles ≥ 1 mm. During the early stages of atresia, thecal cells

remain morphologically and functionally healthy, and continue to express all three studied mRNAs.

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