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Expression of mRNA and protein localization of epidermal growth factor and its receptor in goat ovaries

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Summary

To examine the possibility that epidermal growth factor (EGF) and its receptor (EGF-R) are expressed throughout folliculogenesis, we studied the presence and distribution of EGF and EGF-R in goat ovaries. Ovaries of goats were collected and either fixed in paraformaldehyde for immunohistochemical localization of proteins, or used for the isolation of follicles, luteal cells and ovarian surface epithelium to study mRNA expression for EGF and EGF-R, using the reverse transcriptase polymerase chain reaction. EGF protein and mRNA were found in primordial, primary and secondary follicles as well as in small and large antral follicles and in surface epithelium, but in corpora lutea only the protein could be detected. Antral follicles expressed EGF mRNA in oocyte, cumulus, mural granulosa and theca cells. For EGF-R, both protein and mRNA were present at all stages of follicular development and in all antral follicular compartments. EGF-R protein and mRNA were also found in corpora lutea and surface epithelium. It is concluded that EGF and its receptor are expressed in goat ovarian follicles at all stages of follicle development, in corpora lutea, and in ovarian surface epithelium.

Keywords: EGF, EGF receptor, Follicles, Goat, Oocyte, Ovary

Introduction

It is well known that ovarian activities are regulated not only by endocrine hormones but also by autocrine and paracrine local growth factors (Eppig, 2001; Fortune, 2003; Van den Hurk & Zhao, 2005). Among these factors, epidermal growth factor (EGF) seems to be an important regulator of ovarian physiology. EGF is a protein belonging to the EGF family, which consists of at least eight members (Riese & Stern, 1998). EGF protein has been demonstrated in oocyte and granulosa cells of early and late staged follicles (human: Maruo *et al.*, 1993; Bennett *et al.*, 1996, hamster: Roy & Greenwald, 1990; pig: Singh *et al.*, 1995*a*) while the EGF mRNA has been described only in oocyte and granulosa cells from pig antral follicles (Singh *et al.*, 1995*a*). Both protein and mRNA for EGF were also found in rat (Tekpetey *et al.*, 1995) and porcine corpora lutea (Kennedy *et al.*, 1993; Singh *et al.*, 1995*b*).

The action of EGF in both follicles and luteal cells is mediated by a membrane receptor, ErbB1, which belongs to the ErbB superfamily (Riese & Stern, 1998). This EGF receptor (EGF-R) is a glycoprotein transmembrane receptor with an intrinsic tyrosinase-kinase domain in the cytoplasmic portion of the protein (Carpenter, 1999), and binds to at least six different EGF family members: EGF itself, transforming growth factor-α, heparin binding EGF-like growth factor, amphiregulin, betacellulin and epiregulin (Riese & Stern, 1998). EGF-R mRNA and protein have been identified in oocyte and granulosa cells of early- and late-stage follicles (mouse: Hill *et al.*, 1999; rat: Chabot *et al.*, 1986; Feng *et al.*, 1987; hamster: Garnett *et al.*, 2002; cattle, Lonergan *et al.*, 1996; pig: Singh *et al.*, 1995*a*; human: Maruo *et al.*, 1993; Bennett *et al.*, 1996; Qu *et al.*, 2000), and also in luteal cells of pig (Kennedy *et al.*, 1993; Singh *et al.*, 1995*b*) and rat (Tekpetey *et al.*, 1995).

All this evidence suggests that EGF plays a pivotal role in controlling ovarian activity in mammals. Indeed, *in vitro*, we demonstrated a beneficial effect of EGF on oocyte growth in goat primary follicles (Silva *et al.*,

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2004a). EGF also promotes *in vitro* proliferation of porcine granulosa cells obtained from preantral follicles (Morbeck *et al.*, 1993) and growth of early follicles in cow (Gutierrez *et al.*, 2000), hamster (Roy, 1993), mouse (Boland & Gosden, 1994) and human (Roy & Kole, 1998). In antral follicles, EGF has been shown to stimulate *in vitro* oocyte maturation (mouse: Smitz *et al.*, 1998; De La Fuente *et al.*, 1999; sheep: Guler *et al.*, 2000; cattle: Lonergan *et al.*, 1996; human: Goud *et al.*, 1998; pig: Prochazka *et al.*, 2003; Li *et al.*, 2002), cumulus cells expansion (mouse: O'Donnell *et al.*, 2004), granulosa cell proliferation (pig: May *et al.*, 1992) and estrogen production (human: Misajon *et al.*, 1999).

The goat is an ideal model for the transgenic production of therapeutic recombinant proteins in the milk because of the high yield of purified product and relatively short generation interval (Reggio *et al.*, 2001). Thus, it is very important to understand the mechanisms that control folliculogenesis in this species to produce a large number of *in vitro* matured oocytes either to provide cytoplasts for cloning of transgenic goats or to produce large number of zygotes from valuable animals. With regard to EGF and its receptor, thus far, no information is available on their mRNA expression and protein localization in goat early follicles. Data about expression of both protein and mRNA for EGF in goat antral follicles are also lacking, while expression of EGF-R mRNA and protein has been studied only in oocyte and cumulus cells of goat antral follicles (Gall *et al.*, 2004).

The aim of the present study was to examine the expression of EGF and EGF-R mRNA and protein in goat ovaries obtained from slaughterhouses, with special attention to early and late-stage follicles as possible sources of an EGF/EGF-R system. To this end, mRNA expression was detected by reverse transcriptase polymerase chain reaction (RT-PCR) and protein distribution was evaluated using immunohistochemistry.

Materials and methods

Ovaries

During the breeding season, ovaries ($n = 50$) with large antral follicles and/or corpora lutea from slaughtered adult mixed-breed goats were recovered and transported to the laboratory in a Thermos flask, within 1 h. Ten of the ovaries were fixed overnight at room temperature in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4), and subsequently dehydrated and embedded in paraffin wax (Histoplast, Shandon Scientific, Pittsburgh, USA) in preparation for immunohistochemical studies. The remaining 40 ovaries were used to recover cells and tissues for RT-PCR.

Immunohistochemistry

Immunohistochemical study for EGF and EGF-R was performed on serial 5 μm sections cut from 10 ovaries of five different goats. These sections were mounted on poly-L-lysine coated slides, dried overnight at 37 °C, deparaffinized in xylene and rehydrated in a graded ethanol series. Endogenous peroxidase was blocked by incubating the deparaffinized sections in 3% hydrogen peroxide in methanol for 10 min. The sections were then washed with PBS (pH 7.4) and the epitopes activated by microwaving the sections for 7 min at 900 W in 0.01 M citrate buffer (pH 6.0). Following 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 minimize non-specific binding. The primary antibodies used were: (1) rabbit polyclonal anti-EGF (Z-12, Santa Cruz Biotechnology, Santa Cruz, CA) and (2) rabbit polyclonal anti-EGF receptor (SC-03, Santa Cruz Biotechnology), both diluted 1:100 in PBS containing 5% normal goat serum. 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 goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA), 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, Burlingame, CA). The sections were then washed three times in PBS and stained with diaminobenzidine (DAB; 0.05% DAB in Tris/HCl pH 7.6, 0.03% H_2O_2 – Sigma tablets, St Louis, MO) 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, dehydrated in a graded ethanol series and then xylene, and mounted in Depex. The staining intensity for both EGF and EGF-R immunoreactive protein expression was scored as follows: absent (−), occasionally found (−/+), weak (+), moderate (++) or strong (+++). Sections were analysed in this way 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; (2) incubation with DAB reagent alone to exclude the possibility of non-suppressed endogenous peroxidase activity; and (3)

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preabsorbing the antibody (EGF-R) overnight at 4 °C with its blocking peptide at 20-fold excess (Santa Cruz Biotechnology).

Classification and measurement of follicles

Early follicles were classified as (1) primordial (one layer of flattened/cuboidal granulosa cells), (2) primary (a single layer of cuboidal granulosa cells) and (3) secondary (two or more layers of cuboidal granulosa cells). These follicles were considered either healthy, when a morphologically normal oocyte was surrounded by granulosa cells organized in discrete layers, or atretic, when pyknosis was present in oocyte and/or granulosa cells. Secondary follicles with irregular spaces between the layers of granulosa cells, but without pyknotic granulosa cells or degenerating oocytes were classified as healthy, since these irregular spaces are considered to be early signs of antrum formation (Hirshfield, 1983). Antral follicles were classified into two groups: (1) small antral follicles (<3 mm in diameter; with multiple granulosa cells enclosing an antrum) and (2) large antral follicles (3–6 mm). Among the healthy large antral follicles, it was not possible to distinguish between subordinate and dominant follicles. Antral follicles were classified as healthy when pyknotic cells were absent or occasionally present (less than 5% per follicle), while those follicles having more than 5% of pyknotic granulosa cells were considered atretic. The diameter of follicles was calculated according to the method described by Van den Hurk *et al.* (1994).

Collection of cells and tissues for RT-PCR

The recovered ovaries were rinsed in saline (0.9% NaCl) containing antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin). Then, 10 ovaries were allocated for isolation of early-staged follicles and the others were used to provide antral follicles, oocytes, cumulus cells, mural granulosa cells and samples of corpora lutea and ovarian surface.

Early-stage follicles, i.e. primordial, primary and secondary, were isolated using the mechanical procedure described previously (Lucci *et al.*, 1999). Briefly, ovaries were cut individually into small fragments using a tissue chopper (Mickle Laboratory Engineering, Gomshal, Surrey, UK) adjusted to 75 μm. The fragments were then placed in PBS containing 5% bovine serum albumin (Sigma) at room temperature and aspirated 40 times, using a large Pasteur pipette (diameter ∼1600 μm), and 40 times with a smaller pipette (diameter ∼600 μm). The suspension was then filtered successively through 500 and 100 μm nylon mesh filters. After repeated washing to completely remove the stromal cells, follicles from each of the three different categories (primordial, primary and secondary) were placed in separate Eppendorf tubes in groups of 15. All samples were stored at −80 °C until the RNA was extracted. Previously, we had performed histological analysis to confirm the classification of goat preantral follicles 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 using an 18-gauge needle attached to a tube in line with a vacuum pump. From the follicle content thus 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. After removal of denuded oocytes, the remaining cumulus cells were collected separately and washed four times in PBS. Groups of either 10 denuded oocytes or cumulus cells from 10 COCs were packed in tubes and stored at −80 °C until RNA extraction.

To collect theca cells, small ($n = 10$) and large antral follicles ($n = 10$) were isolated from goat ovaries ($n = 5$) and dissected free of stromal tissue using forceps, as described previously for bovine ovaries (Van Tol & Bevers, 1998). Those follicles that had denuded oocytes and signs of atresia were discarded. The follicles were then bisected and the mural granulosa cells scraped off using a scalpel blade, and then washed and stored until RNA extraction. Next, the theca cell layers were vortexed for 1 min in 1 ml HEPES-buffered M199 (Gibco BRL, Paisley, UK) supplemented with penicillin (100 μg/ml) and streptomycin (100 μg/ml) to remove contaminating mural granulosa cells, transferred to a fresh 1 ml of buffer, vortexed for another minute, 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. Since ovaries collected from slaughterhouses were used it was not possible to determine the exact stage of luteal phase. Three samples of each tissue sample were collected and analysed. Appropriate tests to ensure the purity of samples (oocytes, cumulus cells, mural granulosa cells or theca cells) had previously been performed using gene differential expression. In these previous studies, GDF-9 was demonstrated in granulosa cells but not in the theca, while Kit Ligand was absent in oocytes and present in granulosa cells (Silva *et al.*, 2004*b*, *c*).

Extraction of total RNA and reverse transcription

Isolation of total RNA combined with on-column DNase digestion was performed using the RNeasy mini kit and the RNase-free DNase set (Qiagen, Valencia, USA). Following the manufacturer's instructions,

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

Target gene	Primer sequence (5′ → 3′)	Sense	Position	GenBank accession no.
EGF	CCAGGTTCTCTTAAGTGC	s	48–65	GI: 1706938 (1996)
	ACCAAGAGCTGCTCTCTG	as	151–168	*Ovis aries* EGF
	GCTTCACTTCCATACCACCA	as	165–184	
EGF-R	AGCCTCCAGAGGATGTTCAA	s	392–411	GI: 1628549 (1996)
	CCTCTGATGATCTGCAGGTT	as	551–570	*Homo sapiens* EGF-R

s, sense; a, antisense.

350 µl lysis buffer was added to each frozen sample and the lysate aspirated through a 20-gauge needle before being centrifuged at 10000 *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, Valencia, USA) 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 using RNase-free DNase (340 Kunitz units/ml) for 15 min at room temperature. After washing 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 in a total volume of 20 µl made up of 10 µl of sample RNA, 4 µl 5× reverse transcriptase buffer (Gibco BRL, Breda, The Netherlands), 8 units RNAsin, 150 units Superscript II reverse transcriptase (BRL), 0.036 U random primers (Life Technologies, Leiden, The Netherlands) and containing 10 mM dithiothreitol (DTT) and 0.5 mM of each dNTP. The mixture was successively incubated for 1 hr at 42 °C and 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 by PCR

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 containing 2 mM $MgCl_2$, 200 µM of each dNTP, and 0.5 µM each of primers and 0.625 units *Taq* DNA polymerase (HotStarTaq, Qiagen, Valencia, USA) in 1× PCR buffer. The primers for EGF-R and EGF used for amplification are presented in Table 1.

The thermal cycling profile for EGF and EGF-R during amplification was: 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 50 °C and 45 s at 72 °C. Final extension was for 10 min at 72 °C. During the amplification of EGF cDNA, heminesting was used to increase the specificity and sensitivity. For heminesting, 1 µl of the first round product was transferred to another 200 µl tube containing 24 µl amplification mixture, and amplified for 25 cycles using the same thermal cycling profile. All reactions were performed in a 24-well thermocycler (Perkin-Elmer, Gouda, The Netherlands). Finally, 10 µl of the product was resolved by electrophoresis in 1% agarose gel containing ethidium bromide. A 100 base pair (bp) DNA ladder (Gibco BRL) was included as a reference for fragment size and image of each gel was recorded using a digital camera (Olympus C-4040, New York, USA).

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

Results

Immunohistochemistry

The immunolocalization of EGF and EGF-R proteins in healthy goat follicles is illustrated in Fig. 1 and Table 2. In primordial, primary and secondary follicles, a moderate EGF immunoreaction was generally observed in oocyte and granulosa cells, with the exception of the oocyte of secondary the follicle that had a weak detection (Fig. 1*A*–*C*). In the secondary follicles, a weak reaction was also present in early theca cells (Fig. 1*C*). In small antral follicles, strong EGF immunostaining was localized in oocyte, cumulus cells and mural granulosa cells, but theca cells had weak staining (Fig. 1*D*). In large antral follicles, oocyte and theca cells showed weak EGF immunostaining while cumulus cells and mural granulosa cells had moderate staining (Fig. 1*E*). In addition, moderate to strong and strong EGF immunoreactivity is generally observed in corpora lutea (Fig. 1*F*) and ovarian surface epithelium (Fig. 1*G*), respectively. Both oocyte and granulosa cells from atretic follicles were irregularly stained for EGF (not shown). No specific immunoreaction was observed when control stainings were carried out (Fig. 1*H*).

The EGF-R protein was immunohistochemically demonstrated in healthy oocytes of primordial,

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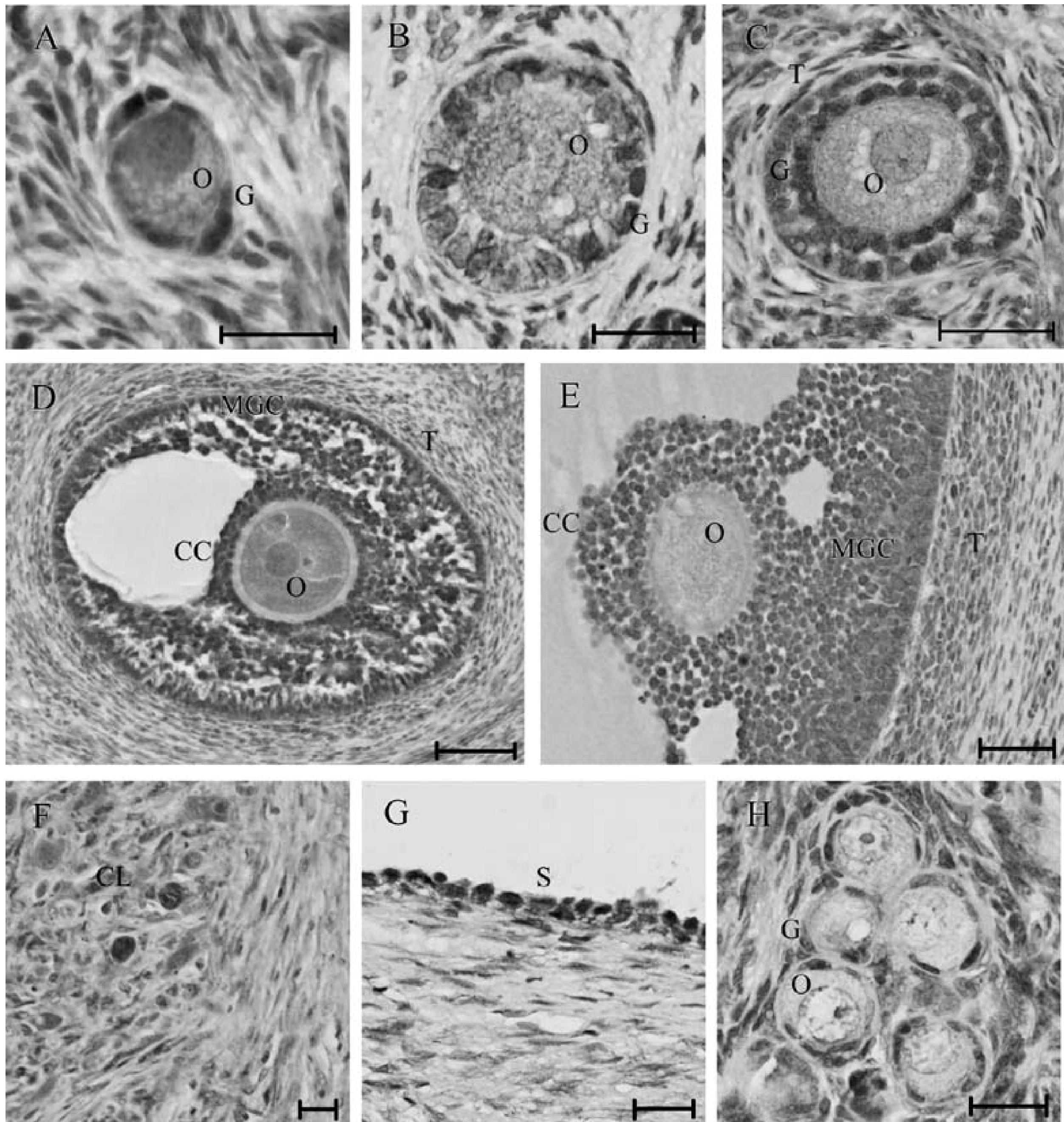

Figure 1 EGF immunoreactivity in healthy ovarian follicles, corpus luteum and ovarian surface epithelium. (*A*) Primordial follicle, (*B*) primary follicle, (*C*) secondary follicle, (*D*) small antral follicle, (*E*) large antral follicle, (*F*) corpus luteum, (*G*) ovarian surface epithelium, (*H*) negative control. 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 (*A*–*C*, *F*–*H*), 50 μm (*D*) and 100 μm (*E*).

primary and secondary follicles (Fig. 2*A*–*C*). The protein was distributed throughout the cytoplasm of the oocytes, while the staining intensity in primordial follicles was stronger than in primary and secondary follicles (Table 2). Occasionally, granulosa cells of primordial follicles were weakly stained for EGF-R (Fig. 2*A*), but a moderate immunoreaction was observed in granulosa cells of primary and secondary follicles (Fig. 2*B*, *C*, Table 2). No EGF-R staining was detected in the theca of secondary follicles (Fig. 2*C*). Oocytes of small antral follicles showed strong EGF-R immunoreactivity, but cumulus cells, mural granulosa cells, and occasionally theca cells were weakly stained (Fig. 2*D*). In large antral follicles, moderate EGF-R immunostaining was present in all follicular compartments, except for the oocytes, which

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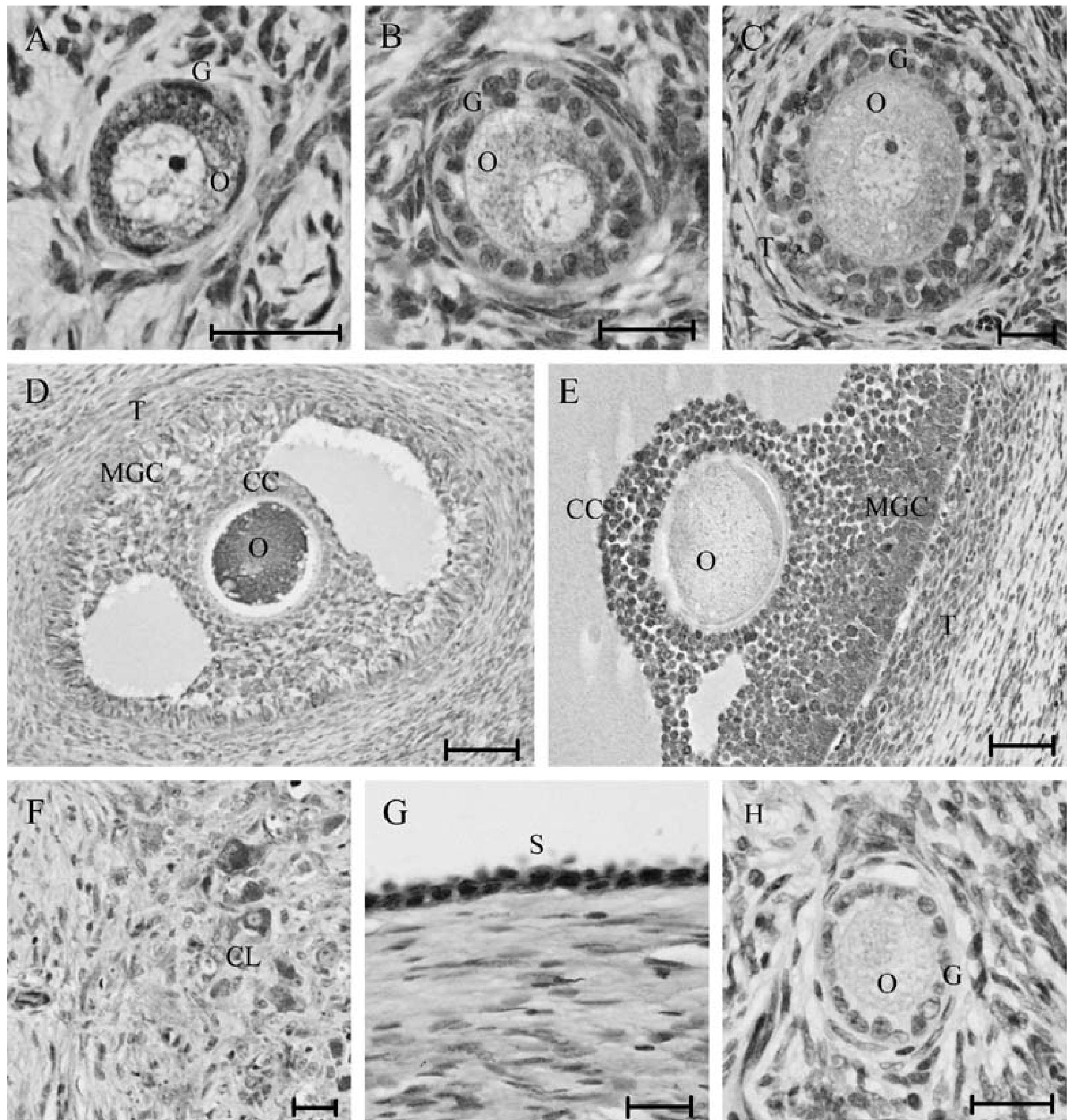

Figure 2 EGF-R immunoreactivity in healthy ovarian follicles, corpus luteum and ovarian surface epithelium. (*A*) Primordial follicle, (*B*) primary follicle, (*C*) secondary follicle, (*D*) small antral follicle, (*E*) large antral follicle, (*F*) corpus luteum, (*G*) ovarian surface epithelium, (*H*) negative control. 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 (*A–C*, *F–H*), 50 μm (*D*) and 100 μm (*E*).

were weakly stained (Fig. 2*E*). Follicles with signs of atresia were irregularly stained for EGF-R (not shown). Apart from follicles, corpora lutea and ovarian surface epithelium immunoreacted with the EGF-R antibody, moderately to strongly (Fig. 2*F*) and strongly (Fig. 2*G*), respectively. No specific immunoreaction was observed when control stainings were carried out (Fig. 2*H*).

Expression of mRNA for EGF and EGF-R in goat ovaries

Using primers for EGF, the first round of amplification yielded abundant products only in samples of cDNA prepared from oocytes collected from small and large antral follicles. After heminesting, however, transcripts for EGF were observed in cDNA from primordial,

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Table 2 Localization of mRNA and relative intensity of immunohistochemical staining for EGF and EGF-R in the ovaries of goats

	EGF		EGF-R	
Ovarian structure	Protein	mRNA	Protein	mRNA
Primordial follicle				
Oocyte	++	+[a]	++	+[a]
Granulosa	++		−/+	
Primary follicle				
Oocyte	++	+[a]	+	+[a]
Granulosa	++		+	
Secondary follicle				
Oocyte	+	+[a]	+	+[a]
Granulosa	++		++	
Theca cells	+		−	
Antral follicle (<3 mm)				
Oocyte	+++	+	+++	+
Cumulus cells	+++	+	+	+
Mural granulosa cells	+++	+	+	+
Theca cells	+	+	−/+	+
Antral follicle (>3 mm)				
Oocyte	+	+	+	+
Cumulus cells	++	+	++	+
Mural granulosa cells	++	+	++	+
Theca cells	+	+	++	+
Corpus luteum	++/+++	−	++/+++	+
Surface epithelium	+++	+	+++	+

[a]Whole follicles.
−, absent; −/+, occasionally found; +, weak; ++, moderate; +++, strong immunoreaction.

primary and secondary follicles as well as from cumulus, mural granulosa or theca cells collected from small or large antral follicles. EGF expression was also detected in ovarian surface epithelium, but no EGF mRNA was detected in corpus luteum (Fig. 3). Amplification of –RT blanks or water controls yielded no specific products in any of the reactions.

With the use of specific primers for EGF-R, amplification of cDNA from primordial, primary and secondary follicles and from oocytes, cumulus cells, mural granulosa and theca cells from small or large antral follicles in all cases resulted in an abundant product after one round of amplification. Expression for EGF-R was also detected in corpus luteum and ovarian surface (Fig. 3). Amplification of –RT blanks or water controls yielded no specific products in any of the reactions.

Figure 4 shows the sequence of the amplified product for EGF and EGF-R. At the nucleotide level, EGF product showed 93% identity with EGF cDNA from ovine species. Similarly, the EGF-R product displayed 97% homology with EGF-R cDNAs from bovine. These results confirmed the specificity of EGF and EGF-R products. A truncated EGF-R has been described in human species, but to our knowledge there is no information about this truncated form in ruminants.

Discussion

The present study examined the distribution of EGF and EGF-R mRNA and protein in goat ovaries, to determine whether EGF signalling may play a role in ovarian functioning in this species, and particularly in folliculogenesis. With regard to EGF, we demonstrated the presence of protein in oocyte and granulosa cells of

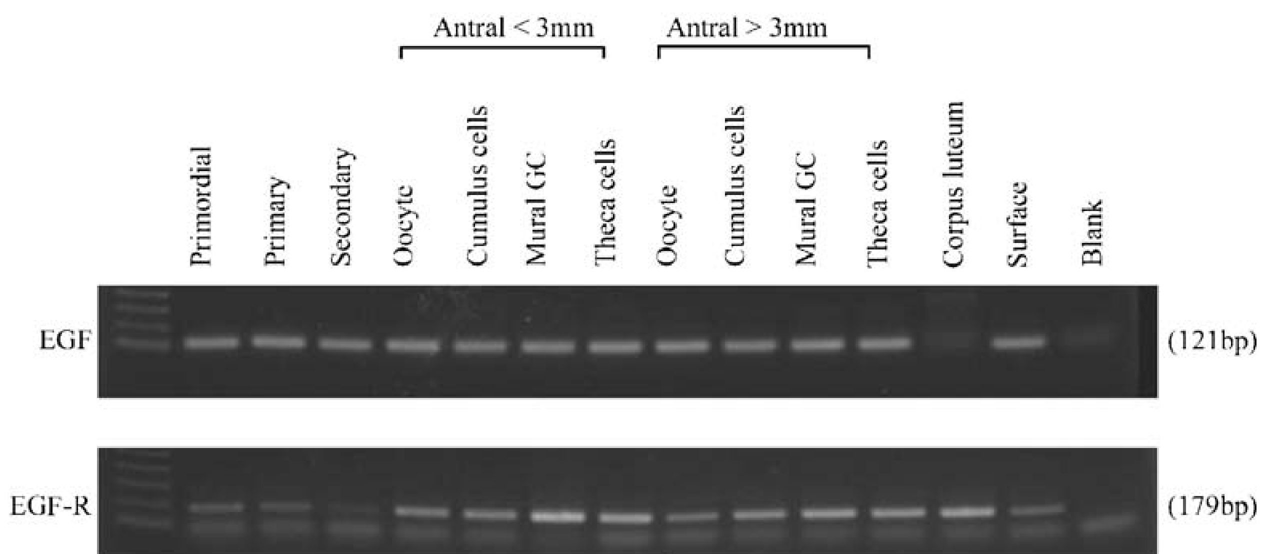

Figure 3 Expression of EGF and EGF-R mRNA in different follicle and cell types in goat ovaries. Follicle and cell types are indicated at the top. One-hundred base pair ladders are included as markers for fragment size.

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A)
gEGF    CTTAAAATAGCCTACAAATACAGCAATTGATCCAGTAGCACGGTACAGGT  -50
oEGF    CTTAAAATATCCTGCAAATATAGCAATTGATCCAGTAGCAAGGTACAGGT

gEGF    TTGTGTTTTGCTCTTCAGAGGTGGCAGGCAGCCTT                 -85
oEGF    TTGTGTTTTGGTCTTCAGAGGTGGCTGGCAGCCTT

B)
gEGF-R  CAACTGTGAGGTGGTCCTTGGGAATTTGGAAATTACCTACATGCAGAGTG  -50
bEGF-R  CAACTGTGAGGTGGTCCTTGGGAATTTGGAAATTACCTACATGCAGAGTA

gEGF-R  GTTACAACCTTTCTTTTCTCAAGACCATCCAGGAGGTTGCCGGCTATGTA  -100
bEGF-R  GTTACAACCTTTCTTTTTTCAAGACCATCCAGGAGGTTGCCGGATATGCA

gEGF-R  CTCATTGCCCTCAACACAGTGGAGAAGATTCCGCTGGAA             -139
bEGF-R  CTCATTGCCCTCAACACAGTGGAGAAGATTCCGCTGGAA
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Figure 4 Nucleotide sequence of the amplified product of (*A*) goat EGF (gEGF) and (*B*) EGF-R (gEGF-R) and their alignment with the corresponding ovine EGF (oEGF) and bovine EGF-R (bEGF-R).

primordial, primary and secondary follicles. A similar distribution of EGF was described previously for pig (Singh *et al.*, 1995*a*) and human (Bennett *et al.*, 1996) early-staged follicles. Using RT-PCR, the current study demonstrated the expression of mRNA for EGF in caprine primordial, primary and secondary follicles and, to our knowledge, is the first study to describe EGF mRNA expression in mammalian early follicles. The mRNA and the protein for EGF-R were both detected in primordial, primary and secondary goat follicles. This supports our previous conclusion that EGF is involved in oocyte growth of goat primary follicles *in vitro* (Silva *et al.*, 2004a). In other species, both protein and mRNA for EGF-R are demonstrated in early follicles (pig: Singh *et al.*, 1995*a*; human: Maruo *et al.*, 1993; Bennett *et al.*, 1996; Qu *et al.*, 2000; hamster: Garnett *et al.*, 2002). *In vitro* studies with preantral follicles showed that EGF promotes growth (cow: Wandji *et al.*, 1996; Saha *et al.*, 2000, Gutierrez *et al.*, 2000; sheep: Hemamalini *et al.*, 2003; buffalo: Gupta *et al.*, 2002) proliferation of their granulosa cells (pig: Morbeck *et al.*, 1993), and that it reduces the rate of atresia (cow: Wandji *et al.*, 1996). Additionally, EGF has been shown to regulate the expression of TGFß receptors (hamster: Yang & Roy, 2001) and connexin 43 (pig: Bolamba *et al.*, 2002; rabbit: Kennedy *et al.*, 2003) in preantral follicles. Connexin 43 is essential for gap junction formation in granulosa cells and plays a critical role in early follicle growth at least in rodents, since the absence of its gene disrupts progression of follicles beyond primary stages in transgenic mouse ovaries (Juneja *et al.*, 1999).

In both small and large caprine antral follicles, mRNA and protein for EGF and its receptor were present in oocyte, cumulus cells, mural granulosa cells and theca cells. This expression pattern argues for autocrine and paracrine roles for EGF in directing the development of goat antral follicles. In goat antral follicles, EGF-R expression was recently described to be present in the oocyte and in cumulus cells (Gall *et al.*, 2004), while in agreement with our present findings, both protein and mRNA have been found in all antral follicle compartments of other species (pig: Singh *et al.*, 1995*a*; hamster: Garnett *et al.*, 2002; human: Maruo *et al.*, 1993). *In vitro* studies with antral follicles have demonstrated that EGF stimulates oocyte maturation (mouse: Smitz *et al.*, 1998; Merriman *et al.*, 1998; De La Fuente *et al.*, 1999; sheep: Guler *et al.*, 2000; cattle: Lonergan *et al.*, 1996; Sakaguchi *et al.*, 2002; human: Goud *et al.*, 1998; pig: Singh *et al.*, 1997, Prochazka *et al.*, 2000, 2003). In cumulus cells, EGF promotes Ca^{2+} efflux and improves their expansion during maturation (Hill *et al.*, 1999; O'Donnell *et al.*, 2004). It was furthermore reported that EGF stimulates granulosa cells proliferation *in vitro* (pig: May *et al.*, 1992) and estrogen production through aromatase activation (human: Misajon *et al.*, 1999; goat: Behl & Pandey, 2001). There is evidence for EGF regulating the *in vitro* cellular activities of granulosa cells either by inhibiting LH receptor production (Hattori *et al.*, 1995) or inhibin secretion (Serta & Seibel, 1993) or by stimulating FSH receptor expression (Luciano *et al.*, 1994) and binding affinity (May *et al.*, 1987). Very recently, Park *et al.* (2004) demonstrated that LH stimulation induces transient expression of the EGF family members amphiregulin, epiregulin and betacellulin in antral follicles. *In vitro*, these growth factors promote the morphological events triggered by LH, including cumulus expansion and oocyte maturation, which supports the idea that EGF-related growth factors are paracrine mediators that propagate the LH

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signal throughout the follicle, via EGF-R (Park *et al.*, 2004).

Apart from follicles, EGF, EGF-R protein and EGF-R mRNA were detected in goat corpora lutea, suggesting a possible role of EGF in luteal activity. The presence of EGF protein but not EGF mRNA in goat corpora lutea points to an EGF origin different from luteal cells. This is in contrast with data from other species, in which EGF mRNA and protein have both been demonstrated in luteal cells (rat: Tekpetey *et al.*, 1995; pig: Kennedy *et al.* 1993, Singh *et al.*, 1995*b*). The importance of EGF/EGF-R for luteal activity is supported by Tekpetey *et al.* (1995), who demonstrated EGF-induced progesterone production by rat luteal cells that had been cultured *in vitro*.

The strong expression of both mRNA and proteins for EGF and EGF-R in goat ovarian surface epithelium (OSE) also suggests a role for EGF/EGF-R at this site. A paracrine effect of EGF from ovarian surface on neighbouring follicles cannot be excluded. It is well established that the OSE must proliferate to repair the ovulatory defects in the ovarian surface and that approximately 90% of the ovarian cancers arise in the OSE (Auersperg *et al.*, 2001). Studies on expression of EGF and its receptor in healthy OSE are very limited but EGF-R has been localized in the malignant OSE (van der Burg *et al.*, 1993). Moreover, EGF and EGF-R-like peptides are expressed in about one-half of all ovarian tumours; yet their role in tumorigenesis is unclear (van Haaften-Day *et al.*, 1996). McClellan *et al.* (1999) demonstrated that EGF regulates proliferation and apoptosis in human ovarian surface epithelial cells. Aberration in the expression of the EGF/EGF-R system in OSE thus may evoke a deviation of the normal proliferation and programmed death of its cells, which may lead to tumour formation.

In conclusion, the present study demonstrates that EGF and its receptor are expressed in goat ovarian follicles at all stages of follicle development, in corpora lutea, and in ovarian surface epithelium. This widespread distribution of EGF and its receptor shows that, in goat ovaries, it may play an important role in various processes, including early and advanced folliculogenesis, luteal activity and surface epithelium behaviour. The demonstrated presence of an EGF/EGF-R system in early-staged follicles supports previous findings of *in vitro* studies, in which we showed a stimulating effect of EGF on oocyte growth in goat primary follicles.

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