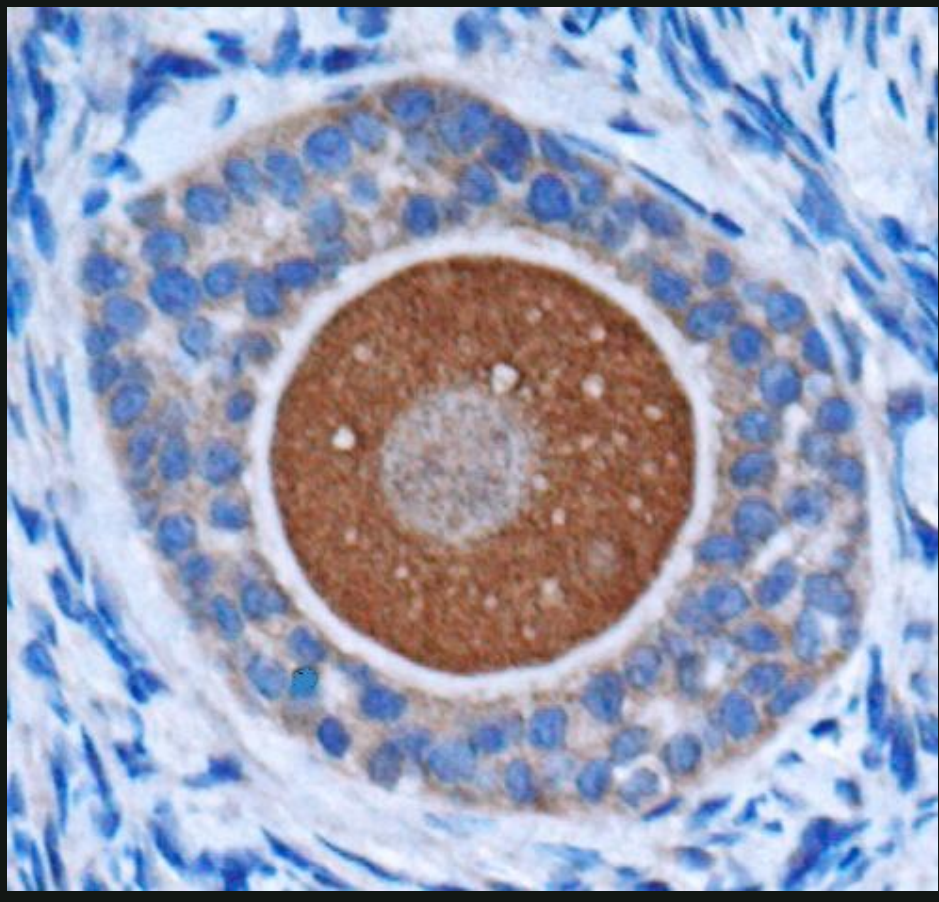
Growth factors in goat ovaries and the role of activin-A in the development of early-staged follicles



José Roberto Viana Silva

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Growth factors in goat ovaries and the role of activin-A in the development of early-staged follicles

Groeifactoren in ovaria van geiten en de rol van activine-A bij de ontwikkeling van vroege follikelstadia

(met een samenvatting in het Nederlands)

Fatores de crescimento em ovários caprinos e o papel da ativina-A no desenvolvimento folicular inicial

(com resumo em Português)

Proefschrift

ter verkrijging van de graad van doctor aan de
Universiteit Utrecht op gezag van de
Rector Magnificus, Prof. Dr. W.H. Gispen,
ingevolge het besluit van het College voor Promoties
in het openbaar te verdedigen op
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José Roberto Viana Silva

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To my grandmother, Helena.

À minha avó Helena, dedico.

Contents

Chapter 1

General Introduction

Goats are among the first mammalian species that have been domesticated. They are economically important because of their production of milk, meat and fiber, being a major source of protein in the tropics. It is well known that goats are seasonally polyestrous, the young being born during the most favorable time of the year, the spring. The length of the sexual season varies with day length, breed, and nutrition. This seasonality is governed by photoperiodicity with estrus activity commencing during a period of decreasing day length. In the tropical zones, where there is less variation in day length, goats tend to breed throughout the year. However, high environmental temperature and lack of food may restrict ovarian cyclicity during some months of the year in the tropics. Shortly after the onset of the rainy season, sexual activity increases, which is probably due to change in food availability (Jainudeen & Hafez 1993). For female goats, sexual activity includes a regular ovarian cycle, culminating in the production of one or more mature (preovulatory) follicles (7 mm) per 21 days, which after a luteinizing hormone (LH) surge may ovulate, thereby producing a fertilizable oocyte.

As in most mammals, the ovaries of a goat are composed of a medulla and a cortex and surrounded by a surface epithelium, commonly known as germinal epithelium. The ovarian medulla consists of irregularly arranged fibroelastic connective tissue and extensive nervous and vascular systems that reach the ovary through the hilus (Fig. 1). The ovarian cortex contains ovarian follicles and corpora lutea at various stages of development or regression. The connective tissue of the cortex consists of fibroblasts, collagen and reticular fibers (Fig. 1). The process of follicular growth in the ovary is termed folliculogenesis. In this process a preovulatory follicle, containing a fully grown oocyte, millions of highly differentiated granulosa cells and a theca consisting of an internal and an external layer, is formed from a primordial follicle which is composed of a small oocyte surrounded by only a few pregranulosa cells.

Folliculogenesis can be divided into the following developmental phases: (1) initiation of primordial follicle development, also termed follicle activation, and formation of primary follicles, (2) transition of primary into secondary follicles, (3) growth of secondary follicles and formation of antral follicles, and (4) growth and differentiation of antral follicles and formation of preovulatory follicles. Understanding the mechanisms that control folliculogenesis in goats is very important to increase their reproductive effectiveness as well as to generate a large number of in-vitro matured oocytes, either to produce embryos from valuable animals or to provide cytoplasts for cloning of transgenic goats. The latter is relevant, because recently, goats have also been considered an ideal model for the transgenic production of therapeutic recombinant proteins in the milk because of their high yield of purified product and relatively short generation interval (Reggio *et al.* 2001).

Most of our present knowledge on these steps of folliculogenesis is restricted to rodents, human and ruminants different from goats. The study in this thesis focuses on the protein and mRNA expression of growth factors in goat ovary and on in vitro culture of primordial and primary follicles. In the first part of this introduction, general information about different developmental phases of folliculogenesis will be given, with emphasis on goats. Then, local growth factors that may control folliculogenesis in goats and in vitro models to study folliculogenesis will be discussed. Finally, an overview is given of the contents of the various chapters in this thesis.

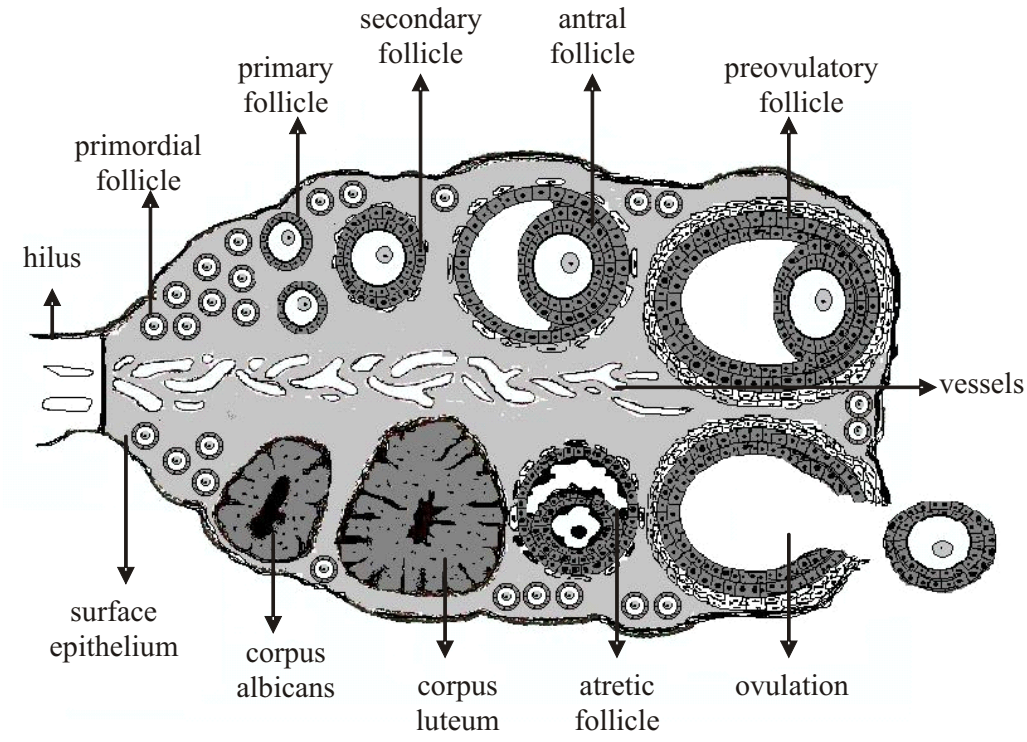

Figure 1. A schematic diagram of the mammalian ovary showing the different structures that can be found in cortex and medulla.

Folliculogenesis

During early fetal development, gonads are colonized by primordial germ cells. After arriving in the primitive gonads, the germ cells are named oogonia. When mitotic activity of the oogonia ceases and meiosis starts, the oogonial germ cells are defined as primary oocytes. Primordial follicles are formed after association of pregranulosa cells with oocytes, which at that stage are arrested in the diplotene stage of the first meiotic division (van den Hurk & Zhao 2005). Thus, a primordial follicle consists of an oocyte surrounded by one layer of flattened pregranulosa cells. In goat ovaries, primordial follicles have a mean diameter of 33 μm and are first observed at day 62 of fetal development (Bezerra *et al*. 1998). As soon as the stock of primordial follicles has been established, some follicles begin to grow, while others remain quiescent. The mechanisms governing initiation of growth of primordial follicles are still not completely understood. Primordial follicles are located in a thin, poorly vascularized layer of the cortex, under the tunica albuginea (van Wezel & Rodger 1996). It is known that early follicle development, including the transition from primordial to primary follicles, is gonadotropin-independent and is regulated mainly by intraovarian factors (van den Hurk

& Zhao 2005). The first trigger for initiation of growth has not been well characterized and might arise in the oocyte itself or in its surrounding cells. Most probably, primordial follicle activation is regulated by a balance between inhibitory and stimulatory factors of local origin. Some studies, especially with rodents, demonstrated that stimulatory factors for primordial follicle activation are Kit ligand (KL - Parrott & Skinner 1999), growth differentiation factor-9 (GDF-9 - Vitt *et al*. 2000), basic fibroblastic growth factor (bFGF- Nilsson *et al*. 2001) and leukemia inhibitory factor (LIF Nilsson *et al.* 2002). In the past few years, evidence has accumulated that points to anti-Mullerian hormone (AMH- Durlinger *et al.* 2002) as a negative regulator of primordial follicle activation.

The initiation of follicular growth is characterized by the start of the proliferation of granulosa cells together with growth of the oocyte. Follicles are called primary when the single layer of granulosa cells surrounding the oocyte becomes cuboidal. Primary follicles have a diameter of approximately 50 μm and are first observed in goat ovaries at day 71 of fetal development (Bezerra *et al.* 1998). During growth of primary follicles, granulosa cells undergo proliferation and the oocyte increases its size and protein content (Picton *et al.* 1998). When two or more layers of granulosa cells have been developed and theca cells can be discerned from the surrounding stroma, secondary follicles are formed. Secondary follicles are observed in goat fetal ovaries at about day 80 of gestation (Bezerra *et al*. 1998). The local growth factors that have been involved in the transition of primary into secondary follicles in species other than goats are activin-A (cow: Hulshof *et al*. 1997), GDF-9 (mice: Dong *et al*. 1996), bone morphogenetic protein-15 (BMP-15) (sheep: Galloway *et al.* 2000), bFGF (cow: Nuttink *et al*. 1996), transforming growth factor-β (TGF- β) (mice: Liu *et al*. 1999) and epidermal growth factor (EGF) (cow: Wandji *et al*. 1996).

Secondary follicles, from a stage with two layers of granulosa cells up to early antrum formation have a diameter from 83 to 130 μm, respectively (Bezerra *et al*. 1998). Local compounds, including intrafollicular factors which are associated with secondary follicle development, in species other than goat are activin-A (rat: Zhao *et al.* 2001, sheep: Thomas *et al*. 2003), keratinocyte growth factor (KGF - rat: McGee *et al.* 1999), growth hormone (GH - rat: Liu *et al.* 1998), TGF-β (mice: Liu *et al.* 1999), EGF (cow: Gutierrez *et al.* 2000), insulin-like growth factor-I (IGF-I, cow: Gutierrez *et al.* 2000), bFGF (Wandji *et al*. 1996), GDF-9 (mice: Hayashi *et al.* 1999) and BMP-15 (sheep: Juengel *et al*. 2002). Gonadotropins like follicle stimulating hormone (FSH, mice: Cortivrindt *et al.* 1997, cow: Gutierrez *et al.* 2000) and LH (mice: Wu *et al.* 2000) can also promote growth of secondary follicles. After intense proliferation of granulosa cells in secondary follicles, the formation of a fluid filled cavity, the antrum, within a multilayered granulosa is characteristic for antral follicles.

Antral follicles (> 130 μm) are first observed in goat ovaries at day 110 of fetal development (Bezerra *et al.* 1998). Antral follicle development is characterized by a phase of basal growth, recruitment, selection and dominance, successively. The early antral follicles possess mRNA for FSH receptors throughout their granulosa, but are relatively independent of gonadotropins during their initial growth period. When FSH secretion was blocked in heifers, antral follicles developed up to approximately 3 mm and

then they stopped growing (Crowe *et al*. 2001). After reaching 3 mm, however, goat and bovine antral follicles become dependent of gonadotropins for continuing of their growth (Crowe *et al.* 2001, Rubianes & Menchaca 2003). Ultrasonographic studies have indicated that the oestrous cycle of goats is characterized by a wavelike pattern of follicle development in the ovaries (de Bulnes *et al.* 1999). A follicle wave involves the emergence of a group of antral follicles from which commonly one or two follicles are selected to grow to more than 5 mm. According to different authors the number of follicular waves per cycle ranges between two and five, but the predominant pattern for goats with oestrous cycles of normal length (19-22 days) is four waves (de Castro *et al.* 1999). The emergence of waves 1, 2, 3 and 4 (ovulatory wave) occur on days 0, 5-6, 10-11, and around day 15 post-ovulation, respectively (Rubianes & Menchaca 2003).

Antral follicle selection takes places during a decrease of FSH levels in the presence of high pulsatile secretion of LH and is a process whereby, dependent on the species, one or more dominant follicles will develop. These latter follicles secrete large amounts of oestradiol and inhibin and have the potency to ovulate. The remaining follicles of the cohort become subordinate and enter atresia. The existence of follicular dominance in small ruminants remained controversial for a long time (Driancourt *et al.* 1991). However, today more data support the concept that dominance is also operative in the goat, especially during the first and the ovulatory wave (Ginther & Kot 1994, Rubianes & Menchaca 2003). Although recruitment, selection and dominance of antral follicles are controlled by gonadrotopins FSH and LH, several locally produced growth factors, like activin, inhibin, follistatin, EGF, IGFs and GDF-9, modulate or mediate the effects of both FSH and LH, resulting in an inhibiting or a stimulatory effect on follicles (reviewed by van den Hurk & Zhao 2005).

Small follicles that do not have an antrum yet are often termed early-staged, preantral, unilaminar or multilaminar follicles. Early-staged follicles refer to primordial, primary and secondary follicles. Such follicles are also frequently called preantral follicles (as we will do in this thesis), although some authors use the term preantral follicle for primary and secondary follicles or even only for the late secondary follicle, which has developed a theca layer capable of steroid production. Unilaminar follicles contain only one layer of granulosa cells around the oocyte and thus include primordial and primary follicles, whereas multilaminar follicles refer to secondary follicles with two or more layers of granulosa cells.

Local growth factors as candidates to control folliculogenesis in goats

During the last decade, the role of growth factors in ovarian folliculogenesis has been extensively studied in mammals, especially in rodents. There is a growing body of evidence that members of the TGF-β superfamily members, such as TGF-β, activins, inhibins, BMPs, GDF-9 and AMH, play a key role in follicular development and atresia (reviewed by Fortune 2003, van den Hurk & Zhao 2005). In addition, other growth factors like IGFs, FGFs and nerve growth factors may play a role in folliculogenesis as do proteins, like LIF and KL. We have chosen to study the possible importance of KL and

the TGF-β superfamily members activin-A, BMP-15, GDF-9 from our studies on folliculogenesis in goats. To this aim we investigated the expression sites of the mRNA and the proteins of these growth factors in goat ovaries as well as those of their receptors and the activin binding protein follistatin. Furthermore, effects of activin-A and follistatin on the in vitro development of goat primordial and primary follicles were studied.

Kit ligand and c-kit

KL is expressed as either membrane-bound or soluble proteins that arise from alternatively spliced mRNAs (Huang *et al.* 1992). Both transcripts, when translated, yield membrane associated products; however, one form, KL-1, is efficiently cleaved due to the presence of a proteolitic cleavage site and is released as a soluble product. The other form, KL-2, lacks this proteolitic cleavage site and therefore remains more stable on the membrane (Fig. 2 - Huang *et al.* 1992). Both the soluble and the transmembrane form of KL are biologically active (Toksoz *et al.* 1992). The receptor for KL, c-Kit, is a member of the tyrosine kinase family. The structure of this receptor includes an extracellular domain and a cytoplasmic domain with tyrosine kynase domain (Fig. 3). The kinase domain is interrupted by a kinase insert sequence that divides the kinase domain into an ATP binding region and a phosphotransferase region (Fig. 3 - Broud 1997). Binding of KL triggers c-Kit receptor homodimerization and intramolecular tyrosine phosphorilation of the receptor, followed by phosphorilation of various substrates and association with signaling molecules, thereby activating distinct signaling cascades (Heldin 1995).

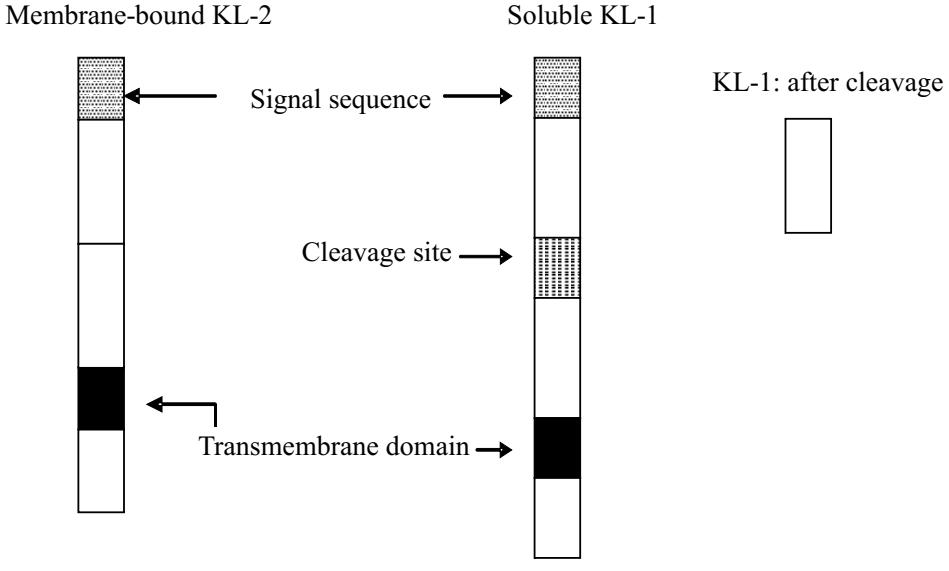

Figure 2. Structure of the membrane-bound (KL-2) and soluble (KL-1) forms of kit ligand. KL-1 is released after cleavage and removal of the signal sequence.

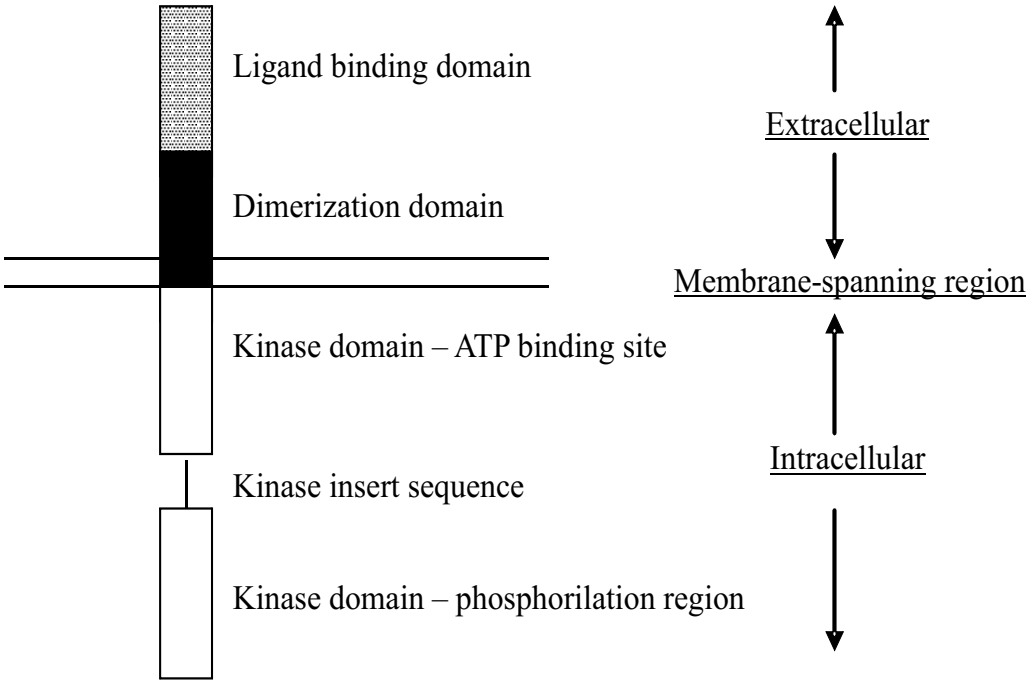

Figure 3. Structure of the c-Kit receptor.

KL is thought to have many roles in follicular development since it is essential for the colonization of the gonad by germ cells in the ovine fetus and has been implicated in the activation and early progression of primordial follicle development in rodents (Yoshida *et al.* 1997, Parrot & Skinner 1999). Importantly, mice lacking KL or c-Kit are severely deficient in primordial germ cells and are sterile (Dolci *et al.* 1991, Kissel *et al.* 2000). It has also been shown in vitro, that KL and its interaction with its receptor, c-Kit, have a role in primordial follicle activation, theca cell recruitment, antrum formation, steroidogenesis and oocyte quality (Parrott & Skinner 1997, 1999, Reynaud *et al.* 2000). In species other than goats, KL is expressed in granulosa cells while its receptor c-Kit is found in oocyte and theca cells (Driancourt *et al.* 2000).

TGF-β superfamily members

The TGF-β superfamily of growth factors include more than 30 structurally related mammalian proteins that have diverse functions during ovarian folliculogenesis. They can be grouped into three sub-families: the TGF-β sub-family, the activin sub-family and the bone morphogenetic protein sub-family that includes GDF-9 (Lin *et al.* 2003). TGF-β ligands interact with type I and type II signaling receptors (Massagé & Chen 2000). Both receptors contain a short extracellular domain that binds ligand, and a

large intracellular serine/threonine kinase domain. Type I and type II receptor kinase domains phosphorylate distinct cellular substrates. While type II receptors are constitutively active, Type I receptors are activated by type II receptors (Massagé 2000). Ligands generally bind type II receptors with high affinity followed by recruitment of a lower affinity type I receptor into a complex, although certain BMP ligands can bind directly to type I receptors without requiring prior type II receptor interaction (Shimasaki *et al.* 2004). Activated type I receptors phosphorylate intracellular receptor-regulated signal transducers R-SMAD proteins, which associate with a cytoplasmic common-mediator (Co-SMAD) forming a complex that translocates to the nucleus to mediate transcription (Massagé & Wotton 2000).

- Activin-A and follistatin

Activins and inhibins are structurally related and form a distinct sub-family of the TGF-β superfamily. Inhibins are made up of α-β dimers, whereas the activins are made up of β-β dimers. There are two forms of inhibins known which are composed of different β-subunits, leading to inhibin-A (α-βA) and inhibin-B (α-βB) formation. Similarly, combination of β subunits lead to activin-A (βA-βA), activin-B (βB-βB) and activin AB (βA-βB) (Fig. 4). Four types of activin receptors have been identified and they were named activin receptors type IA, IB, IIA and IIB (Table 1). Activin-A first binds to ActR-IIA or ActR-IIB and then, preferentially to ActR-IB. Follistatin is structurally unrelated to the activins and inhibins, but binds with high affinity to the β-subunits and so is able to neutralize the activity of inhibin, and more particularly, activin forms (Phillips 2005). Otsuka *et al.* (2001) have also demonstrated that follistatin can inhibit the action of BMP-15. There are two forms of follistatins, i.e., follistatin-315 and follistatin-288, and the only difference in structure is the additional 27 amino acids at the carboxyl terminal end of follistatin 315 (Fig. 4 - Lin *et al.* 2003). Recently, a separate gene product, follistatin like-3, has been isolated and shares some of the biological properties of follistatin and is structurally similar to some extent, but lacks a consensus heparin-binding sequence or other means for binding to cell surfaces (Schneyer *et al.* 2003). Unfortunately, current knowledge on the biological role of the different follistatin isoforms is far from complete.

Several studies, in species other than goats, have demonstrated the importance of the activin-follistatin system in the control of ovarian function (reviewed by van den Hurk & Zhao 2005). In vitro, activin-A stimulates granulosa cell proliferation in preantral / early antral follicles and up-regulates FSH receptors and FSH-induced aromatase activity (rat: Xiao *et al.* 1992). In addition, activin-A delays luteinization and atresia in large antral follicles and enhances oocyte maturation. On the other hand, follistatin attenuates the above action of activin-A and may promote luteinization and atresia of large antral follicles. The expression of activin-A and follistatin is, dependent of the species, found in oocytes, granulosa and/or theca cells (Knight & Glister 2003).

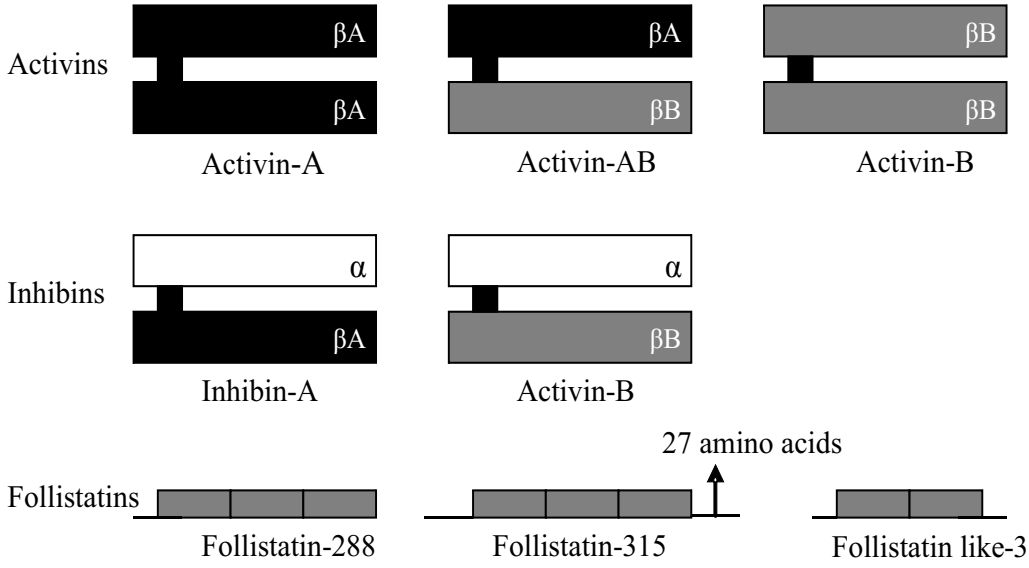

Figure 4. Schematic structure of activins and follistatins 288, 315 (with additional 27 amino acids), and follistatin like-3 that lacks a consensus heparin-binding sequence.

- GDF-9 and BMP-15

Like other members of the TGF-β superfamily, GDF-9 and BMP-15 are synthesized as preproproteins comprised of a signal peptide, a prodomain and a mature domain (Laitinen *et al.* 1998). GDF-9 and BMP-15 possess a proteolitic cleavage site between the pro and mature domain where a specific protease binds and separate these two domains as an important part of the post-translational processing of these molecules (Dube *et al.* 1998). Importantly, all of the evidence shows that for the proteolytic cleavage of the TGF-β superfamily member proproteins to occur, the proproteins must first dimerize (Hogan 1996). Some studies have shows that GDF-9 and BMP-15 form noncovalently linked homodimers, and when coexpressed also form heterodimers (Fig. 5, Liao *et al.* 2003). Three types of BMP receptors have been identified, i.e., BMP receptors type IA, IB and II (Table 1). Although the pattern of BMP receptor oligomerization is not well understood, it has been proposed that BMP-15 may first bind to BMP receptor type IB, followed by recruitment of BMP receptor type II (Shimasaki *et al.* 2004). Recent evidence suggests that GDF-9 signals through known receptors and intracellular SMADs of the TGF-β superfamily. GDF-9 binds to type II BMP receptor (Vitt *et al.* 2002) and subsequently seems to activate TGF-β type I receptor (Mazerbourg *et al.* 2004).

The crucial role of GDF-9 in follicle development and female fertility has been demonstrated by loss-of-function studies in mice in which it was shown that female mice lacking GDF-9 are infertile due to a block in folliculogenesis at one-layer primary follicle stage (Dong *et al.* 1996). Female mice lacking BMP-15 are subfertile and exhibit only minimal histopathological defects (Yan *et al*. 2001), but ewes homozygous for

inactivating mutations in BMP-15 are sterile, because of the arrest of follicular development at the primary stage (Galloway *et al*. 2000). In the species studied so far, GDF-9 and BMP-15 are expressed mainly in the oocyte from primary follicles onward (Findlay *et al.* 2002). In vitro studies have demonstrated that both GDF-9 and BMP-15 promote granulosa cell proliferation and stimulate primordial and primary follicle development in rodents (Vitt *et al.* 2000, Fortune 2003).

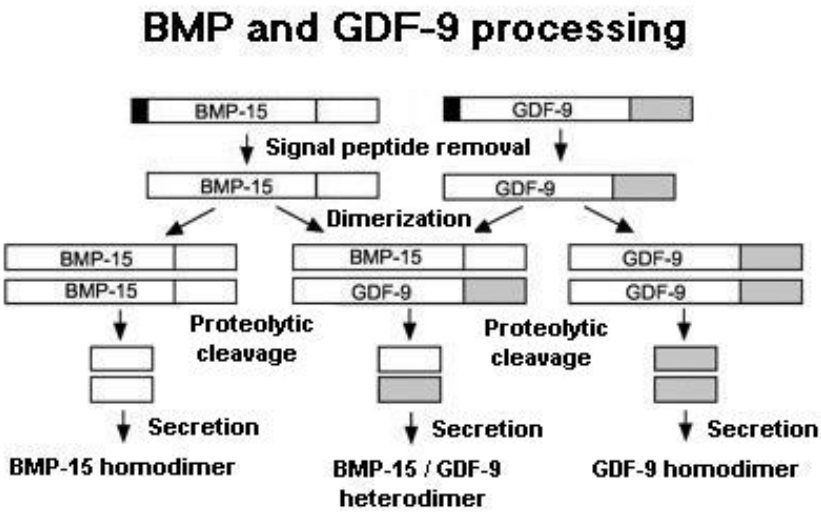

Figure 5. Schematic structure of bone morphogenetic potein-15 (BMP-15) and growth differentiation factor-9 (GDF-9).

Table 1. Receptors types I and II and intracellular Smads proteins for activin-A, GDF-9 and BMP-15.

Ligands	Type II receptor	Type I receptor	Smads	Reference
Activin-A	ActR-IIA ActR-IIB	ActR-IB	Smad 2/3	Ten Dijke *et al.* 1993
GDF-9	BMPR-II	TGFβR-IB	Smad 1/5/8	Vitt *et al.* 2002 Mazerbourg *et al.*2004
BMP-15	BMPR-II	BMPR-IB	Smad 1/5/8	Moore *et al*. 2003

In vitro models to study folliculogenesis

A variety of methods have been developed for maintaining viability and promoting growth of preantral follicles in vitro (Hartshorne 1997). In rodents, the whole ovary is small enough to be kept alive in organ culture and this method is useful for studying factors that may affect the entry of primordial follicles into the growing pool (termed follicle activation). In vitro, some primordial follicles in newborn rodent whole ovaries activate spontaneously and progress to primary and then secondary follicles in a

fashion that appear temporally and qualitatively normal (Eppig & O´Brien 1996) and this model has been used to test factors that might control primordial follicle activation. In contrast, the size of ovaries of large mammals does not allow their culture as intact units. However, methods for culturing small pieces of ovarian cortex, the area where the pool of resting follicles is located, have been developed for studies of primordial follicle activation and growth of activated primary follicles in several species: cattle (Braw-Tal & Yossefi 1997), baboons (Wandji *et al.* 1997), woman (Hovatta *et al.* 1997) and goats (Silva *et al.* 2004). Although most primordial follicles are activated within in-vitro cultured cortical pieces and develop into primary follicles (Wandji *et al.* 1997), few follicles are able to progress to the secondary stage. Mechanical methods have been developed to isolate and purify large numbers of intact primary / secondary follicles from ovaries of goats (Lucci *et al.* 1999), sheep (Cecconi *et al.* 1999), cows (Figueiredo *et al.* 1993), rats (Zhao *et al.* 2000) and mice (Lenie *et al.* 2004). Such isolated follicles can be cultured as intact spherical units to study the in vitro effects of hormones and growth factors on primary or secondary follicles.

Several groups have used these models to study the development of early-staged follicles in rodents and ruminants (reviewed by Smitz & Cortvrindt 2002, Fortune 2003). The yields of good-quality oocytes from these in vitro cultures have been rather limited, especially in ruminants, and much effort is still required to improve culture conditions. Only with the mouse model in vitro culture of primordial follicles resulted in the birth of 59 descendants so far. In this report, 66% of the oocytes obtained from in vitro cultured follicles were competent to undergo germinal vesicle breakdown (GVB). Then, 53% of the oocytes that underwent GVB cleaved to the two-cell stage after in vitro insemination. Twenty-two percent of two-cell stage embryo developed to the blastocyst stage, being 8% the proportion of blastocysts to oocytes developed in vitro. Of 1160 transferred embryos derived from in-vitro grown oocytes, 59 pups were born and survived successfully (O´Brien *et al.* 2003). Additionally, experiments by the group of Smitz in Brussels (Belgium) have established a mouse culture system in which primary (<100μm) and secondary follicles (100-130μm) were cultured as single units up to the mature, fertilizable stage and which gave rise to live young (Cortvrindt *et al.* 1996, Smitz & Cortvrindt 2002, Lenie *et al*. 2004). In domestic animals and human, however, this technology is still far from successful. Several independent experiments have demonstrated transition from primordial to primary follicle stage (bovine: Braw-Tal & Yossefi 1997, caprine: Silva *et al*. 2004), growth of primary / early secondary follicles (bovine: Figueiredo *et al.* 1994, Hulshof *et al.* 1995) and transition from late secondary to early antral follicles (bovine: Gutierrez *et al.* 2000, ovine: Cecconi *et al.* 1999). There are only a few reports of limited success using in vitro culture of large secondary follicles that have progressed to advanced antral stages. The oocytes in the in vitro formed antral follicles were almost fully grown, but their meiotic competence remained low, indicating that the culture methods still require further improvement (ovine: Cecconi *et al.* 1999).

In the experiments reported in this thesis, we incubated ovarian cortical pieces and mechanically isolated primary follicles to study the in-vitro development of goat primordial and primary follicles, respectively.

Overview of the thesis

The studies described in this thesis aim to increase the understanding of ovarian folliculogenesis in goats by studying gene expression and protein localization for selected growth factors and by culturing early follicles in vitro. In *Chapter 2*, we examined the possible existence of an intrafollicular activin-follistatin-activin receptor system and its distribution in ovaries of goats to find evidence for a possible role of this system in ovarian folliculogenesis. Therefore, mRNA expression of activin-A (βA subunit), its type I and type II receptors and its binding protein follistatin were investigated, using reverse transcription polymerase chain reaction (RT-PCR), while the presence and cellular localization of the corresponding proteins were analyzed by use of immunohistochemistry. In *Chapter 3,* experiments were carried out to examine the possible effects of activin-A and follistatin on primordial follicle activation during culture of goat ovarian cortical slices, and on the in vitro growth of mechanically isolated goat primary follicles. Parameters such as oocyte and follicle diameter, follicular atresia and DNA fragmentation, and expression of activin-A, follistatin, GDF-9, BMP-15 and KL were studied before and after culture. In *Chapter 4*, the mRNA expression and protein localization of GDF-9, BMP-15 and BMP receptors type II, IA and IB in ovarian structures were investigated, while in *Chapter 5*, a similar approach was used for KL and c-Kit to obtain evidence for the synthesis and presence of these factors in the various-staged follicles, to find evidence for their role in goat folliculogenesis. Finally, in *Chapter 6*, a general discussion summarizes the results of the study and reflects on possible future studies on folliculogenesis in goats.

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Chapter 2

Gene Expression and Protein Localisation for Activin-A, Follistatin and Activin Receptors in Goat Ovaries

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Abstract

We studied the protein and mRNA expression for activin-A, follistatin and activin receptors in goat ovaries to find evidence of their possible role in ovarian activity, particularly in the various stages of follicle development. Ovaries of cyclic goats were collected and then either fixed in paraformaldehyde for immunohistochemical localisation of activin-A, follistatin, activin receptors IIA/B (ActR-IIA/B) and IA (ActR-IA) proteins or used to obtain samples to demonstrate mRNA expression of activin-A (*ß*A subunit), follistatin, ActR-IIA, -IIB, -IA and -IB, using RT-PCR. For this latter goal, primordial, primary and secondary follicles were isolated mechanically, washed to remove the stromal cells and then used for RT-PCR. In addition, oocytes, cumulus, mural granulosa and theca cells from small (< 3 mm) and large (3-6 mm) antral follicles, luteal cells and surface epithelium were collected to study mRNA expression. Activin-A and follistatin proteins were found in oocytes of all follicle classes, granulosa cells from the primary follicle stage onwards, theca cells of antral follicles, corpora lutea and ovarian surface epithelium. In antral follicles, these proteins were detected both in cumulus and mural granulosa cells. ActR-IIA/B protein was found at the same follicular sites, and additionally in granulosa cells of primordial follicles onward. The localisation of ActR-IA corresponded with that of ActR-IIA/B, but the former protein was absent in the theca of large antral follicles. The mRNAs for activin-A (βA subunit), follistatin, and ActR-IIA, -IIB, -IA and -IB were detected at all follicular and cellular types studied, except that ActR-IIB was not found in follicles that had not developed an antrum yet. In conclusion, in goat ovaries, transcripts of activin-A (βA subunit), its receptors and its binding protein follistatin are expressed and their proteins formed at all follicular stages and in corpora lutea. These findings implicate a role of activin-A in the local regulatory system during the entire follicular development and during luteal activity.

Keywords: goat, follicles, activin-A, follistatin

Introduction

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

Activin was originally identified as a peptide growth factor from the ovarian follicular fluid that stimulates FSH secretion in cultured anterior pituitary cells (Vale *et al*. 1986). It is a homodimer or heterodimer of two similar but distinct β subunits (βA and βB). The dimerization of activin β subunits gives rise to three forms of activin, i.e., activin A (βAβA), activin B (βBβB), and activin AB (βAβB). Both ßA and βB subunits can also combine with the subunit and form inhibin-A (αβA) and and -B (αβB), respectively. In

the ovary, activin-A is the most studied type (Tisdall *et al*. 1994, Yokota *et al*. 1997, Zhao *et al*. 2001, Thomas *et al*. 2003) and plays an important role in ovarian follicle development (Ohshima *et al*. 2002, Lovell *et al*. 2003), but activin-B has also been described (Bristol & Woodruff 2004). The activities of activin are modulated by its binding protein follistatin, which was originally purified from mammalian follicular fluid as a FSH inhibitor (Phillips & de Krester 1998). Follistatin binds activin with high affinity, and its binding effectively neutralises the bioactivities of activin in a variety of target tissues (Knight & Glister 2001, Fisher *et al*. 2003).

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

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

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

Materials and Methods

Ovaries

During the breeding season, ovaries (n=56) were recovered from slaughtered cyclic adult mixed-breed goats and transported to the laboratory in a thermos flask, within 1h. Sixteen of those 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 Ltd, Pittsburgh, USA) in preparation for immunohistochemical studies. The remaining 40 ovaries were used to recover cells and tissues for RT-PCR.

Immunohistochemistry

Localisation of activin-A, follistatin and ActR-IIA/B and -IA was performed on serial 5-µm sections cut from ovaries of eight 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 and the epitopes were 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 were: (1) rabbit anti-activin-A (Innogenetics, Ghent, Belgium), (2) mouse anti-follistatin (R&D System Europe, Abingdon, Oxon, UK), (3) rabbit anti-act-RIIA that also cross-reacts with ActR-IIB (Celgen, Leuven, Belgium) and mouse anti-ActR-IA (R&D System Europe, Abingdon, Oxon, UK). All antibodies were diluted 1:50. Unfortunately, the available anti-ActR-IB is not suitable for use in goat tissue. The sections were incubated overnight at 4ºC in appropriate dilutions of the antibodies. All other incubations and washes were performed at room temperature. After incubation with an antibody, sections were washed three times with PBS-T and incubated for 45 min with biotinylated secondary antibody (goat 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 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, USA). 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, USA) for a maximum of 20 min. The stained sections were rinsed in PBS and water, and counterstained for 10 sec in Mayer's haematoxylin. Finally, the sections were washed for 10 min in running tap water, and subsequently dehydrated in a graded ethanol series followed by xylene treatment and mounting in Pertex (Cellpath Ltd., Hemel Hempstead, UK). The staining intensity was scored as follows: absent (-), weak (+), moderate (++), or strong (+++). Sections were analysed by two independent researchers. Controls for non-specific staining were performed by (1) replacing the primary antibody with IgGs from the same species in which the specific antibody was raised, at the same concentration, and (2) incubation with diaminobenzidine reagent alone to exclude the possibility of non-suppressed endogenous peroxidase activity.

Classification and measurement of follicles

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

Collection of cells and tissues for RT-PCR

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

Early-stage follicles, i.e. primordial, primary and secondary, were isolated using the mechanical procedure described previously (Lucci *et al*. 1999). After isolation, these follicles were washed repeated times to completely remove the stromal cells, and then placed per category into separate Eppendorf tubes in groups of 15. This procedure was completed within 2 h and all samples were stored at -80ºC until the RNA was extracted. In previous work from our group, histological analysis was performed to confirm goat preantral follicle classification after isolation (Lucci *et al*. 1999).

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

either 10 denuded oocytes, cumulus cells from 10 COCs or samples of mural granulosa, and stored at -80°C until RNA extraction.

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

Extraction of total RNA and reverse transcription

Isolation of total RNA combined with on-column DNAse digestion was performed using the RNeasy mini kit and the RNAse-free DNAse set (Qiagen, Valencia, USA). As per the manufacturer's instructions, 350 µl lysis buffer was added to each frozen samples and the lysate was 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 a proteinase K treatment (6,7 mAU/ml, Qiagen) at 55°C for 10 min. Thereafter, all lysates were diluted 1:1 with 70% ethanol and introduced to a mini column. After binding of the RNA to the column, DNA digestion was performed 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 70^0C, and chilled on ice. Reverse transcription was then performed in a total volume of 20µl made up of 10µl of sample RNA, 4µl 5X reverse transcriptase buffer (Gibco BRL, Breda, The Netherlands), 8 units RNAsin, 150 units Superscript II reverse transcriptase (Gibco BRL, Breda, The Netherlands), 0.036 U random primers (Life Technologies BV, Leiden, The Netherlands) and containing 10 mM DTT and 0.5 mM of each dNTP. The mixture was incubated for 1 h at 42^0C, for 5 min at 80^0C and then stored at -20^0C. Minus RT blanks were prepared under the same conditions, but without inclusion of reverse transcriptase.

Amplification of cDNA

PCR reactions were carried out in 200 µl tubes (Biozym, Landgraaf, The Netherlands), using 1 µl cDNA as template in 25 µl of a mixture 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). The primers used for amplification of inhibin/activin A subunit, follistatin, Act-RIIA, -RIIB, -RIA, -RIB, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are presented in Table 1.

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

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

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

The thermal cycling profile for the first round of PCR was: initial denaturation and activation of the polymerase for 15 min at 94^{0}C, followed by 40 cycles of 15 sec at 94^{0}C, 30 sec at 55^{0}C and 45 sec at 72^{0}C. Final extension was for 10 min at 72^{0}C. During the amplification of inhibin/activin A subunit and ActR-IIB cDNA, hemi-nesting was used to increase the specificity and sensitivity. For hemi-nesting, 1 µl of the first round product was transferred to another 200 µl tube with 24 µl PCR amplification mixture containing the second antisense primer, and amplified for 25 cycles using the same thermal cycling profile. All reactions were performed in a 24-well thermocycler (Perkin-Elmer, Gouda, The Netherlands). Finally, ten µl of the product was resolved by electrophoresis in 1% agarose gels containing ethidium bromide. A 100 base pair (bp) DNA ladder (Gibco BRL) was included as a reference for fragment size and images of each gel were recorded 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

Protein localisation for activin-A, follistatin and activin receptors

Activin-A and follistatin proteins were detected in oocytes of primordial follicle stage onward (Fig. 1A, J); and in granulosa cells of primary (Fig. 1B, K), and secondary follicles (Fig. 1C, L). Occasionally, activin-A was found in granulosa cells of primordial follicles (Fig. 1A), but no reaction for both activin-A and follistatin was found in theca cells from secondary follicles (Fig. 1C, L). ActR-IIA/B and ActR-IA proteins were found

in oocyte and granulosa cells of primordial (Fig. 2A, J), primary (Fig. 2B, K) and secondary follicles (Fig. 2C, L), but not in theca cells of the latter follicles.

In antral stages, all follicular compartments, i.e. the oocyte, cumulus cells, mural granulosa cells and thecal cells generally had a moderate to strong reaction for both activin-A (Fig. 1D, E, F) and follistatin (Fig. 1M, N, O), except for small antral follicles that had a weak reaction for activin-A (Fig. 1D, Table 2). Additionally, these same sites had a positive reaction for both ActR-IIA/B (Fig. 2D, E, F) and ActR-IA (Fig. 2M, N, O), except that theca cells of large antral that did not show reaction for ActR-IA (Fig. 2O). Apart from follicles, immunoreactions for activin-A, follistatin, ActR-IIA/B and ActR-IA were observed in corpora lutea (Fig. 1G, P) and in ovarian surface epithelium (Fig. 1H, Q). The relative intensity of immunohistochemical staining for activin-A, follistatin, ActR-IIA/B and ActR-IA in all cell types studied is illustrated in Table 2. For all antibodies tested, control reactions (Fig. 1I, R and 2I, R) confirmed the absence of non-specific staining.

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

Amplification of cDNA from primordial, primary and secondary follicles resulted in specific products for inhibin/activin βA subunit, follistatin, ActR-IIA, -IA and IB, but not for ActR-IIB (Fig. 3). Transcripts for both βA subunit of activin (653 bp), follistatin (566 bp) and all activin receptors (IIA [527 bp], IIB [184 bp], IA [431 bp] and IB [315 bp]) were also detected in cDNA from oocytes, cumulus, mural granulosa and theca cells collected from small and large antral follicles as well as from corpus luteum and ovarian surface epithelium (Fig. 3). Sequence analysis of the amplified βA subunit of activin, follistatin, ActR-IIA, -IIB, -IA and -IB products confirmed their specificity. Amplification of -RT blanks or water controls yielded no specific products in any of the reactions.

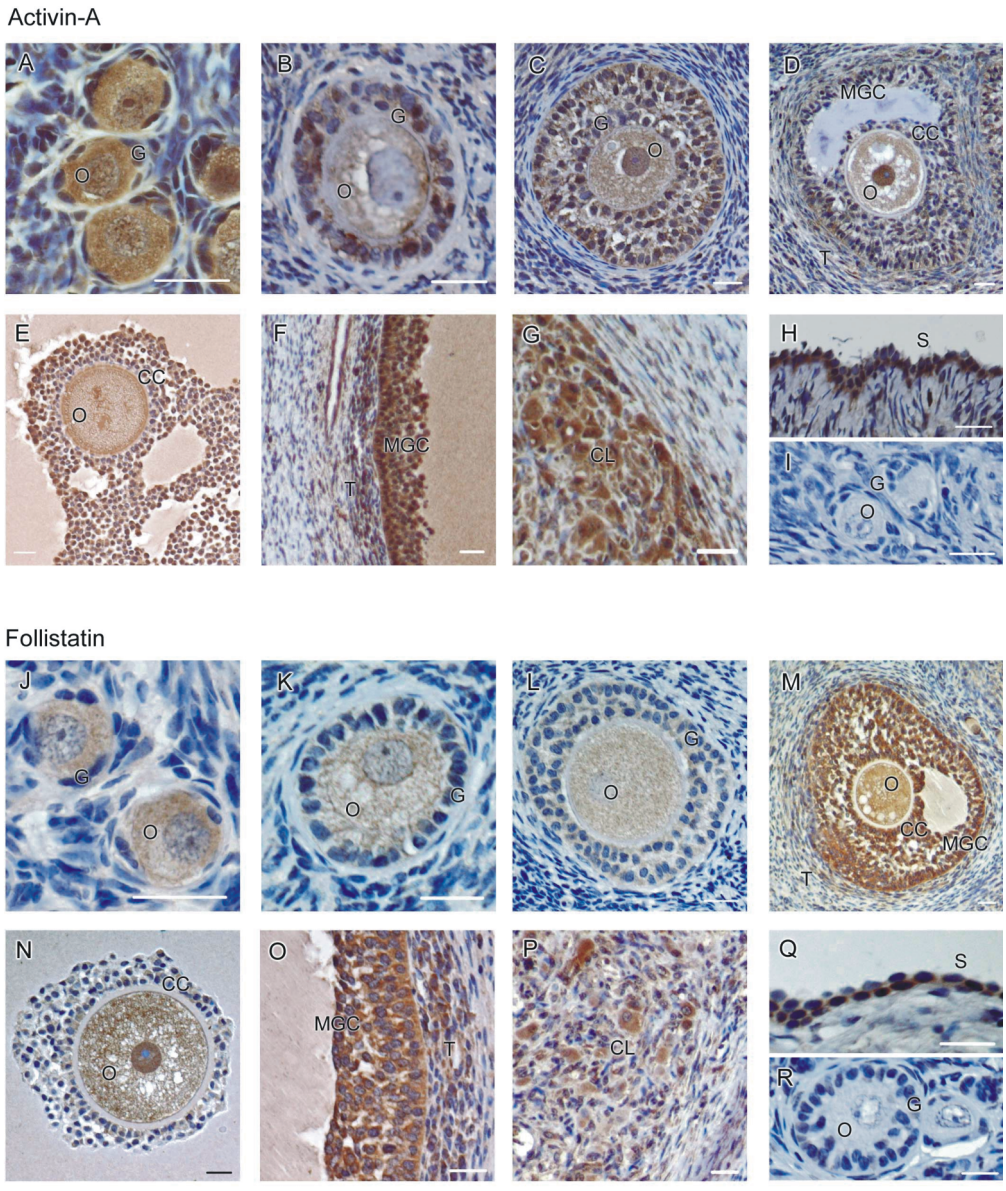

Figure 1. Activin-A and follistatin immunoreactivity in the different structures found within goat ovaries. (A, J) Primordial follicle, (B, K) Primary follicle, (C, L) Secondary follicle, (D, M) Small antral follicle, (E, N) COC of a large antral follicle, (F, O) Mural granulosa and theca cells from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

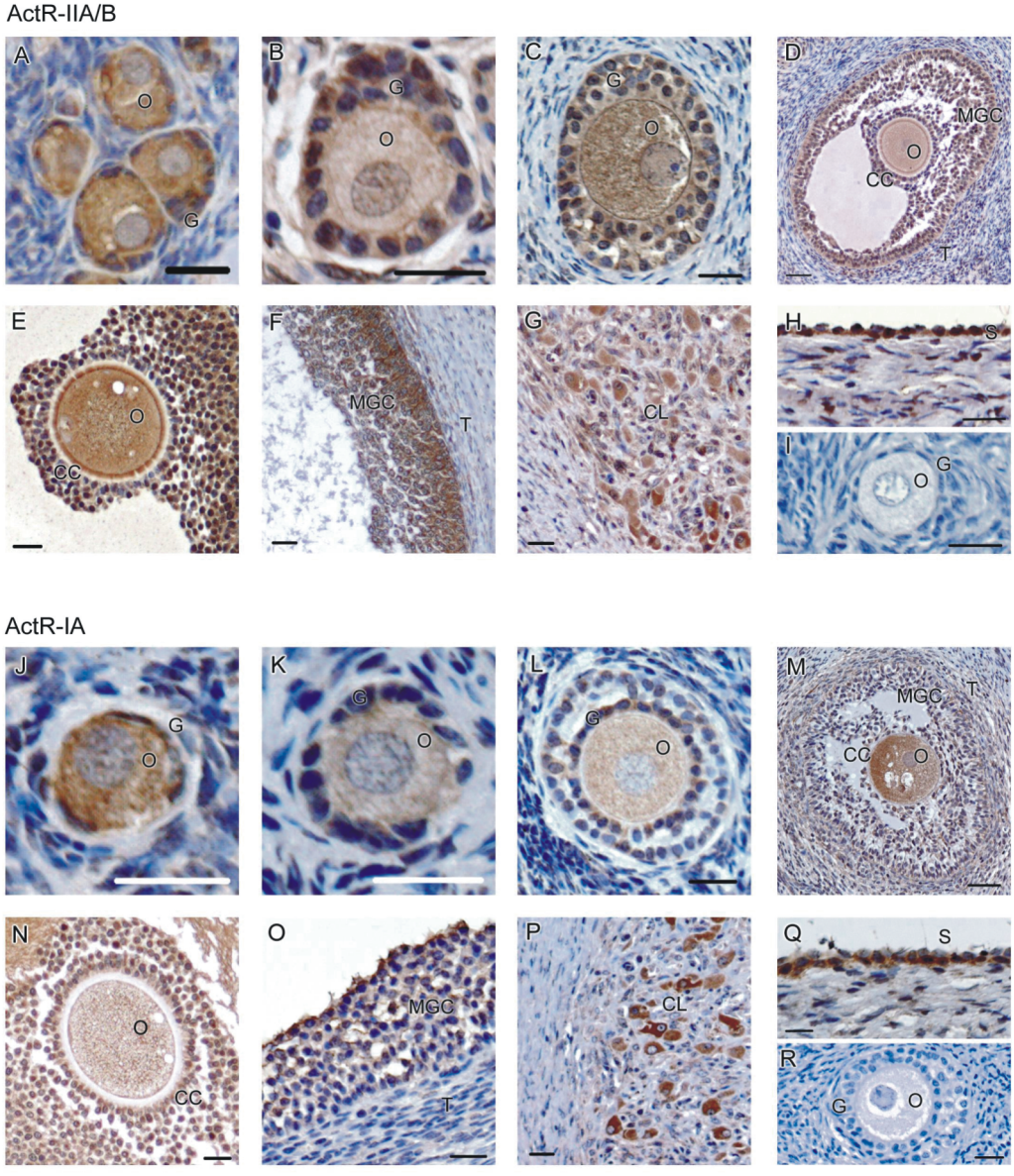

Figure 2. ActR-IIA/B, and ActR-IA immunoreactivity in the different structures found within goat ovaries. (A, J) Primordial follicle, (B, K) Primary follicle, (C, L) Secondary follicle, (D, M) Small antral follicle, (E, N) COC of a large antral follicle, (F, O) Mural granulosa and theca cells from a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

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

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

* whole follicles, (/ +) occasionally found; (-) absent, (+) weak, (++) moderate and (+ ++) strong immunoreaction

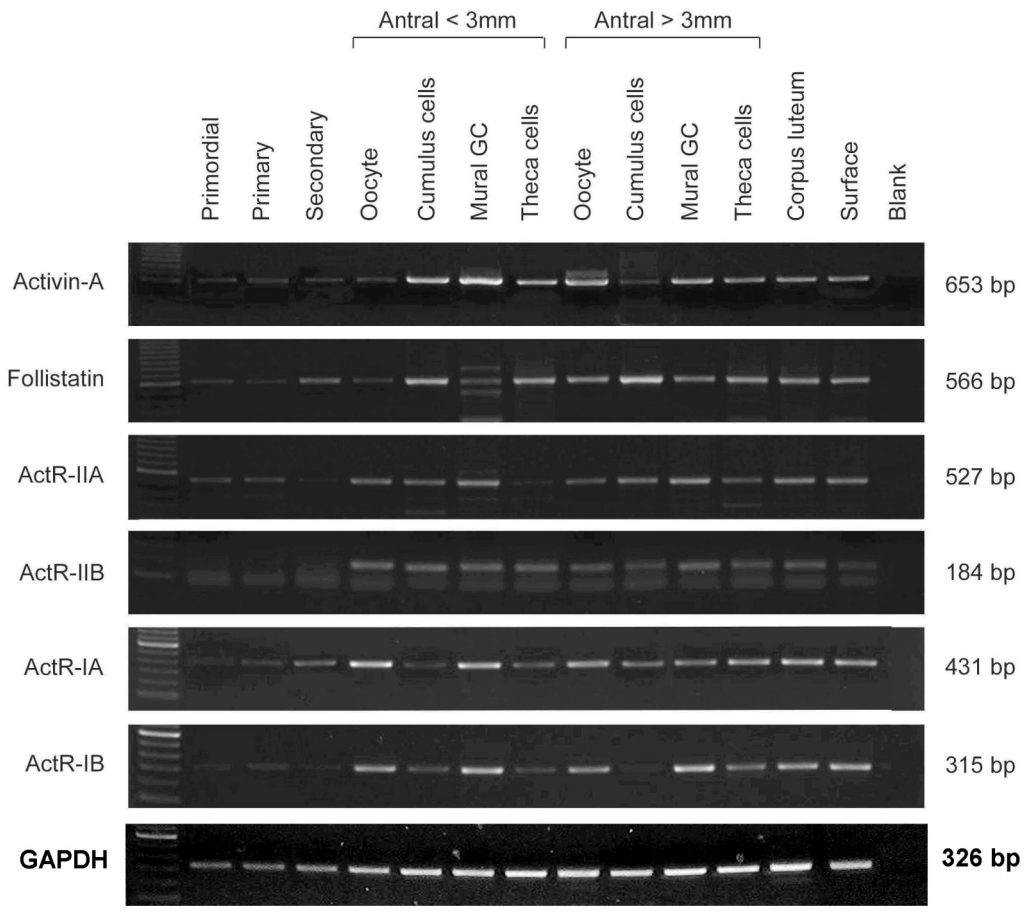

Figure 3. Expression of activin-A, follistatin, ActR-IIA, ActR-IIB, ActR-IA and ActR-IB mRNA in different follicle and cell types in goat ovaries. Follicle and cell types are indicated at the top. One-hundred base pair ladders are included as markers for fragment size. Tests to assure sample purity were preformed in previous work (Silva *et al*. 2004)

Discussion

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

follistatin protein was previously demonstrated in sheep oocytes of primordial follicles and in oocytes and granulosa cells of primary and secondary follicles (McNatty *et al*. 1999), but its mRNA was only detected in granulosa cells of secondary follicles (Braw-Tal 1994, Tisdall *et al*. 1994). Because follistatin binds and neutralises activin-A (de Winter *et al*. 1996, Sidis *et al*. 2001, 2002, Fisher *et al*. 2003), co-expression of both compounds in goat primordial, primary and secondary follicles suggests that follistatin may control the biological action of activin-A during early follicle growth. To support possible autocrine and/or paracrine actions of activin-A in such goat follicles, we demonstrated the proteins for ActR-IIA/B and ActR-IA as well as the mRNA for ActR-IIA, -IA and -IB. The only exception was ActR-IIB, since no cDNA amplification from primordial, primary and secondary follicles was detected. Previous studies have shown ActR-IIA/B protein in early follicles from cow (Hulshof *et al*. 1997), while those of cat and rat have ActR-IIA, -IIB, -IA and -IB (Drummond *et al*. 2002, Bristol & Woodruff 2004). In contrast, ActR-IIA protein was not found in mouse early follicles (Pangas *et al*. 2002). In pig, both protein and mRNA for ActR-IIA were detected in these follicle types (van den Hurk & van de Pavert 2001). In-vitro studies have shown that activin-A stimulates early follicle development in cows (Hulshof *et al*. 1997), sheep (Thomas *et al*. 2003) and rodents (Liu *et al*. 1998, Smitz *et al*. 1998, Zhao *et al*. 2001).

In caprine antral follicles, both activin-A and follistatin proteins were present in the oocyte, cumulus cells, mural granulosa cells and theca cells. These compounds apparently are formed in these cells, since their mRNA was also detected at the same locations. Transcription of βA subunit can also form inhibin-A, but this will occur only when there is an excess of βA subunit production (Findlay 1993, Knight & Glister 2001). The role of inhibin on folliculogenesis has been recently reviewed by Knight & Glister 2001. Activin-A protein has been detected in oocytes and granulosa cells in rat (Ogawa *et al*. 1991), human (Yamoto *et al*. 1992, Roberts *et al*. 1993, Wada *et al*. 1996), bovine (Izadyar *et al*. 1998, Silva *et al*. 2003) and pig (van den Hurk & van de Pavert 2001) antral follicles. In human (Roberts *et al*. 1993) and pig (van den Hurk & van de Pavert 2001), theca cells of antral follicles also immunoreacted for the activin-A protein. Thus far, expression of activin-A mRNA in antral follicles appeared restricted to the granulosa cells in rat (Meunier *et al*. 1988), primate (Roberts *et al*. 1993), sheep (Braw-Tal 1994, Tisdall *et al*. 1994) and pig (van den Hurk & van de Pavert 2001), while in the bovine it was also detected in oocytes (Izadyar *et al*. 1998). Follistatin protein and mRNA have been demonstrated in oocyte and granulosa cells from bovine antral follicles (Izadyar *et al*. 1998, Silva *et al*. 2003). Expression of follistatin mRNA in antral follicles has also been reported in rat (Nakatani *et al*. 1991), human (Roberts *et al*. 1993, Sidis *et al*. 1998) and sheep (Braw-Tal 1994, Tisdall *et al*. 1994), and appeared confined to granulosa cells.

We furthermore demonstrated ActR-IIA/B and ActR-IA proteins and the mRNAs for ActR-IIA, -IIB, -IA and -IB in oocyte, cumulus cells, mural granulosa cells and theca cells, in the goat. Previously, our group reported the expression of ActR-IIA mRNA and protein in oocyte and granulosa cells of pig (van den Hurk & van de Pavert 2001) and cow (Izadyar *et al*. 1998) antral follicles, while in those of the human, ActR-IIA and -IIB were detected in granulosa cells and theca cells (Pangas *et al*. 2002). In mouse and human antral follicles, mRNAs for all four activin receptor subtypes were expressed

in both oocyte and granulosa cells (Sidis *et al.* 1998). In-vitro, activin-A stimulated antrum formation (rat: Zhao *et al*. 2001), FSH receptor and FSH-induced LH receptor production (rat: Minegishi *et al*. 1999, Tsuchiya *et al*. 1999), and cytochrome P-450 aromatase activity, thus estrogen synthesis, in granulosa cells (human: Mukasa *et al*. 2003). Activin also suppressed androgen production in theca cells from different species (human: Hillier *et a*l. 1991, bovine: Wrathall & Knight 1995, ovine: Campbell & Baird 2001). Consequently, activin is thought to play a crucial role in basal growth, recruitment and selection of antral follicles through stimulation of proliferation and FSH receptor expression in granulosa cells and modulation of steroidogenesis in granulosa and theca cells, its actions being time and concentration dependent and regulated by follistatin (reviewed by Findlay *et al*. 1993, 2002, Driancourt 2001, Knight & Glister 2001). Activin-A is also involved in the regulation of oocyte maturation in several species (bovine: Silva & Knight 1998, human: Alak *et al*. 1998, mouse: Sidis *et al*. 1998). The distribution pattern of activin-A, its binding protein follistatin and activin receptors in goat antral follicles point to an important role of these proteins in antral follicle development in this species.

Apart from follicles, we found evidence for the presence of an activin-follistatin-activin receptor system in corpora lutea and ovarian surface epithelium, because of the presence of both mRNA and protein for activin-A, follistatin and all four types of activin receptors at these sites. With regard to corpora lutea, expression of protein and mRNA for activin-A and follistatin were previously demonstrated in human (Roberts *et al*. 1993, Wada *et al*. 1996) and cow (Singh & Adams 1998). Both Act-RIIA protein and mRNA were detected in pig luteal tissue (van den Hurk & van de Pavert 2001) and, in vitro, activin-A suppressed progesterone production by primate luteal cells (Brannian *et al*. 1992). Expression of activin-A protein and mRNA in ovarian surface epithelium has previously been demonstrated in pig (van den Hurk & van de Pavert 2001) and cow (Hulshof *et al*. 1997). Choi *et al.* (2001) demonstrated that activin inhibits growth and induces apoptosis in cultured ovarian surface epithelial cells. As mentioned above, the activin-follistatin system has been demonstrated to control growth and differentiation of antral follicles by affecting gonadotropin receptor synthesis and steroidogenesis. The activin-follistatin system presence in goat corpora lutea and surface epithelium may therefore point to a regulatory function in or fine tuning of their activity.

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

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Chapter 3

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 Minimum Essential Medium (MEM) supplemented with activin-A (0, 10 or 100 ng/mL), follistatin (0, 10 or 100 ng/mL) and their combinations. After culture, the number 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. 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 cultured goat follicles maintained their expression of proteins and mRNA for activin-A, follistatin, KL, GDF-9 and BMP-15. From the atretic follicles in cultured cortical tissue less than 30% 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 indicates incomplete blockage of activin in these experiments. In contrast to cortical enclosed atretic follicles, all atretic follicles that had arisen in cultures with isolated primary follicles had TUNEL-positive cells, which points to differences between isolated and ovarian tissue enclosed follicles with regard to the followed pathways leading to their degeneration. In summary, this in vitro study has demonstrated that cultured goat primordial follicles are activated to grow and develop into intermediate and primary follicles. During in vitro culture, the follicles maintain their ability to express activin-A, follistatin, KL, GDF-9 and BMP-15. The in vitro growth and survival of activated cortical tissue enclosed primordial follicles and the in vitro growth of isolated primary follicles are stimulated by activin-A.

Keywords: goat, early follicles, activation, growth, activin-A, follistatin

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, and 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 in vitro cultured for 20 days (Wandji *et al.* 1997, Fortune *et al.* 1998). Only when mice ovarian tissue was used, primordial follicles could 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). Such a 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, GDF-9, BMP-15 and Kit ligand (for reviews, see van den Hurk *et al.* 2000, Findlay *et al.* 2002, Fortune 2003, van den Hurk & Zhao 2005).

Activins are a heterodimer (ßAßB activin AB) or two homodimers (ßAßA - activin-A, ßBßB - activin B) of the ß 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 internet site, including the five studies from our group (Izadyar *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 ß subunits and so is able to neutralise the activities of activin in a variety of target tissues, inclusive ovary (Phillips & de Krester 1998).

Activins signal through two types of closely related receptors designated type I and type II, each represented by two isoforms, i.e., activin receptors type IA (ActR-IA), IB (ActR-IB), IIA (ActR-IIA) and IIB (ActR-IIB). Activin first binds to a type II receptor, which in turn recruits and activates a type I receptor by phosphorylation (Pangas & Woodruff 2000), with ActR-IB being the predominant type I receptor for activin (Massagué & Chen 2000). Expression of protein and mRNA for activin-A and activin receptors have been localised in both the oocyte and the granulosa cells of goat early follicles, as are 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 small-sized preantral follicles have demonstrated that, in these species, activin-A is involved in their development (bovine: Hulshof *et al.* 1997, rodents: Smitz *et al.* 1998, Zhao *et al.* 2001). There are, however, also reports that showed an age-dependent effect of activin-A on mouse isolated medium-sized preantral follicles (Mizunuma *et al.* 1999) and even 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 caprine, 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 seen 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 (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 presence or absence of activin A and/or follistatin and 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. One pair of ovaries was used in each of five different replicates.

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 x 3 x 1 mm. 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 aliquots of culture medium in 24 well culture dishes at 39°C in an atmosphere of 5% CO_2-in-air. Control culture medium was alpha Minimum Essential Medium Eagle (Sigma Chemicals, Poole, Dorset, UK) supplemented with SPIT (5 ng/mL selenium, 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. 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 ng/mL activin-A

plus either 10 or 100ng/mL of follistatin as well as 100 ng/mL activin-A plus 10 or 100ng/mL of follistatin. The ovarian slices from each animal were cultured for 5 days and, at days 2 and 4, the culture medium was replaced with fresh medium.

Protein localization for activin-A, follistatin, GDF-9, BMP-15 and Kit ligand 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, 5-µm sections of each slice were cut and mounted on glass microscope slides. Immunohistochemistry was performed as described by Silva *et al.* (2004b). In brief, the epitopes were activated by microwaving 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, Abingdon, Oxon, UK), (3) mouse anti-kit ligand (1:20, Santa Cruz Biotechnology Inc, 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 4ºC 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 an avidin-biotin complex (1:600, Vectastain Elite ABC kits; Vector laboratories, Burlingame, CA, USA). Protein localization was determined with diaminobenzidine (DAB; 0.05% DAB in Tris/HCl pH 7.6, 0.03% H_2O_2 - Sigma Chemicals, Poole, Dorset, UK). The sections were counterstained with haematoxylin, dehydrated and mounted in Pertex (Cellpath Ltd., Hemel Hempstead, 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 diaminobenzidine reagent alone to exclude the possibility of non-suppressed endogenous peroxidase activity. Specificity of the antibodies were previously tested (Silva *et al.* 2004bc)

The early-staged 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 hematoxylin-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 were calculated before (Day 0) or after 5 days of culture in the various media. Oocyte and follicle diameters were measured at 40x magnification using a light microscope (Zeiss, German) fitted with an eyepiece micrometer.

Gene expression for activin-A, follistatin, GDF-9, BMP-15 and Kit ligand 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 RNeasy mini kit and the RNAse-free DNAse set (Qiagen, Valencia, USA) according to the manufacturer's instructions and described previously (Silva *et al.* 2004b). Prior to the reverse transcription reaction, the eluted RNA samples were incubated for 5 min at 70^0C. Reverse transcription was then performed in a total volume of 20 µl, made up of 10 µl of sample RNA, 4 µl 5X reverse transcriptase buffer (Gibco BRL, Breda, The Netherlands), 8 units RNAsin (Promega, Southamptom, UK), 150 units Superscript II reverse transcriptase (Gibco BRL, Breda, The Netherlands), 0.036 U random primers (Life Technologies BV, Leiden, The Netherlands), and containing 10 mM DTT and 0.5 mM of each dNTP. The mixture was incubated for 1 h at 42^0C, for 5 min at 80^0C and then stored at -20^0C. Minus RT 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 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). The primers used for amplification are presented in Table 1. After initial denaturation and activation of the polymerase for 15 min at 94^0C, 40 cycles of 15 sec at 94^0C, 30 sec at 55^0C and 45 sec at 72^0C were carried out. Final extension was for 10 min at 72^0C. During the amplification, heminesting was used to increase the specificity and sensitivity. For heminesting, 1 l of the first round product was transferred to another 200 l tube containing 24 l PCR amplification mixture, and amplified for 25 cycles using the same thermal cycling profile.

Assessment of DNA fragmentation in follicles within cortical slices

The TUNEL assay for DNA fragmentation was performed using an in situ cell death detection kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. In brief, sections were deparaffinized, rehydrated and endogenous peroxidase was blocked with 3% (v/v) H_2O_2 in methanol for 20 min. After a PBS wash, terminal deoxynucleotidyltransferase (TdT) enzyme solution was added to each section to label single and double DNA strand breaks with fluorescein, and the reaction was incubated for 1 h in a humidified chamber at 37◦C. Then, anti-fluorescein antibody Fab fragments conjugated with horseradish peroxidase were added to each section and incubated for 30 min in a humidified chamber at 37◦C. A negative control without the addition of the TdT enzyme was also included. The sections were washed in PBS, covered with diaminobenzidine (DAB; 0.05% DAB in Tris/HCl pH 7.6, 0.03% H_2O_2) and then, counterstained with haematoxylin, dehydrated and mounted.

Table 1. Oligonucleotide primers used for PCR analysis of ovarian cortical tissues.

Target gene	Primer sequence (5' → 3')	Sense	Position	Genbank accession number
Activin-A	ACCTCGGAGATCATCACGTT	s	357-376	GI: 563747 (2002)
	CAGTCATTCCAGCCAATGTC	as (R2)	990-1009	*Bos taurus*
	CACGACTTGAGGTTGGCGAA	as (R1)	1146-1165	Act-ßA
Follistatin	TGTGAGAACGTGGACTGTGG	s	289-308	GI: 404023 (1994)
	ATACTGTTCAGAGGAGGTGG	as (R2)	602-621	*Bos taurus*
	ACAGGCTCCTCAGACTTACT	As (R1)	835-854	Follistatin
GDF-9	ACAACACTGTTCGGCTCTTC	s (R1)	332-351	GI: 6715598 (2003)
	CGGCAGAGCTCCATACTCAT	s (R2)	570-589	*Homo sapiens*
	TAAGCCTGAGCACTTGTGTC	as	805-824	GDF-9
BMP-15	GATTCAGGAGCTGCTAGAAG	s	129-148	GI: 8925958 (2000)
	AGTGGCTCTGACTAGTTGGT	as (R2)	399-418	*Ovis aries*
	GAGTTAGGTGAAGCTGATGG	as (R1)	430-449	BMP-15
KL	GATCTGCAGGAATCGTGTGA	s (R1)	81-100	GI: 4505174 (2003)
	AGTCGATGACCTTGTGGAGT	s (R2)	321-340	*Homo sapiens*
	ACTGGAAGAAGAGACAGCCA	as KL1	707-726	Kit Ligand
		as KL-2	623-642	
GAPDH	AGGCCATCACCATCTTCCAG	s	179-198	GI: 2285902 (1997)
	GGCGTGGACAGTGGTCATAA	as	485-504	*Bos taurus* GAPDH

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 adult goats were colleted in a slaughterhouse and transported to the laboratory. Primary follicles were isolated 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, and then used for in vitro culture.

Primary Follicle Culture

For the control group, the follicles (n = 60) were cultured in groups of 3 to 5 in 5 well plates (Bibby Sterilin Ltd. Stone, Staffs, UK) containing 250 µl of culture medium (alpha Minimum Essential Medium Eagle (Sigma Chemicals, Poole, Dorset, UK) supplemented with SPIT (5 ng/mL selenium, 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 obtained from Sigma Chemicals. Experimental media consisted of the above described control media to which recombinant human activin-A (R&D Systems; n=66) or follistatin (R&D Systems; n=63) was added at a concentration of 100 ng/ml, or both activin A and follistatin (n=62) were added at concentrations of 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 air atmosphere with 5 % CO_2 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. From morphologically normal follicles, i.e., follicles that do not show signs of oocyte retraction or those of granulosa cell disorganization, follicle diameters were measured using a crossed micrometer under 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. First, 4% paraformaldehyde fixed follicles were incubated twice for 15 min in PBS containing 150 mM glycine and 1 mg/ml of 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 of PVA (PBS-PVA; pH 7.4) before being incubated in 20-µl drops of fluorescein-conjugated dUTP and TdT (Roche Mannheim, Germany) 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 of 4,6-diamino-2-phenyl-indole (DAPI) in PBS for 10 min.

Stained follicles were mounted on glass slides with an antifading medium (Vectashield, Vector Laboratories, Burlingame, CA) 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 Analyses

In the first experiment, one-way ANOVA and Tukey HSD test were used to compare: (1) the number of primordial and developing follicles, (3) the follicle and oocyte diameter, (4) the number of granulosa cells in normal follicles and (5) the number 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, ANOVA and Tukey HSD test were also used to compare the diameter of isolated primary follicles, the number of granulosa cells and the number 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 number 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±12.4 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 to culturing in MEM, 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.

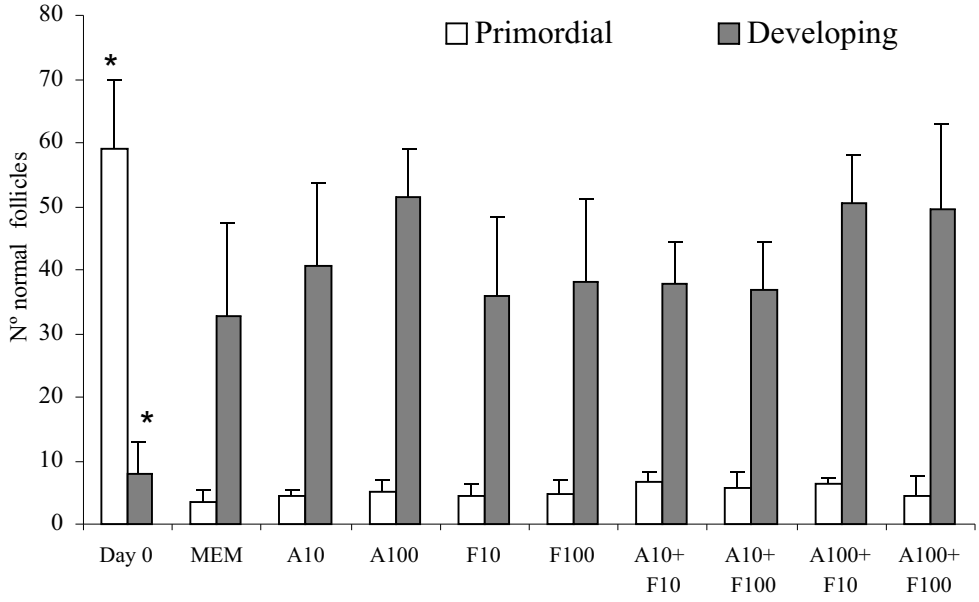

Figure 1. Number of morphologically normal primordial and developing follicles in non-cultured tissue (day 0) and tissue cultured for 5 days in MEM plus 10 (A10) or 100 ng/ml activin-A (A100), 10 (F10) or 100 ng/ml follistatin (F100), or a combination of both. N= 5 replicates for each treatment.
* - Day 0 differs significantly from in vitro cultured tissue

The follicle and oocyte diameters as well as the number of follicular granulosa cells per section were evaluated and 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 to 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 of activin-A. Unilaminar follicle diameters but not their oocyte diameters had also increased when ovarian slices were cultured in 100 ng/mL of activin-A plus 10 ng/mL of 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, MEM + 10ng/mL activin-A and MEM + 10ng/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.

Table 2. Follicle / oocyte diameters (μm) and number of granulosa cells per section for follicles with one (unilaminar follicles) or two or more layers of granulosa cells (multilaminar follicles).

	Diameter (mean ± SD) Unilaminar follicles			Diameter (mean ± SD) Multilaminar follicles		
	Follicle	Oocyte	Nº GC	Follicles	Oocyte	Nº GC
Uncultured	30.6±4.7	26.9±2.9	8.2±4.5	108.4±47.1	54.0±19.5	69.2±26.4
Treatment	After 5 days culture					
MEM	31.7±3.7a	26.3±3.1a	11.9±4.1ab	99.0±45.6	46.7±16.0	63.2±25.3
A10	33.4±7.0abc	27.9±4.4abc	13.0±7.2ab	104.7±27.3	51.7±22.4	86.4±46.3
A100	35.5±6.0*b	29.6±4.9*b	12.4±3.7*ab	110.0±25.7	58.5±14.6	92.8±42.5
F10	32.8±4.6abc	27.2±2.8abc	12.0±3.1*ab	105.0±21.5	46.2±10.9	81.2±21.3
F100	32.3±4.5abc	27.0±3.2abc	12.2±5.6*ab	87.5±33.7	46.2±14.3	69.8±28.7
A10+F10	31.4±3.8ac	26.2±2.1ac	11.8±5.5*ab	81.5±52.4	46.5±16.0	57.4±37.4
A10+F100	30.8±4.2ac	25.9±2.6ac	10.9±4.7a	94.2±47.0	48.7±18.3	76.0±35.1
A100+F10	35.5±5.5*b	29.3±4.2bd	15.2±5.3*b	95.9±34.0	55.5±17.1	97.4±56.3
A100+F100	32.2±3.6ab	26.8±2.6acd	13.2±4.2*ab	91.0±44.9	49.7±19.4	84.0±64.6

* Denotes values that differ significantly from uncultured control (day 0).
a b c - Values with different letters denote significant differences among culture media within a given column ($P < 0.05$).

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 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 culture, among the tested media, the one containing 100 ng/mL of activin-A significantly reduced the number of atretic developing follicles ($P<0.05$), the effect not being counteracted when either 10 or 100 ng/mL of follistatin was added to activin-A. In none of the normal follicles within uncultured cortical slices DNA fragmentation could 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 absence (F) and presence of TUNEL reactivity in a granulosa cell (G) and an oocyte (H) nucleus.

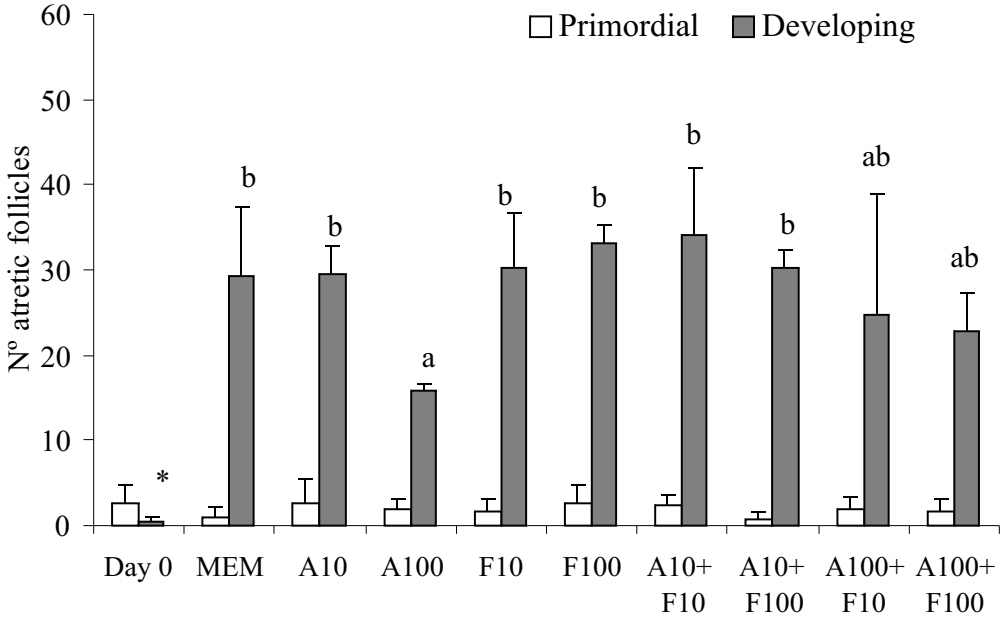

Figure 2. Number of atretic (B) primordial and developing follicles in non-cultured tissue (day 0) and tissue cultured for 5 days in MEM plus 10 (A10) or 100 ng/ml activin-A (A100), 10 (F10) or 100 ng/ml follistatin (F100), or a combination of both.
N= 5 replicates for each treatment
* - Day 0 differs significantly from in vitro cultured tissue
ab: Values with different letters denote significant difference between treatments after in vitro culture ($P < 0.05$)

Both before and after 5 days culture and independent of the in vitro treatment applied, all or the great majority of morphologically normal follicles (98 to 100%) expressed the proteins for activin-A, follistatin, GDF-9, BMP-15 and KL (Fig. 3A,B,C,D,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 strong 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 of those factors were synthesized during the in vitro culture period. Amplification of -RT blanks or water controls yielded no specific products in any of the reactions (Fig. 4).

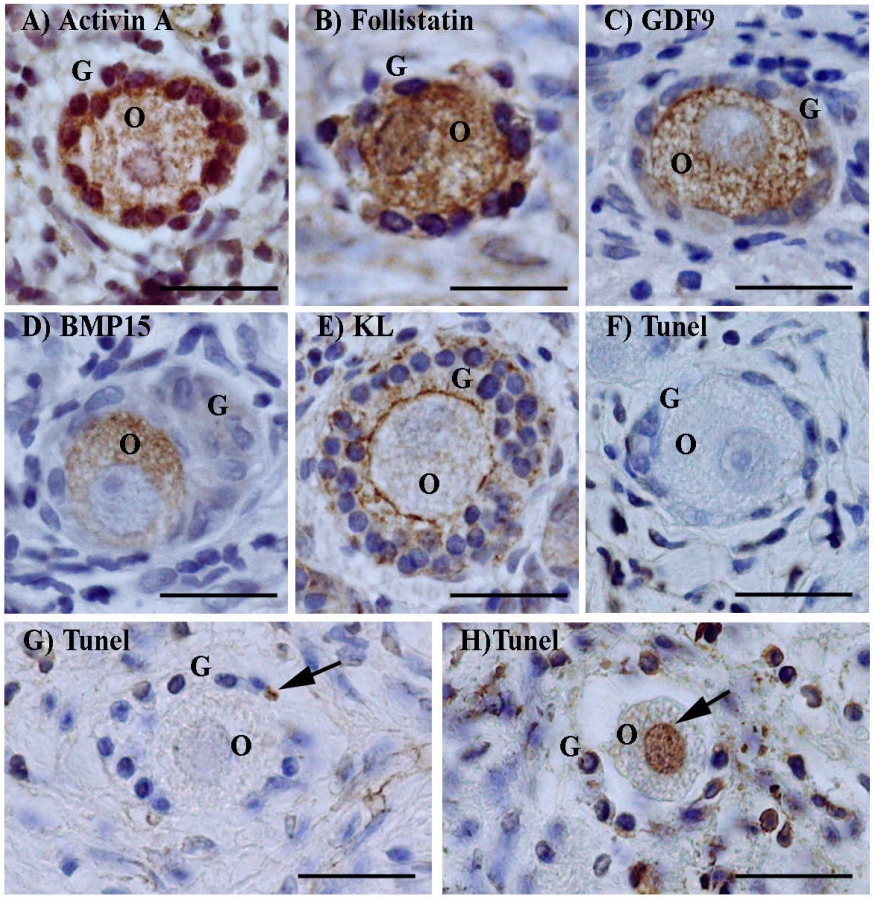

Figure 3. Protein expression for activin-A (A), follistatin (B), GDF-9 (C), BMP-15 (D), KL (E) and TUNEL staining / DNA fragmentation (F, G, H) in goat ovarian follicles after 5 days culture. O: oocyte, G: granulosa cells. Arrow shows DNA fragmentation in granulosa cell (G) and oocyte (H) detected by TUNEL. Bars: 25µm

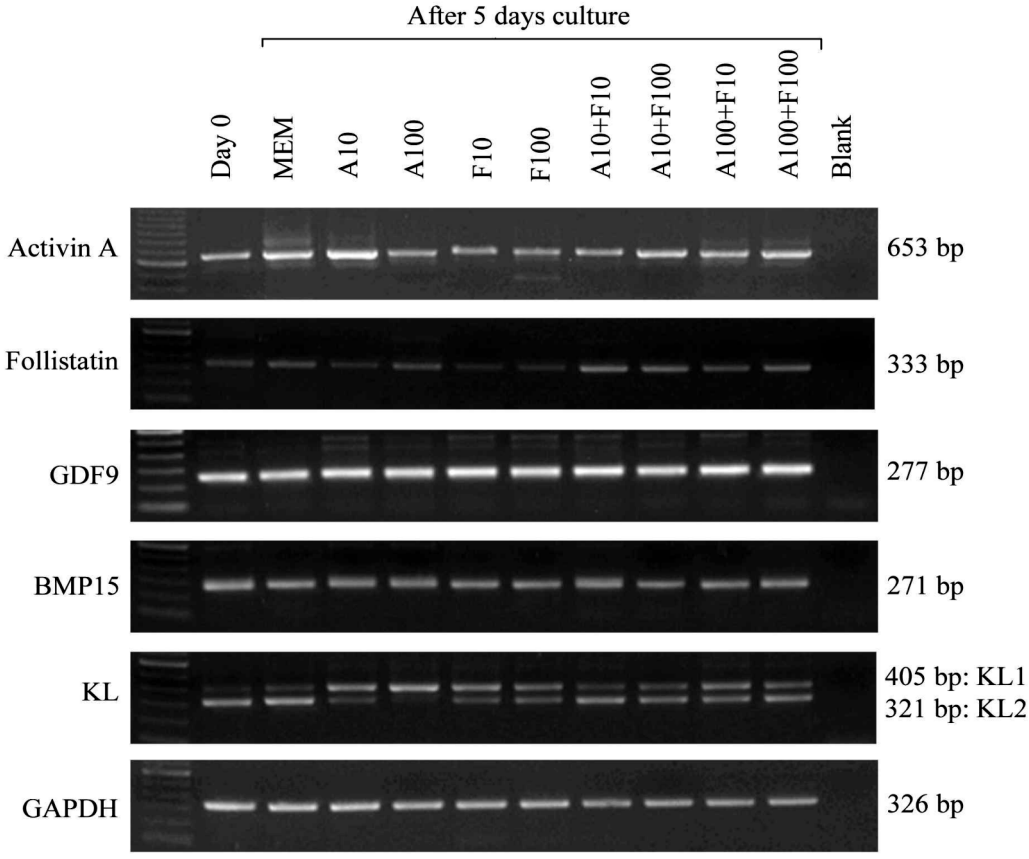

Figure 4. Messenger RNA expression for activin-A, follistatin, GDF-9, BMP-15, KL and GAPDH in goat ovarian cortical tissue after 5 days culture.

Experiment 2

In vitro growth of isolated primary follicles

A total of 251 isolated primary follicles were cultured in vitro (from 60 to 66 follicles in each treatment). As illustrated in Table 3, isolated primary follicles that were cultured for 6 days in medium containing 100ng/mL activin-A showed a significant increase ($P<0.05$) in follicle diameter when compared with control medium (MEM), MEM + 100ng/mL follistatin or MEM + 100ng/mL activin-A + 200ng/mL follistatin. Follicles showing signs of atresia were not included in these diameter calculations. No significant differences in the number of granulosa cells per follicles were observed among the treatments, but (normal) follicles cultured in activin-A containing medium had higher number of granulosa cells when compared to uncultured follicles ($P<0.05$, Table 4). From the follicles that were cultured for 6 days, 30.0 % (18/60) showed signs of atresia in their oocytes or granulosa cells after culture in 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 culture, which contains a clear oocyte surrounded by granulosa cells, and an atretic follicle with a dark retracted oocyte and disorganized granulosa cells.

Table 3. Follicular growth of goat isolated primary follicles after 6-day culture in MEM or in MEM plus activin-A (Act.-A) and/or follistatin. Only non-atretic follicles are included; their numbers are given between brackets.

Treatment	Follicle diameter (mean ± SD-μm) day 0	Follicle diameter (mean ± SD-μm) day 6	increase in diameter at day 6 (mean ± SD-μm)
MEM (n=60)	46.7 ± 8.5	50.0 ± 9.9	3.3 ± 3.1 [a]
MEM+Act.-A (n=66)	45.5 ± 7.9	53.9 ± 9.8	8.5 ± 4.8 [b]
MEM+follistatin (n=63)	50.2 ± 10.4	53.4 ± 11.7	3.2 ± 3.1 [a]
MEM+Act.-A+follistatin (n=62)	51.1 ± 9.4	53.3 ± 9.1	2.2 ± 2.1 [a]

a b - Values with different letters denote significant differences among culture media ($P < 0.05$).

Table 4. Number (mean ± SD) of granulosa cells and percentages (mean ± SD) of TUNEL-positive cells in morphologically normal follicles after 6-day culture in MEM or in MEM plus activin-A (Act.-A) and/or follistatin (n=10).

Treatment	Number of granulosa cells per follicle	% of TUNEL-positive granulosa cells per follicle
Control–day 0	61.6 ± 6.0 [a]	0
MEM–day 6	65.0 ± 13.7 [ab]	3.3 ± 1.5 [ab]
MEM+Act.-A (100ng/mL)–day 6	78.4 ± 10.1 [b]	1.6 ± 0.6 [a]
MEM+follistatin (100ng/mL)–day 6	66.6 ± 11.3 [ab]	3.5 ± 1.7 [b]
MEM+Act.-A+follistatin–day 6	69.8 ± 13.6 [ab]	2.0 ± 1.8 [ab]

a b - Values with different letters denote significant differences among treatments ($P < 0.05$).

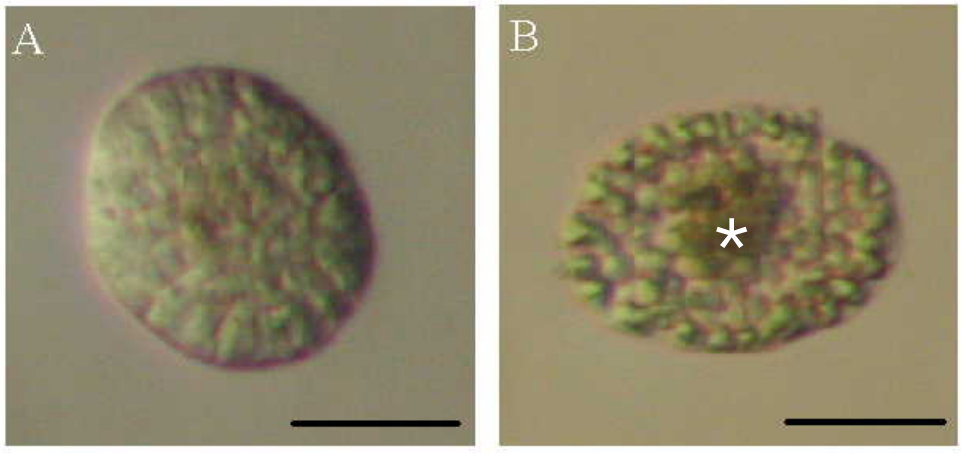

Figure 5. A morphologically normal (A) and a degenerated (B) isolated preantral follicle after 6-day culture. Bars: 25µm, (*) shrunken oocyte

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 culture, normal follicles generally had few (1 to 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 granulosa cells per follicle when compared to 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 6C shows confocal images of primary follicles with TUNEL- fluorescence in both granulosa and oocyte nuclei.

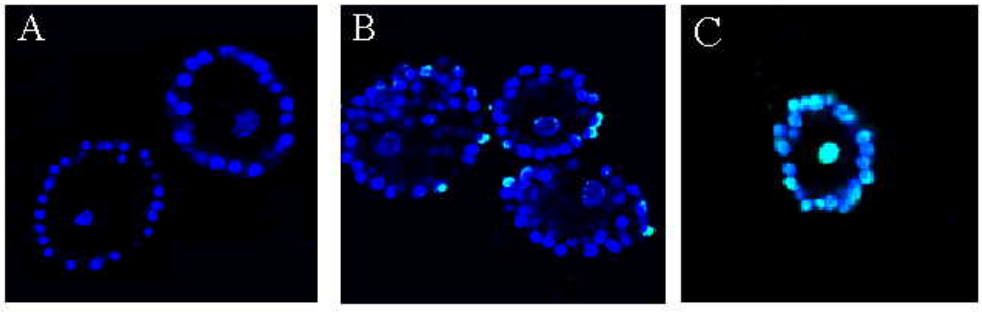

Figure 6. Confocal images of morphologically normal follicles before (A) and after 6 days culture (B), and of a follicle that became atretic during in vitro culture (C). Note the green TUNEL-positive fluorescence in granulosa cell nuclei in B and C and oocyte nucleus in C. Normal nuclei are blue (Dapi staining).

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 occurs '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 (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 both before and after (5 days) culture the expression of their mRNAs in these tissues, while the respective proteins were immunocytochemically demonstrated in the enclosed early ovarian follicles. From these proteins, activin-A was found to be involved in germ cell proliferation in human (Martins da Silva *et al.* 2004), secondary follicle development in bovine (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 follistatin in primordial follicle activation. Kit Ligand 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 Dong *et al.* (1996) and Galloway *et al.* (2000), which showed that GDF9-deficient mice and BMP15-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 mice and are probably produced within the ovarian cortex.

When compared to control medium, the diameter of unilaminar follicles had increased in the current studies in which ovarian tissue was cultured in presence of activin-A (100ng/mL). The effect is not due to an increase in the number of granulosa

cells, but rather to an increase in volume of follicular cells during transition from flattened to cuboidal shape, which is indicated by the observed increase in follicle diameter. Correspondingly, Activin-A stimulated the growth of cultured goat isolated primary follicles. In contrast with the cultured cortical tissue enclosed follicles, follistatin (100 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. 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 neutralises 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 (Smitz *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 of 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. From the atretic follicles in our culture experiments with cortical slices less than 30% 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 lesser 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 Ca^+, which on its 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 in vitro culturing of ovarian cortical slices, large numbers of goat primordial follicles have been transformed into more advanced staged, especially primary follicles, and that addition of activin-A and follistatin to the culture medium had no effect on this process. Activin-A (100ng/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 and the incomplete block of available activin by follistatin. 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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Chapter 4

Expression of Growth Differentiation Factor 9 (GDF-9), Bone Morphogenetic Protein 15 (BMP-15) and BMP Receptors in the Ovaries of Goats

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Abstract

The process of ovarian folliculogenesis is composed of proliferation and differentiation of the constitutive cells in developing follicles. In goats, relatively little information is available on the local factors that regulate this process. We studied the presence and distribution of GDF-9, BMP-15 and BMP receptors types II (BMPR-II), IA (BMPR-IA) and IB (BMPR-IB) in goat ovaries to find evidence for their possible roles in folliculogenesis. Ovaries of cyclic goats were collected and fixed in paraformaldehyde for immunohistochemical localization of GDF-9 and BMP-15 proteins or used to collect follicles and luteal tissue to study the mRNA expression of GDF-9, BMP-15 and BMP receptors using reverse transcriptase polymerase chain reaction. GDF-9 and BMP-15 proteins were found in oocytes of all types of follicles and granulosa cells of primary, secondary and antral but not primordial follicles. The mRNAs for GDF-9, BMP-15, BMPR-II, BMPR-IA and BMPR-IB were detected in primordial, primary and secondary follicles as well as in oocyte and granulosa cells of antral follicles. Transcripts for BMPR-II, BMPR-IA, BMPR-IB and GDF-9, and GDF-9 protein were furthermore found in corpora lutea. It is concluded that, the mRNAs and proteins of GDF-9 and BMP-15 and the mRNAs of BMP receptors are expressed in goat ovarian follicles at all stages of their development, and that they form a complex intrafollicular regulatory system during folliculogenesis. Expression of all BMP receptor mRNAs and GDF-9 mRNA and protein in luteal tissue additionally points to a role of GDF-9 in corpus luteum function.

Key words: follicles, oocyte, cumulus, mural granulosa cells, corpora lutea

Introduction

Ovarian folliculogenesis is a complex process consisting of follicular development through primordial, primary, secondary and antral stages, after which ovulation occurs and the residual cells luteinize to form corpora lutea. Regulation of cell proliferation, differentiation, and atresia associated with folliculogenesis is brought about through complex interaction mainly between local growth factors and hypophyseal gonadotropins from the pituitary. Since the actions of the gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH) have been well documented, considerable recent research focuses on the regulatory intraovarian proteins.

The transforming growth factor-ß (TGF-ß) superfamily consists of over forty members of secreted growth factors grouped together upon structural homology (Chang *et al.* 2002). Recent studies on the expression patterns and biological functions of two recently identified members of this superfamily (growth differentiation factor-9 [GDF-9] and bone morphogenetic protein 15 [BMP-15]) have demonstrated that they play a critical role in ovarian function (Findlay *et al.* 2002, Juengel *et al.* 2002, Lin *et al.* 2003, Knight & Glister 2003). Mice and sheep lacking GDF-9 and BMP-15, respectively, have folliculogenesis arrested at primary follicles and are sterile (Dong *et al.* 1996, Galloway *et al.* 2000). Within the ovary, the oocyte expresses both mRNA and protein for GDF-9 and BMP-15 in various staged follicles (rodents: McGrath *et al.* 1995, Elvin *et al.* 1999a,

2000a, Jaatinen *et al.* 1999, primates: Aaltonen *et al.* 1999, Gougeon & Busso 2000). In primates (Sidis *et al.* 1998, Duffy 2003) GDF-9 mRNA and protein are also expressed in granulosa cells of antral follicles. In rodents (McGrath *et al.* 1995, Fitzpatrick *et al.* 1998) and human (Aaltonen *et al.* 1999) GDF-9 is detectable in oocytes of follicles from the primary stage onward, but not in primordial follicles. In possum (Eckery *et al.* 2002), ovine and bovine (Bodensteiner *et al.* 1999) oocytes, however, expression of GDF-9 mRNA begins at the primordial follicles stage. The expression of BMP-15 mRNA closely resembles that of GDF-9 in rodents (Laitinen *et al.* 1998, Dube *et al.* 1998, Elvin *et al.* 2000a, Otsuka *et al.* 2000, Erickson & Shimasaki 2003) and possum (Eckery *et al.* 2002), but in sheep BMP-15 is detected only in oocytes from the primary follicle stage onward. In human, BMP-15 mRNA expression begins shortly later, i.e., in the oocytes of late primary follicles (Aaltonen *et al.* 1999).

Recent studies have shown that exposure to GDF-9 enhances primary and preantral follicular growth in vitro and in vivo (Hayashi *et al.* 1999, Vitt *et al.* 2000a), and promotes granulosa cell proliferation and differentiation in vitro (Vitt *et al.* 2000b). In addition, treatment with GDF-9 enables cumulus expansion (Elvin *et al.* 1999a) and increases thecal cell androgen production (Solovyeva *et al.* 2000). Furthermore, BMP-15 partially mimics GDF-9 effects on granulosa cell proliferation and progesterone production (Otsuka *et al.* 2000).

Members of the TGF-ß superfamily exert their biological function by forming heteromeric complexes with a type I and a type II receptor on the cell surface (Massagué & Chen 2000). Studies on TGF-ß proteins and activins have shown that these ligands bind to their respective type II receptors before associating with type I receptors (Wrana *et al.* 1994), leading to type I receptor phosphorylation followed by phosphorylation of intracellular transcription factors. These factors are then translocated to the nucleus to regulate target gene transcription (Massagué 2000). BMPs, which are related to GDF-9, appear to interact with specific BMP types II (BMPR-II), -IA (BMPR-IA) and -IB (BMPR-IB) receptors. Recent studies demonstrated that interaction of BMPR-IB and BMPR-II elicits BMP-15 biological activity (Moore *et al.* 2003), and that BMPR-II is essential for GDF-9 signaling in granulosa cells (Vitt *et al.* 2002). BMP receptor pathway is very important for ovarian function, since mutations in BMPR-IB is associated with increased ovulation rate in ewes (Mulsant *et al.* 2001, Souza *et al.* 2001, Fabre *et al.* 2003) and impaired follicular development in mice (Yi *et al.* 2001).

Although there is convincing evidence that GDF-9 and BMP-15 signaling is important for ovarian function, information on their localization and function is mainly obtained from rodents, sheep and human. Since goats have been used for many purposes, i.e., meat, milk, fibre and skin production, they are, economically seen, highly attractive live-stock animals. Therefore, it is desirable to improve the knowledge on their reproductive physiology, especially on the control of ovarian folliculogenesis. To explore the possible existence of an intrafollicular modulatory system of GDF-9 and BMP-15 in ovaries of cyclic goats, the present study was undertaken to detect mRNA expression of GDF-9, BMP-15 and BMP receptors, using reverse transcriptase polymerase chain reaction (RT-PCR), and to analyze the presence and cellular localization of GDF-9 and BMP-15 protein, using immunohistochemmistry.

Materials and Methods

Ovaries

During the breeding season, ovaries (n=54) were recovered from slaughtered cyclic adult mixed-breed goats and transported to the laboratory in a thermos flask, within 1hr. Fourteen of those ovaries were fixed overnight at room temperature in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS), and subsequently dehydrated and embedded in paraffin wax (Histoplast, Shandon Scientific, Ltd, Pittsburgh) in preparation for immunohistochemical studies. The remaining 40 ovaries were used to recover cells and tissues for RT-PCR.

Immunohistochemical localization of GDF-9 and BMP-15

Localization of GDF-9 and BMP-15 was performed on serial 5-µm sections cut from ovaries of seven 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 minutes. The sections were then washed with PBS and the epitopes were activated by microwaving the sections for 7 minutes 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 minutes with 5% normal goat serum in PBS to minimize non-specific binding. The primary antibodies were mouse anti-GDF-9 (Wyeth Research, Cambridge, USA) diluted 1:100 (0.4 µg/mL) and mouse anti-BMP-15 (Wyeth Research, Cambridge, USA) diluted 1:50 (0.3 µg/mL). Unfortunately the available anti-BMPR-II, -IA and -IB antibodies are not suitable for using in goat tissue. The sections were incubated overnight at 4ºC in appropriate dilutions of the antibodies. All other incubations and washes were performed at room temperature. After incubation with antibody, sections were washed three times with PBS-T and incubated for 45 minutes with biotinylated secondary antibody (goat anti-mouse IgG from Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), diluted 1:200 in PBS containing 5% normal goat serum. Next, the sections were washed three times in PBS-T before being incubated for 45 minutes 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) for a maximum of 20 minutes. The stained sections were rinsed in PBS and water, and counterstained for 10 seconds in Mayer's haematoxylin. Finally, the sections were washed for 10 minutes in running tap water, dehydrated in a graded ethanol series and then xylene, and mounted in Depex. The staining intensity for both GDF-9 and BMP-15 immunoreactive protein expression was scored as follows: absent (-), weak (+), moderate (++), or strong (+++). Sections were analyzed by two independent researchers.

Controls for non-specific staining were performed by (1) replacing either the

primary or the secondary antibody with IgGs from the same species in which the specific antibody was raised, at the same concentration; (2) incubation with diaminobenzidine reagent alone to exclude the possibility of non-suppressed endogenous peroxidase activity and; (3) western blot analysis to confirm the specificity of both antibodies. For western blot analysis, goat (n=3) ovaries were homogenized in lysis buffer (20 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 10% glycerol and 1% protease inhibitor cocktail). After centrifugation at 13 000 x *g* for 15 minutes, the supernatant was removed and used for analysis. For each sample, 20 µl was boiled in the presence of 2ß-mercaptoethanol and electrophoresed in a 12% polyacrylamide gel. Following electrophoresis, gels were electrotransferred for 1 h to nitrocellulose membranes (Amersham Pharmacia, Piscataway, NJ, USA). Membranes were then blocked in Tris-buffered saline (50 mM Tris [pH 7.4] and 150 mM NaCl) with 5% nonfat dry milk, incubated with primary antibodies (mouse anti-GDF-9 diluted 1:500 or mouse anti-BMP-15 diluted 1:200), washed twice with blocking buffer, and incubated with secondary antibody (goat anti-mouse conjugated with horse-radish peroxidase, Santa Cruz Biotechnology Inc), at 1:2000 dilution. After washing three times, detection was performed using DAB (0.05% DAB in Tris/HCl pH 7.6, 0.03% H_2O_2 - Sigma, St. Louis, MO, USA).

Classification and measurement of follicles

Ovarian follicles were classified as (1) primordial (one layer of flattened/cuboidal granulosa cells), (2) primary (a single layer of cuboidal granulosa cells), (3) secondary (two or more layers of cuboidal granulosa cells), (4) small antral follicles (< 3 mm in diameter; with multiple granulosa cells enclosing an antrum), and (5) large antral follicles (3 - 6 mm). The diameter of follicles was calculated 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). Ten ovaries were dedicated to preantral follicle isolation and the others were used to provide antral follicles, oocytes, cumulus cells, mural granulosa cells and samples of corpora lutea.

Early-stage follicles, i.e. primordial, primary and secondary, were isolated using a mechanical described procedure (Lucci *et al.* 1999). After isolation, follicles were washed repeated times to completely remove the stromal cells. Then, follicles from each of the three different categories (primordial, primary and secondary) were placed into separate Eppendorf tubes in groups of 15. All samples were stored at -80ºC until the RNA was extracted. In a previous work from our group, histological analysis was performed to confirm goat preantral follicle classification after isolation (Lucci *et al.* 1999).

From a second group of ovaries (n=20), cumulus-oocyte complexes (COCs) were aspirated from small (1-3 mm) and large (3-6 mm) antral follicles using an 18-gauge

needle attached to a tube in line with a vacuum pump. From the follicle content, so collected, compact COCs were selected as described by van Tol & Bevers (1998). Thereafter, the cumulus was separated from the oocyte by a combination of vortexing and aspiration via a narrow-bore Pasteur pipette. Denuded oocytes, cumulus and mural granulosa cells were separated, washed four times in PBS, packed in tubes in groups of either 10 denuded oocytes, cumulus cells from 10 COCs, or samples of mural granulosa, and then stored at -80°C until RNA extraction.

To collect theca cells, small (n=10) and large antral follicles (n=10) were isolated from ovaries (n=5) and dissected free of stromal tissue using forceps, as described previously (van Tol & Bevers 1998). The follicles were then bisected and the granulosa cells were scraped off using a scalpel blade. Next, the theca cell layers were vortexed for 1 minute in 1 ml HEPES buffered M199 (Gibco BRL, Paisly, UK) supplemented with penicillin/streptomycin, 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 were collected and stored at -80°C until RNA extraction. Three samples of each tissue sample were analyzed.

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). As per the manufacturer's instructions, 350 µl lysis buffer was added to each frozen samples and the lysate was aspirated through a 20-gauge needle before being centrifuged at 10000 g for 3 minutes at room temperature. The lysates of theca cells and corpus luteum were then subjected to a proteinase K treatment (6.7 mAU/ml, Qiagen, Valencia, USA) at 55°C for 10 minutes. 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 minutes at room temperature. After washing the column three times, the RNA was eluted with 30 µl RNAse-free water.

Prior to reverse transcription, the eluted RNA samples were incubated for 5 minutes at 70^0C, 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 5X reverse transcriptase buffer (Gibco BRL, Breda, The Netherlands), 8 units RNAsin (Promega), 150 units Superscript II reverse transcriptase (BRL), 0.036 U random primers (Life Technologies BV, Leiden, The Netherlands) and containing 10 mM DTT and 0.5 mM of each dNTP. The mixture was incubated for 1 hour at 42^0C, for 5 minutes at 80^0C and then stored at -20^0C. Minus RT blanks were prepared under the same conditions, but without inclusion of reverse transcriptase.

Amplification of GDF-9, BMP-15 and BMP receptors 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 µM polymerase (HotStarTaq, Qiagen, Valencia, USA) in 1xPCR buffer. The primers used for amplification were predicted to span an intron based on the other species (Table 1).

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

Target gene	Primer sequence (5' → 3')	Sense	Position	Genbank accession number
GDF-9	ACAACACTGTTCGGCTCTTC	s	332-351	GI: 6715598 (2003)
	TAAGCCTGAGCACTTGTGTC	as	805-824	*Homo sapiens* GDF-9
BMP-15	GATTCAGGAGCTGCTAGAAG	s	129-148	GI: 8925958 (2000)
	AGTGGCTCTGACTAGTTGGT	as	399-418	*Ovis aries* BMP-15
BMPR-II	GATATGCAGGTTCTGGTGTC	s	20-39	GI: 26985548 (2002)
	AGTTCAGCCATCCTCTCTTC	as	170-189	*Bos taurus* BMPR-II
BMPR-IA	TCGTCGTTGTATTACAGGAG	s	1603-1632	GI: 6753193 (2003)
	AAACGCTTGCGGCCAATCGT	as	1719-1738	*Mus musculus*
	TTACATCCTGGGATTCAACC	as	1952-1971	BMPR-IA
BMPR-IB	ACGGAGCAGTGATGAGTGTCT	s	1554-1574	GI: 6680801 (2003)
	CAGAGTTTAATGTCCTGGGAC	as	1674-1694	*Mus musculus* BMPR-IB

The thermal cycling profile for the first round of PCR was: initial denaturation and activation of the polymerase for 15 minutes at 94^{0}C, followed by 40 cycles of 15 sec at 94^{0}C, 30 sec at 55^{0}C and 45 sec at 72^{0}C. Final extension was for 10 minutes at 72^{0}C. During the amplification of BMPR-IA 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, ten 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

Expression of GDF-9 and BMP-15 in goat ovaries

The ovarian sections contained primordial, primary, secondary and antral follicles as well as corpora lutea. Both GDF-9 and BMP-15 proteins were detected in oocytes from the primordial follicle stage onward (Fig. 1, Table 2). All primordial oocytes

were positive for both GDF-9 and BMP-15. A positive immunoreaction for both GDF-9 (Fig. -1A, B, C) and BMP-15 (Fig. 1I, J, K) was also observed in granulosa cells from primary and secondary, but not from primordial follicles. Amplification of cDNA from primordial, primary and secondary follicles resulted in specific products for both GDF-9 (493 base pairs [bp]) and BMP-15 (302 bp) as shown in Fig. 2.

Occasionally, in small antral follicles, cumulus and mural granulosa cells showed a weak reaction for GDF-9 (Fig. 1D), and a more prominent reaction for BMP-15 (Fig. 1L). In large antral follicles, cumulus and mural granulosa cells respectively had moderate and strong reaction for GDF-9 (Fig. 1 E, F). These large follicles did not show a positive reaction for BMP-15 in cumulus cells, but a strong reaction was observed in mural granulosa cells (Fig. 1 M, N). GDF-9 and BMP-15 immunoreactions were absent in theca cells, except in large antral follicles where they occasionally showed GDF-9 staining (Fig. 1F). Both GDF-9 and BMP-15 mRNA expression was detected in oocytes, cumulus and mural granulosa cells collected from small and large antral follicles. Theca cells from both small and large antral follicles showed no specific mRNA for both GDF-9 and BMP-15.

GDF-9 protein and mRNA expression was furthermore detected in corpora lutea (Fig. 1G, 2). BMP-15 protein and mRNA, however, could not be demonstrated at these sites (Fig. 1O, 2). No immunoreaction was observed in any follicle or corpus luteum when control tests were carried out (GDF-9 [Fig. 1H] and BMP-15 [Fig. 1P]). Additionally, western blot analysis showed single bands with molecular size of approximately 54 and 46 kDa (Fig. 3), which are consistent with the sizes of GDF-9 and BMP-15 propeptides (Duffy 2003), respectively. Sequence analysis of the amplified GDF-9 and BMP-15 products confirmed their specificity. Amplification of -RT controls yielded no specific products (results not shown).

Expression of mRNA for BMP receptors in goat ovaries

In all replicates, an abundant message for BMPR-II (169 bp), BMPR-IA (135 bp) and BMPR-IB (140 bp) was clearly observed after amplification of cDNA from primordial, primary and secondary follicles, as well as from oocytes, cumulus, mural granulosa cells and theca tissue from small or large antral follicles. BMPR-II, BMPR-IA and BMPR-IB mRNA expression was also detected in corpora lutea (Fig. 2). Amplification of -RT controls yielded no specific products (results not shown). Sequence analysis of the amplified BMPR-II, BMPR-IA and BMPR-IB products confirmed their specificity.

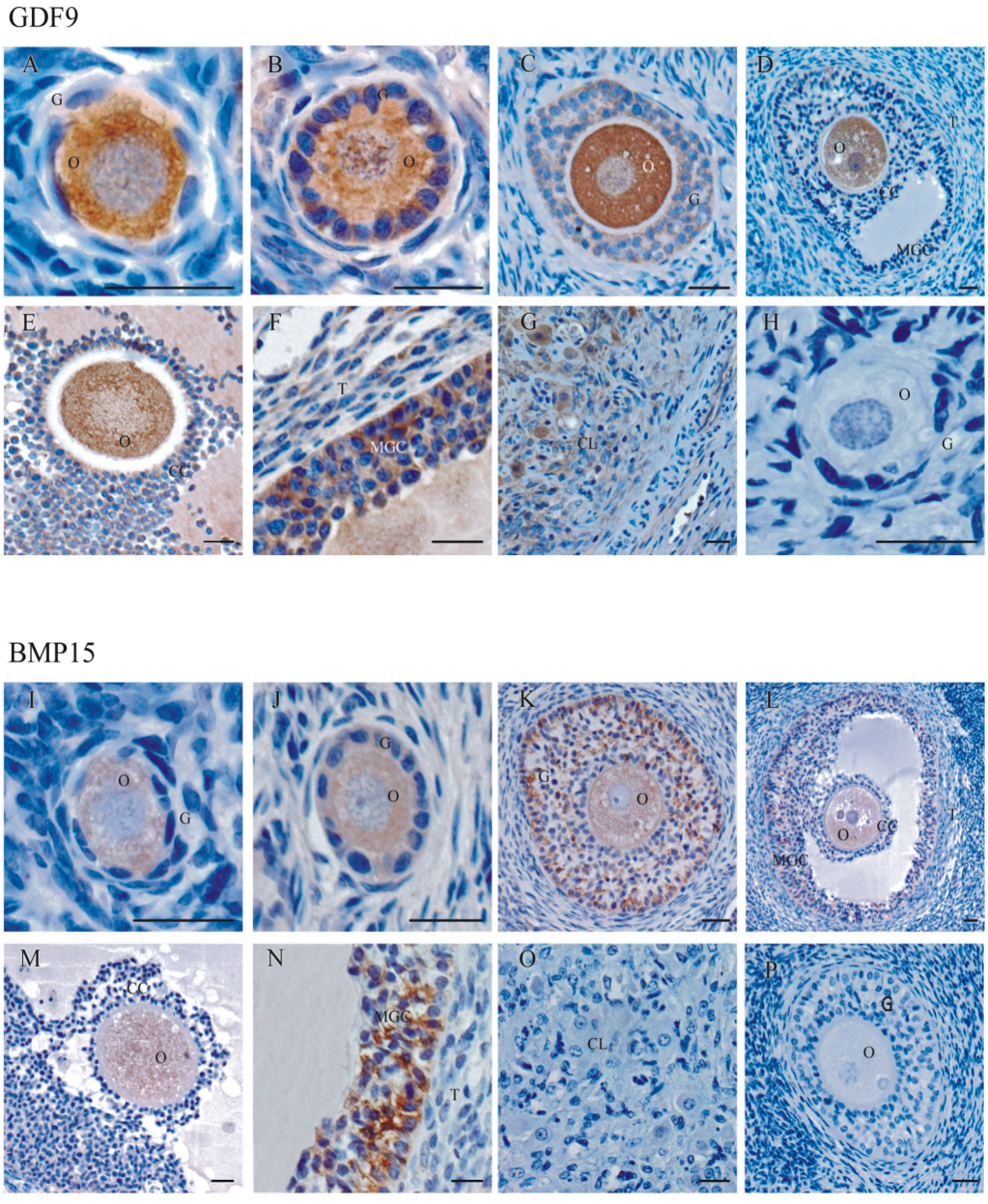

Figure 1. GDF-9 and BMP-15 immunoreactivity in the different structures found within goat ovaries. (A, I) Primordial follicle, (B, J) primary follicle, (C, K) secondary follicle, (D, L) small antral follicle, (E, M) COC of a large antral follicle, (F, N) mural granulosa and theca cells from a large antral follicle, (G, O) corpus luteum and (H, P) negative control. O: oocyte, G: granulosa cells, CC: cumulus cells, MGC: mural granulosa cells, T: theca cells, CL: corpus luteum. Scale bars represent 25 μm.

Table 2. Relative intensity of immunohistochemical staining for GDF-9 and BMP-15, and localization of mRNA for GDF-9, BMP-15, BMPR-II, BMPR-IA and BMPR-IB in the ovaries of goats.

Structure	GDF-9		BMP-15		BMPR-II	BMPR -IA	BMPR-IB
	Protein	mRNA	Protein	mRNA	mRNA	mRNA	mRNA
Primordial follicle							
Oocyte	+ + +	+ *	+ +	+ *	+ *	+ *	+ *
Granulosa	–		–				
Primary follicle							
Oocyte	+ + +	+ *	+ +	+ *	+ *	+ *	+ *
Granulosa	+		+				
Secondary follicle							
Oocyte	+ + +		+ +				
Granulosa	+	+ *	+ +	+ *	+ *	+ *	+ *
Theca cells	–		–				
Antral follicle<3mm							
Oocyte	+ + +	+	+ +	+	+	+	+
Cumulus cells	+	+	+	+	+	+	+
Mural GC	+	+	+ +	+	+	+	+
Theca cells	–	–	–	+	+	+	+
Antral follicle>3mm							
Oocyte	+ + +	+	+ +	+	+	+	+
Cumulus cells	+ +	+	– / +	+	+	+	+
Mural GC	+ + +	+	+ + +	+	+	+	+
Theca cells	– / +	–	–	+	+	+	+
Corpus luteum	+ +	+	–	–	+	+	+

* whole follicles, GC: granulosa cells

(/ +) occasionally found, (-) absent, (+) weak, (+ +) moderate and (+ + +) strong immunoreaction

Discussion

The current study evaluated the distribution of GDF-9 and BMP-15 mRNA and protein as well as BMP receptor mRNA in goat ovaries to determine whether these TGF-ß members may play a role in follicular development in the goat.

We demonstrated the protein expression of both GDF-9 and BMP-15 in oocytes of primordial, primary and secondary follicles. Using RT-PCR we were able to demonstrate that caprine primordial, primary and secondary follicles express mRNA for both GDF-9 and BMP-15 confirming that these follicles synthesize both proteins. These results are similar to those reported for brushtail possum (Eckery *et al.* 2002), sheep and cow (Bodensteiner *et al.* 1999, 2000) where both GDF-9 and BMP-15 mRNA were found as early as in oocytes of primordial follicles. This is earlier than that found for the mouse, rat and human (McGrath *et al.* 1995, Laitinen *et al.* 1998, Dube *et al.* 1998, Aaltonen *et al.* 1999, Jaatinen *et al.* 1999, Elvin *et al.* 2000a, Erickson & Shimasaki 2003) where they are first observed in oocytes of primary follicles. The expression of both GDF-9 and BMP-15 in the very early, non-growing, follicles is intriguing and it is possible that they play an

important role in the maintenance of primordial follicles in goats. However, we cannot exclude the possibility that an inhibitory factor, for instance follistatin (Otsuka *et al.* 2001a), that blocks their biological activity is present. GDF-9 and BMP-15 may be retained within the oocyte of primordial follicles, since we detected both proteins in granulosa cells of primary and secondary, but not in primordial follicles. Presence of these growth factors in granulosa cells of early

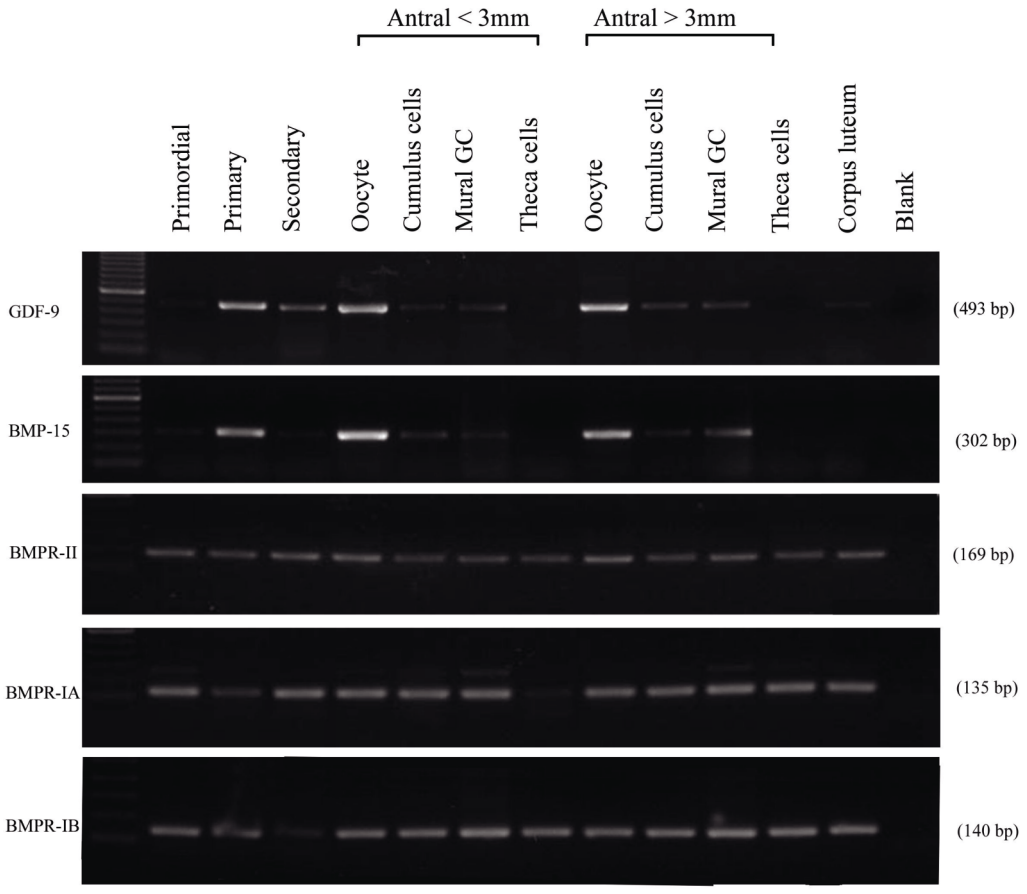

Figure 2. Expression of GDF-9, BMP-15, BMPR-II, BMPR-IA and BMPR-IB in different follicle and cell types in goat ovaries. Follicle and cell types are indicated at the top. One-hundred base pair ladders are included as markers for fragment size.

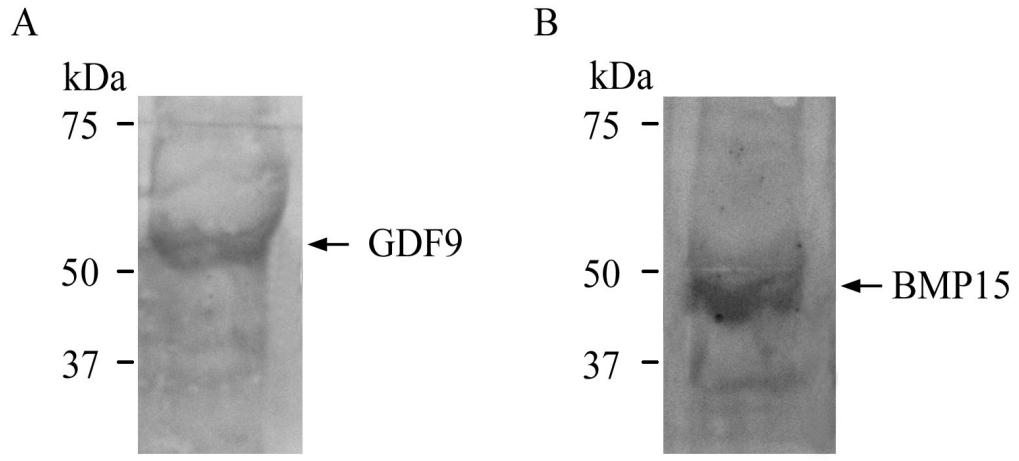

Figure 3. Western blot analysis of GDF-9 (A) and BMP-15 (B) expression in goat ovaries. Bands of approximately 54 and 46 kDa are consistent with the sizes of GDF-9 and BMP-15 propeptides, respectively.

follicles has not been described before. GDF-9 and BMP-15 may play different roles in regulating early follicular development in different species. Yan *et al.* (2001) showed that mice lacking BMP-15 are subfertile, while GDF-9 null are infertile (Dong *et al.* 1996, Carabastos *et al.* 1998, Elvin *et al.* 1999b). In contrast, ewes that have naturally occurring inactivating mutations in the BMP-15 gene showed follicular development arrested at primary follicle stage and are infertile (Galloway *et al.* 2000).

The mRNA of the BMP receptors type -IA, -IB and II were all detected in primordial, primary and secondary goat follicles. Expression of BMP receptors, in such early follicles has also been described in rodents (Shimasaki *et al.* 1999, Erickson & Shimasaki 2003) and cattle (Souza *et al.* 2002). Despite the proteins for BMP receptors were not demonstrated because suitable antibodies are lacking, our findings suggest that, in the goat, early follicular development could be controlled by the ligands of these receptors, with GDF-9 and BMP-15 being likely candidates. Both GDF-9 and BMP-15 are known to stimulate granulosa cell mitosis and early follicular development in rodents (Vitt *et al.* 2000a, Otsuka *et al.* 2000, Nilsson & Skinner 2002). Furthermore, the importance of GDF-9 and BMP-15 for early folliculogenesis is confirmed by the findings of Dong *et al.* (1996) and Galloway *et al.* (2000), that showed that GDF-9-deficient mice and BMP-15-deficient sheep are infertile because follicle development does not proceed beyond the primary stage.

In caprine antral follicles, both GDF-9 and BMP-15 proteins were present in the oocyte, cumulus and mural granulosa cells. The mRNAs for these growth factors were also detectable at these sites. The expression pattern of GDF-9 in antral follicles is similar to that described for primates (Sidis *et al.* 1998, Yamamoto *et al.* 2002, Duffy 2003) but not for rodents (Dong *et al.* 1996, Jaatinen *et al.* 1999), bovine and sheep (Bodensteiner *et al.* 1999). In contrast to our findings BMP-15 has not been demonstrated in granulosa

cells in other species studied so far (mice: Dube *et al.* 1998, sheep: Galloway *at al.* 2000, human: Aaltonen *et al.* 1999). We furthermore detected the types -IA, -IB and II BMP receptors in goat oocyte and both cumulus and mural granulosa cells of antral follicles. These latter observations correspond to previously reported findings with rodents (Erickson & Shimasaki 2003). Some in-vitro studies have showed the importance of GDF-9 and BMP-15 for granulosa cell activity. In cultured granulosa cells, recombinant GDF-9 induced hyaluronan synthase-2, cyclooxigenase-2 and steroidogenic acute regulator (STAR) protein, suppressed LH receptor synthesis (Elvin *et al.* 1999a) and thus stimulated steroidogenesis (Yamamoto *et al.* 2002). In a subsequent study, Elvin *et al.* (2000b) found that, in vitro, GDF-9 stimulated the synthesis of EP2 prostaglandin E2 receptor and progesterone by granulosa cells as well as the expansion of cumulus cells. Furthermore, GDF-9 stimulated pentraxin 3, TNF-induced protein 6 (mice: Varani *et al.* 2002), and inhibin production by cultured granulosa cells (rat: Roh *et al.* 2003, human: Kaivo-Oja *et al.* 2003). BMP-15 alone did not affect estradiol production in in-vitro cultured rat granulosa cells, but it significantly inhibited FSH-stimulated progesterone production through its ability to suppress FSH receptor expression (Otsuka *et al.* 2001b). In addition, treatment with BMP-15 alone stimulated granulosa cell proliferation (Otsuka *et al.* 2001b) but did not affect the expression of STAR, P450scc, P450 aromatase, 3-ß-hydrosteroid dehydrogenase and LH receptor (Wu & Matzuk 2002). BMP-15, however, markedly decreased FSH-induced expression of these factors (Otsuka *et al.* 2001b). Our data suggest that, in goats, GDF-9 and BMP-15 may function not only as oocyte-secreted paracrine factors but also as autocrine factors that may regulate several key biological functions in granulosa cells.

Occasionally we detected GDF-9 protein, but not its mRNA in theca tissue of the more advanced goat antral follicles, which suggests that this protein is derived from other sources such as the oocyte or granulosa cells. At this site, GDF-9 may act as a paracrine factor because of the presence of BMP receptors in theca cells. The importance of GDF-9 for theca development has been demonstrated in GDF-9 null mice (Dong *et al.* 1996), that fail to form theca layers and lack several theca cell marker genes (i.e., LH receptor, 17-α-hydroxylase [CYP17], and c-kit). However, the effect of GDF-9 on theca cell formation appears to be indirect, since inhibin α is highly upregulated in GDF-9 null ovaries (Elvin *et al.* 1999b). In double mutant mice for GDF-9 and inhibin α, recruitment of thecal cells occurs, but theca cell markers are not expressed (Wu *et al.* 2004). In mice that did not express inhibin α, but in which GDF-9 expression is fairly normal, there is no detectable CYP17 expression in the thecal cells (Wu *et al.* 2004). This contradicts previous findings that application of GDF-9 directly led to an increase in ovarian CYP17 content (Vitt *et al.* 2000b), and increased steroidogenesis (Otsuka *et al.* 2000) and androstenedione production by cultured theca cells (Solovyeva *et al.* 2000).

Surprisingly, we detected the GDF-9 protein and both its mRNA and those of all BMP receptors in goat corpora lutea, suggesting a possible role of GDF-9 in their activity. Thus far, the expression of GDF-9 in mammalian corpora lutea was not described. Based on the knowledge that granulosa cells are precursors of large luteal cells, that GDF-9 stimulates progesterone production by cultured granulosa cells (Elvin *et al.* 2000b), and that currently and previous researchers demonstrated both mRNA and protein expression

in granulosa cells (Yamamoto *et al.* 2002, Duffy 2003), we speculate that GDF-9 promotes progesterone secretion by luteal cells in an autocrine manner.

In conclusion, it is currently demonstrated that the mRNAs and proteins of GDF-9 and BMP-15, and the mRNA of BMPR-IA, BMPR-IB and BMPR-II are expressed in goat ovarian follicles at all stages of their development. Antral follicles express the mRNA for GDF-9 and BMP-15 in their oocyte and granulosa cells, and BMPR-IA, BMPR-IB and BMPR-II also in the theca. These findings show the presence of a complex intrafollicular regulatory system, consisting of GDF-9, BMP-15 and BMP-receptors, in goat ovaries. Expression of GDF-9 protein and mRNA and BMP receptors mRNA in corpora lutea indicates that GDF-9 may also be involved in luteal activity. The data furthermore show that the initial concept that GDF-9 and BMP-15 are exclusively produced by the oocyte can not be maintained for the goat.

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Chapter 5

The Kit Ligand / c-Kit Receptor System in Goat Ovaries: Gene Expression and Protein Localisation

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Abstract

Relatively little information is available on the local factors that regulate folliculogenesis in goats. To examine the possibility that the Kit Ligand (KL) system is expressed throughout the folliculogenesis, we studied the presence and distribution of KL and its receptor, c-Kit, in goat ovaries. Ovaries of goats were collected and either fixed in paraformaldehyde for immunohistochemical localisation of KL and c-Kit proteins, or used for the isolation of follicles, luteal cells, surface epithelium and medullary samples to study mRNA expression for KL and c-Kit, using the reverse transcriptase polymerase chain reaction. KL protein and mRNA was found in follicles at all stages of development, i.e., primordial, primary, secondary, small and large antral follicles, as well as in corpora lutea, surface epithelium and medullary tissue. Antral follicles expressed both KL-1 and KL-2 mRNAs, while earlier staged follicles expressed KL-1 transcript only. KL protein was demonstrated in granulosa cells from the primordial follicle onward. Its mRNA could be detected in granulosa cells isolated from antral follicles and occasionally in their theca cells. c-Kit mRNA was expressed in all antral follicular compartments and at all stages of follicular development. c-Kit protein was predominantly found in oocytes from the primordial follicle stage onwards, in theca cells of antral follicles, as well as in corpora lutea, surface epithelium and medulary tissue, particularly in the wall of blood vessels, which may indicate these cells as main action sites of KL. It is concluded that the KL / c-Kit system, in goat ovaries, is widespread and that it may be involved in the regulation of various local processes, including folliculogenesis and luteal activity.

Keywords: goat, ovary, follicles, kit ligand, c-kit

Introduction

The adult mammalian ovary is a complex organ composed of various cell types including oocytes, granulosa, theca, stroma and surface epithelial cells. These cell types are further subdivided into various subtypes. For example, the granulosa cells can be further differentiated into mural, cumulus, corona radiata or luteal cells, while theca cells develop into internal, external, and luteal cells. The coordinated control of proliferation, differentiation and apoptosis of these cell types forms the underlying basis for menstrual or estrous cycles in mammals. The mechanism by which each cell type obtains its state of proliferation and/or differentiation is the subject of intense study and it has been shown that, as well as endocrine compounds, locally produced factors can regulate or modulate these developmental processes (Eppig 2001).

Kit ligand (KL), encoded by the *Steel* (*Sl*) gene, is a locally produced factor that is thought to have many roles in ovarian function (Yoshida *et al.* 1997). KL mRNA expression in follicles is, however, localised to granulosa cells in all species studied so far (Ismail *et al.* 1996, Laitinen *et al.* 1995, Manova *et al.* 1990, Motro & Bernstein 1993, Tisdall *et al.* 1997, 1999), and can be expressed as either a membrane-bound or a soluble protein, depending on how the mRNA is spliced (Huang *et al.* 1992). Both transcripts, when translated, yield membrane-associated products, but KL-1 is efficiently cleaved

and released as a soluble product due to a proteolytic cleavage site encoded by an 84-base pair exon. The other form, KL-2, lacks this cleavage site and therefore remains membrane-bound (Huang *et al.* 1992). The membrane-bound KL is the more potent of the two forms with regard to its ability to induce the proliferation of primordial germ cells (Dolci *et al.* 1991, Allard *et al.* 1996). Both membrane-bound and soluble forms of KL are present in the mouse ovary (Manova *et al.* 1993).

The receptor for KL is c-Kit, a member of the tyrosine kinase receptor family encoded by a proto-oncogene at the *W* locus. During postnatal ovarian development, both c-Kit mRNA and protein are found in oocytes at all stages of follicle development, at least in mice (Manova *et al.* 1990, Horie *et al.* 1991, Motro & Bernstein 1993) and sheep (Clark *et al.* 1996). In addition, c-Kit expression is found in interstitial and theca cells of antral follicles in mice and sheep (Manova *et al.* 1990, Motro & Bernstein 1993, Clark *et al.* 1996). In sheep, c-Kit mRNA has also been found in granulosa cells (Clark *et al.* 1996, Juengel *et al.* 2000), suggesting that its full range of functions may differ between species. In vitro studies with rodents and sheep showed that the KL / c-Kit system has been implicated in proliferation of primordial germ cells, activation of primordial follicles, oocyte growth, proliferation of granulosa cells and recruitment of theca cells (reviewed by Driancourt *et al.* 2000, van den Hurk & Zhao 2005).

Although there is evidence for an intraovarian KL / c-Kit system that is important for ovarian function, information on its localisation and function is mainly obtained from murine, ovine and primate models. The distribution of KL and c-Kit in the goat ovary has not yet been described. Knowledge of the factors that control folliculogenesis in goats is important to improving the effectiveness of in vitro techniques such as culture of early follicles, maturation and fertilisation of oocytes, which facilitate the production of large numbers of embryos from genetically valuable animals.

The aim of the present study was to examine the expression of KL and c-Kit mRNA and protein in the ovaries of goats, to obtain evidence for the presence of a KL / c-Kit system that may play an important role during folliculogenesis. 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=60) were recovered from slaughtered adult mixed-breed goats and transported to the laboratory in a thermos flask, within 1hr. Twenty ovaries from ten randomly chosen goats 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, Ltd, Pittsburgh, USA) in preparation for immunohistochemical studies. The remaining 40 ovaries were used to recover cells and tissues for RT-PCR.

Immunohistochemical localisation of KL and c-Kit

Immunohistochemical study for KL and c-Kit was performed on serial 5-μm sections cut from ovaries of ten 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 minutes. The sections were then washed with PBS (pH 7.4) and the epitopes were activated by microwaving the sections for 7 minutes at 900 W in 0.01 M citrate buffer (pH 6.0). Following microwave treatment, the sections were washed in PBS / 0.05% Tween (Merck, Darmstadt, Germany) before being incubated for 30 minutes with 5% of either normal goat or horse serum in PBS to minimize non-specific binding. The primary antibodies used were: (1) rabbit polyclonal anti-c-Kit antibody (C-19, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) diluted 1:50 (4μg/mL), and (2) mouse monoclonal anti-KL antibody (G-3, Santa Cruz Biotechnology Inc) diluted 1:20 (10μg/mL) in PBS containing 5% normal goat or horse serum. The mentioned antibodies have been successfully used in human tissues (LaFevre *et al.* 2004). The sections were incubated overnight at 4ºC 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 / 0.05% Tween and incubated for 45 minutes with an appropriate biotinylated secondary antibody, i.e. goat anti-rabbit IgG for c-Kit and either goat or horse anti-mouse IgG for the KL antibody (both from Vector laboratories, Burlingame, CA, USA), diluted 1:200 in PBS containing 5% normal goat or horse serum. Next, the sections were washed three times in PBS / 0.05% Tween before being incubated for 45 minutes with an avidin-biotin complex (1:600, Vectastain Elite ABC kits; Vector laboratories, Burlingame, CA, USA). 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, USA) for a maximum of 20 minutes. The stained sections were rinsed in PBS and water, and counterstained for 10 seconds in Mayer's haematoxylin. Finally, the sections were washed for 10 minutes in running tap water, dehydrated in a graded ethanol series and then xylene, and mounted in Depex. The staining intensity for both KL and c-Kit immunoreactive protein expression was scored as follows: absent (-), weak (+), moderate (++), or strong (+++). Five randomly chosen sections from each ovary (n=8) from eight different goats were analyzed 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 diaminobenzidine reagent alone to exclude the possibility of non-suppressed endogenous peroxidase activity; (3) preabsorbing the c-Kit antibody overnight at 4ºC with its blocking peptide at 20-fold excess (Santa Cruz Biotechnology Inc); and (4) western blotting analysis to confirm the specificity of KL antibody, since a respective blocking peptide is not available. For western blotting, goat (n=3) and mouse (n=2, positive control; specific reaction according to manufacturer) ovaries were homogenised in lysis buffer (20 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton

X-100, 10% glycerol and 1% protease inhibitor cocktail). After centrifugation at 13 000 x g for 15 minutes, the supernatant was removed and used for analysis. For each sample, 20 µl was boiled in the presence of 2ß-mercaptoethanol and electrophoresed in a 12% polyacrylamide gel. Following electrophoresis, gels were electrotransferred for 1 h to nitrocellulose membranes (Amersham Pharmacia, Piscataway, NJ, USA). Membranes were then blocked in Tris-buffered saline (50 mM Tris [pH 7.4] and 150 mM NaCl) with 5% nonfat dry milk, incubated with primary antibody (same used for immunohistochemistry) diluted 1:200, washed twice with blocking buffer, and incubated with secondary antibody (goat anti-mouse conjugated with horse-radish peroxidase, Santa Cruz Biotechnology Inc), at 1:2000 dilution. In the negative control, the primary antibody was replaced with IgGs from the same species in which the specific antibody was raised. After washing three times, detection was performed using DAB (0.05% DAB in Tris/HCl pH 7.6, 0.03% H_2O_2 - Sigma, St. Louis, MO, USA).

Classification of follicles and statistical analysis

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

Oneway ANOVA and Duncan test were used to compare the number of follicles of different categories with oocyte, granulosa or theca cells positive for either KL or c-Kit among four different ovaries. The differences were considered significant when $P < 0.05$.

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) and dried with paper towel. Ten ovaries were dedicated to preantral follicles isolation and the others were used to provide antral follicles, oocytes, cumulus cells, mural granulosa cells and samples of corpora lutea, medulla 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 (The Mickle Laboratory Engineering Co., Gomshal, Surrey, UK) adjusted to 75 µm. The fragments were then placed in PBS containing 5% bovine serum albumin (Sigma) 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, 15 primordial (Fig. 1a), primary (Fig. 1b) or secondary (Fig. 1c) follicles were carefully selected based on morphological shape and number of granulosa cell layers around the

oocyte and placed into separate Eppendorf tubes. All samples were stored at 80ºC until the RNA was extracted.

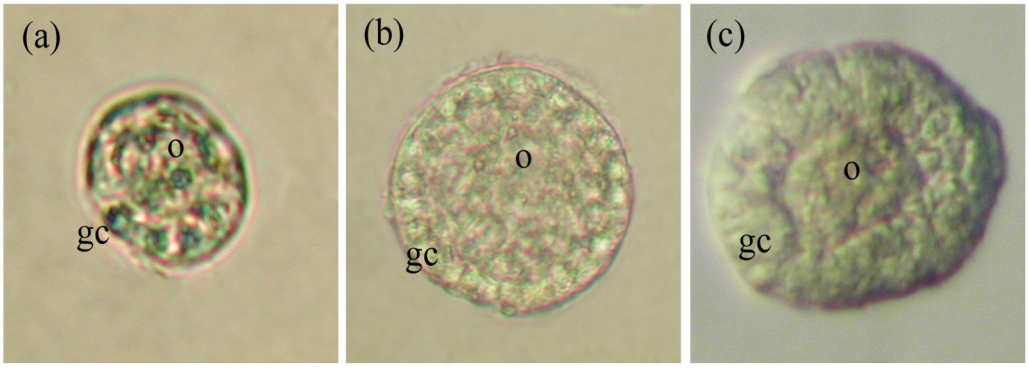

Figure 1. Isolated goat ovarian follicles. (a) Primordial follicle, an oocyte surrounded by single layer of flattened / cuboidal granulosa cells, (b) primary follicle with one layer of cuboidal granulosa cells and (c) secondary follicle with more than two layers of granulosa cells. o: oocyte, gc: granulosa cells.

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, so collected, compact COCs were selected as described by van Tol & Bevers (1998). Thereafter, the cumulus was separated from the oocyte by a combination of vortexing and aspiration via a narrow-bore Pasteur pipette. Mural granulosa cells were scraped off from follicular walls recovered from dissected antral follicles in which the COCs had been removed to avoid contamination by cumulus cells. Denuded oocytes, cumulus and mural granulosa cells were washed four times in PBS and packed in tubes in groups of either 10 denuded oocytes, cumulus cells from 10 COCs, or samples of mural granulosa and stored at -80°C until RNA extraction.

To collect theca cells, small (n=10) and large antral follicles (n=10) were isolated from goat ovaries (n=5) and dissected free of stromal tissue using forceps, as described previously for bovine ovaries (van Tol & Bevers 1998). The follicles were then bisected and the granulosa cells were scraped off using a scalpel blade. Next, the theca cell layers were vortexed for 1 minute in 1 ml HEPES buffered M199 (Gibco BRL, Paisly, UK) supplemented with penicillin/streptomycin, 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. To investigate the possibility of theca cells contamination by adhering mural granulosa cells we used specific primers (Table 1) to detect growth differentiation factor-9 (GDF-9) that is expressed in goat mural granulosa cells but not in the theca (Silva *et al.* 2004). From another group of ovaries (n=5), small pieces of corpus luteum, medula and surface epithelium were collected and stored at -80°C until RNA extraction. Three samples of each tissue sample were recovered and analysed.

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). As per the manufacturer's instructions, 350 µl lysis buffer was added to each frozen samples and the lysate was aspirated through a 20-gauge needle before being centrifuged at 10000 g for 3 minutes at room temperature. The lysates of theca cells, corpus luteum, medulla and ovarian surface samples were then subjected to a proteinase K treatment (6,7 mAU/ml, Qiagen, Valencia, USA) at 55°C for 10 minutes. 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 minutes 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 minutes at 70^0C, 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 5X 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 BV, Leiden, The Netherlands) and containing 10 mM DTT and 0.5 mM of each dNTP. The mixture was incubated for 1 hour at 42^0C, for 5 minutes at 80^0C and then stored at -20^0C. Minus RT blanks were prepared under the same conditions, but without inclusion of reverse transcriptase.

Amplification of KL and c-Kit 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 1xPCR buffer. The primers used for amplification of KL, c-Kit, GDF-9 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are presented in Table 1. GAPDH was used as an internal control, since transcripts for this gene are expected to be present at the same level in all cell types. For KL, the primers spanned the alternatively spliced exon, thus enabling simultaneous detection of mRNA for both the soluble form, KL-1, and the membrane bound form, KL-2. Since the position of the lacking exon (KL-2) in the goat sequence of KL is not known, the primers for KL were designed based in the human sequence.

The thermal cycling profile for the first round of PCR was: initial denaturation and activation of the polymerase for 15 minutes at 94^0C, followed by 40 cycles of 15 sec at 94^0C, 30 sec at 55^0C and 45 sec at 72^0C. Final extension was for 10 minutes at 72^0C. During the amplification of KL cDNA, heminesting was used to increase the specificity and sensitivity, using a different sense primer. For heminesting, 1 µl of the first round product was transferred to another 200 µl tube containing 24 µl amplification mixture, and amplified for 30 cycles using the same thermal cycling profile. All reactions were performed in a 24-well thermocycler (Perkin-Elmer, Gouda, The Netherlands). Finally,

ten µl of the product was resolved by electrophoresis in 1% Agarose gels containing ethidium bromide. A 100 base pair (bp) DNA ladder (Gibco BRL) was included as a reference for fragment size.

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

Target gene	Primer sequence (5' → 3')	sense	Position	Genbank accession number
Kit Ligand	GATCTGCAGGAATCGTGTGA	s (R1)	81-100	GI: 4505174 (2003)
	AGTCGATGACCTTGTGGAGT	s (R2)	321-340	*Homo sapiens* Kit
	ACTGGAAGAAGAGACAGCCA	as (R1,2)	707-726(KL-1) 623-642(KL-2)	Ligand
c-Kit	CACTGCTCAGCGAATCAGAA	s	610-629	GI: 633053 (1999)
	TCCACATAGAGTCCACGGAA	as	777-796	*Capra hircus* c-Kit
GDF-9	ACAACACTGTTCGGCTCTTC	s	332-351	GI: 6715598 (2003)
	TAAGCCTGAGCACTTGTGTC	as	805-824	*Homo sapiens* GDF-9
GAPDH	AGGCCATCACCATCTTCCAG	s	179-198	GI: 2285902 (1997)
	GGCGTGGACAGTGGTCATAA	as	485-504	*Bos taurus* GAPDH

R1 = round 1, R2 = round 2 or heminesting

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

Results

Immunohistochemistry

All stages of follicle development (primordial, primary, secondary and antral follicles) and corpora lutea were identified within the ovarian sections. KL was detected in granulosa cells of follicles from the primordial stage onward (Fig. 2, Table 2) and, in primordial, primary and secondary follicles a moderate reaction was observed at the junction of the granulosa and the oocyte (Fig. 2a,b,c). Occasionally, oocyte from primordial follicles also stained positively for KL (Fig. 2a). In small antral follicles, KL staining intensity was weaker than in preantral follicles and distributed equally across the cumulus and mural granulosa cells (Fig. 2g). In large antral follicles, both cumulus and theca cells showed a weaker immunoreaction than the corresponding mural granulosa cells (Fig. 2h,i). In addition, strong KL immunoreactivity is observed in corpora lutea (Fig. 3a), ovarian surface epithelium (Fig. 3b) and vascular smooth muscle (Fig. 3c). In the control sections for KL protein (Fig. 3d), replacing the specific antibody with normal IgG, no positive staining was observed. Additionally, western blot analysis showed, in goat and mouse ovaries, a band with molecular size of approximately 25kDa (Fig. 4), which is consistent with the size of KL protein.

c-Kit was immunohistochemically demonstrable in the oocytes of follicles at all stages, i.e. primordial, primary, secondary, small and large antral follicles (Fig. 2; Table 2). The protein was distributed throughout the cytoplasm of the oocytes, while the staining intensity in primordial and primary follicles was stronger than in late-staged follicles. In addition, a moderate to strong c-Kit staining was observed in theca cells of

late secondary, small and large antral follicles (details in Fig. 2f,j,k,l). Occasionally, weak c-Kit immunoreaction was observed in the granulosa cell of early or late-staged follicles (Fig. 2e,f,j,k). Finally, immunoreactivity for c-Kit was clearly visible in corpora lutea (Fig. 3e), ovarian surface epithelium and vascular smooth muscle and endothelial cells (Fig. 3f,g). Table 3 shows the number of follicles from different categories analysed in eight different ovaries. No significant inter-ovary variation ($P>0.05$) in the immunohistochemical staining for either KL or c-Kit was observed (Table 3). Control sections for c-Kit, replacing the specific antibody by normal IgG showed absence of staining (Fig. 3h). When the antibody was preabsorbed with its blocking peptide, only a weak background staining was observed (Fig. 3i).

Expression of mRNA for KL and c-Kit in goat ovaries

The first round of amplification using primers for KL yielded specific products for both KL-1 and KL-2 only in samples of cDNA prepared from mural granulosa cells of antral follicles and corpora lutea. After heminesting, however, amplification of cDNA from primordial, primary and secondary follicles resulted in specific products for soluble KL-1 mRNA, but not for KL-2 mRNA. When cDNA from either cumulus, mural granulosa, or theca cells collected from small or large antral follicles was amplified, products for both KL-1 and KL-2 were observed after heminesting. KL mRNA expression was not detected in oocytes from either small or large antral follicles (Fig. 5a) which confirms the absence of contaminating cumulus cells. In addition, we detected GDF-9 mRNA in mural granulosa cells but not in the theca (Fig. 5b) confirming the purity of theca samples. KL-1 and KL-2 mRNA expression were also detected in corpus luteum, ovarian medulla and ovarian surface epithelium (Fig. 5a). Amplification of -RT blanks or water controls yielded no specific products in any of the reactions (results not shown).

Amplification of cDNA from primordial, primary and secondary follicles and from oocytes, cumulus, mural granulosa and theca cells from small or large antral follicles using specific primers for c-Kit, resulted in an abundant product after one round of amplification in all cases. c-Kit expression was also detected in corpus luteum, ovarian surface epithelium and medullary tissue (Fig. 5a). The expression of the housekeeping gene (GAPDH) is also illustrated in Fig. 5a. Amplification of -RT blanks or water controls yielded no specific products in any of the reactions (not shown).

Sequence analysis of the amplified c-Kit and KL products confirmed their specificity when compared with the published c-Kit (GI: 633053) and KL-1 (GI: 16580734) mRNA from goats. Sequencing of KL-2 showed that, compared with the sequence of KL-1 (GI: 16580734), a 84-base pair exon is absent between nucleotides 680 and 764.

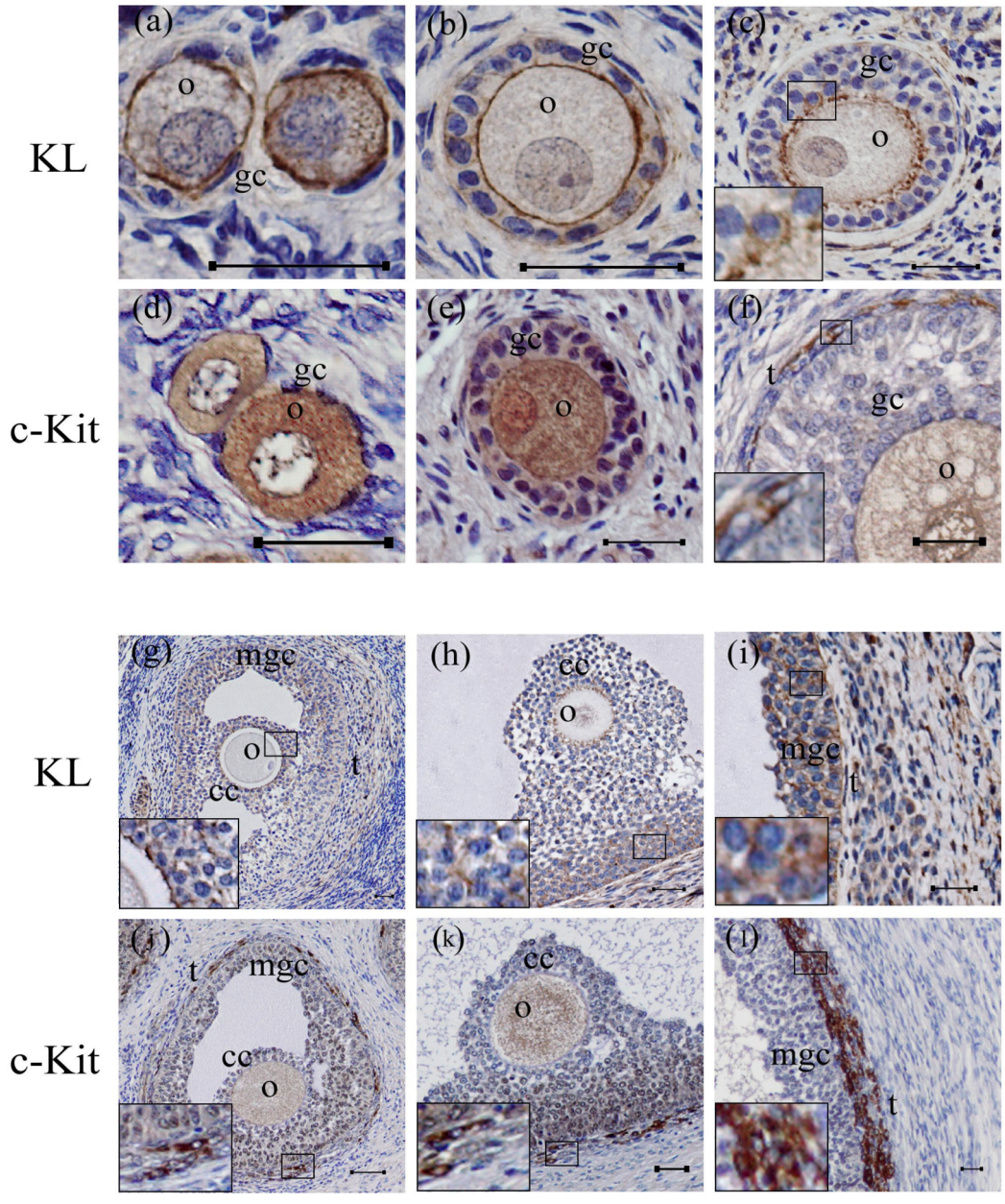

Figure 2. KL and c-Kit immunoreactivity in goat ovarian follicles. (a, d) Primordial follicle, (b, e) primary follicle, (c, f) secondary follicle, (g, j) small antral follicle, (h, k) COC of a large antral follicle, (i, l) mural granulosa and theca cells from a large antral follicles. Inserts: higher magnification showing immunoreaction in the cell cytoplasm. o: oocyte, gc: granulosa cells, mgc: mural granulosa cells, cc: cumulus cells, t: theca cells, Scale bars represent 25 µm.

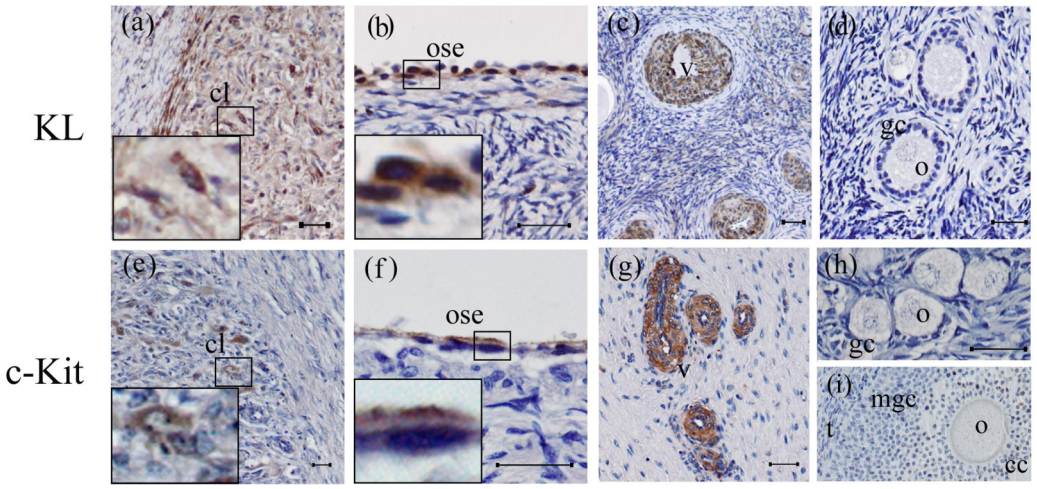

Figure 3. KL and c-Kit immunoreactivity in goat (a, e) corpus luteum, (b, f) ovarian surface epithelium, (c, g) blood vessels and (d, h, i) negative control. Inserts: higher magnification showing immunoreaction in the cell cytoplasm. o: oocyte, gc: granulosa cells, mgc: mural granulosa cells, cc: cumulus cells, t: theca cells, cl: corpus luteum, ose: ovarian surface epithelium and v: vessels. Scale bars represent 25 μm.

Discussion

Over the last decade, several papers have demonstrated the presence of a functional KL / c-Kit system in mammalian ovaries, in particular those of the mouse and the sheep (for review, see Driancourt *et al.* 2000, van den Hurk & Zhao 2005). The present study examined the distribution of KL and c-Kit mRNA and protein in goat ovaries, to determine whether the KL / c-Kit system may also play a role in folliculogenesis in the goat. With regard to KL, we demonstrated the presence of protein in granulosa cells of primordial, primary and secondary follicles, and in particularly at the junction of oocyte and granulosa cells, which suggests that granulosa cells secrete the protein. A similar distribution of KL was described previously for monkey primordial follicles (Gougeon & Busso 2000). In some occasions we detected KL protein in the oocyte of caprine primordial follicles, a finding that confirmed the previous descriptions of KL in murine oogonia and oocytes from primordial follicles (Doneda *et al.* 2002, Kang *et al.* 2003). Using RT-PCR, the current study demonstrated the expression of mRNA for the soluble KL-1 in caprine primordial, primary and secondary follicles. The detection of KL mRNA in granulosa cells of early-stage follicles was previously described in sheep (Tisdall *et al.* 1997, MacNatty *et al.* 1999), mouse (Motro & Bernstein 1993) and human (Laitinen *et al.* 1995).

Table 2. Localisation of mRNA and relative intensity of immunohistochemical staining for KL and c-Kit in the ovaries of goats.

Structure	Kit Ligand		c-Kit	
	Protein	mRNA	Protein	mRNA
- Primordial follicle				
Oocyte	– / +	+ *	+ + +	+ *
Granulosa	+ + §		–	
- Primary follicle				
Oocyte	–	+ *	+ + +	+ *
Granulosa	+ + §		– / +	
- Secondary follicle				
Oocyte	–		+	
Granulosa	+ + §	+ *	– / +	+ *
Theca cells	–		+ +	
- Antral follicle (<3mm)				
Oocyte	–	–	+	+
Cumulus cells	+	+	+	+
Mural granulosa cells	+	+	+	+
Theca cells	– / +	+	+ + +	+
- Antral follicle (>3mm)				
Oocyte	–	–	+	+
Cumulus cells	+	+	+	+
Mural granulosa cells	+ +	+	+	+
Theca cells	– / +	+	+ + +	+
- Corpus luteum	+ +	+	+	+
- Ovarian surface	+ +	+	+	+
- Medullary vessels	+ + +	+	+ + +	+

* whole follicles
(/ +) occasionally found, ( ) absent, (+) weak, (+ +) moderate and (+ + +) strong immunoreaction
§ reaction mainly observed at the junction of granulosa cells and oocyte

c-Kit mRNA and protein were both detected in the oocyte cytoplasm of both early and later-staged goat follicles. This suggests that, in the goat, the oocyte is a target for granulosa cell-derived KL, as has been proposed in the sheep (Clark *et al.* 1996, Tisdall *et al.* 1999), mouse (Motro & Bernstein 1993) and monkey (Gougeon & Busso 2000). In vitro, KL has been shown to be essential for mouse primordial follicle activation (Parrott & Skinner 1999). Similarly, injection of a KL antibody into the ovaries of mice severely retarded early folliculogenesis (Yoshida *et al.* 1997), while ovaries of mice carrying a mutation in the *steel* gene (Kuroda *et al.* 1988, Huang *et al.* 1993, Bedell *et al.* 1995) contained only follicles arrested at early stages of development. Granulosa cell-derived KL also appeared to promote the formation of theca cell layers around mouse primary and secondary follicles (Parrott & Skinner 1997, 2000), which suggests that KL may act as a theca cell organizer.

Table 3. Mean number of follicles (± SD) per section with oocytes, granulosa or theca cells positive for Kit Ligand or c-Kit in eight different ovaries.

Mean number (± SD) of follicles per section with cells positive for Kit Ligand

	Primordial		Primary		Secondary			Antral < 3mm				Antral > 3mm			
O	oocyte	GC	oocyte	GC	oocyte	GC	theca	oocyte	CC	MGC	theca	oocyte	CC	MGC	theca
O 1	45.0±21.0	82.5±35.4	0	4.7±1.7	0	1.5±1.3	0	0	1.3±0.9	1.3±0.9	0.3±0.5	0	0.8±0.5	0.8±0.5	0.5±0.6
O 2	34.3±19.0	76.0±25.0	0	2.8±1.7	0	1.3±0.5	0	0	0.8±0.5	0.8±0.5	0.5±0.6	0	0.8±0.5	0.8±0.5	0.3±0.5
O 3	46.7±14.5	87.0±9.6	0	2.8±0.5	0	1.5±0.6	0	0	0.5±0.5	0.5±0.5	0.5±0.6	0	0.5±0.6	0.5±0.6	0.5±0.6
O 4	39.0±18.4	75.7±23.0	0	2.5±1.7	0	1.2±0.5	0	0	0.8±0.9	0.8±0.9	0.5±0.6	0	0.8±0.9	0.8±0.9	0.5±0.6

Mean number (± SD) of follicles per section with cells positive for c-Kit

	Primordial		Primary		Secondary			Antral < 3mm				Antral > 3mm			
O	oocyte	GC	oocyte	GC	oocyte	GC	theca	oocyte	CC	MGC	theca	oocyte	CC	MGC	theca
O 5	61.0±3.9	0	1.5±0.5	0.2±0.5	1.5±0.6	1.0±0.8	1.5±0.6	1.5±0.6	1.2±0.5	1.2±0.5	1.5±0.6	1.2±0.5	1.2±0.5	1.2±0.5	1.2±0.5
O 6	73.5±31.6	0	5.0±3.1	1.5±1.3	2.0±0.8	1.0±0.8	2.0±0.8	0.8±0.5	0.8±0.5	0.8±0.5	0.8±0.5	0.7±0.5	0.7±0.5	0.7±0.5	0.7±0.5
O 7	62.4±34.0	0	3.2±1.9	1.8±1.3	0.8±0.4	0.6±0.5	0.8±0.4	0.8±0.8	0.8±0.8	0.8±0.8	0.8±0.8	1.4±1.6	1.4±1.6	1.4±1.6	1.4±1.6
O 8	53.2±36.5	0	1.5±0.5	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.8	1.0±0.8	1.0±0.8	1.0±0.8	0.8 0.9	0.8±0.9	0.8±0.9	0.8±0.9

O: ovary, GC: granulosa cells, MGC: mural granulosa cells

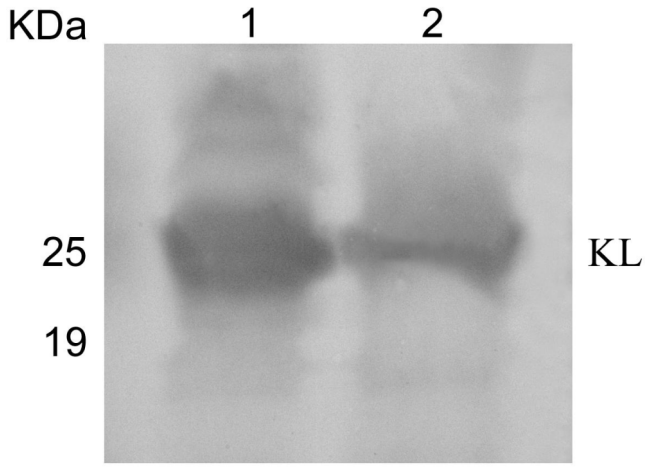

Figure 4. Western blot analysis of KL expression in mouse (1) and goat ovaries (2). Band of approximately 25kDa is consistent with the size of KL protein.

In caprine antral follicles, KL protein was present in cumulus and mural granulosa cells, and occasionally in theca cells. At these sites, mRNA for both the soluble and membrane-bound KL subtypes were also detectable. With regard to its presence in theca cells it is unclear whether this mRNA is derived from theca cells per se or from blood vessel tissue (endothelium or smooth muscle) present within the thecal layer. The expression of c-Kit by oocytes, cumulus, mural granulosa and thecal cells of both small and large antral follicles argues for autocrine and paracrine roles for KL in directing the development of goat antral follicles. Patterns of c-Kit expression were similar in sheep (Clark *et al.* 1996, Tisdall *et al.* 1999, Juengel *et al.* 2000), but different in mice (Motro & Bernstein 1993) and brushtail possums (Eckery *et al.* 2002), in which c-Kit was not expressed by granulosa cells. In vitro studies with antral follicles have demonstrated that KL can promote mouse oocyte growth (Nilsson & Skinner 2001, Eppig 2001) and inhibits bone morphogenetic protein-15 mRNA expression in mice (Otsuka & Shimasaki 2002) and meiotic maturation of rat oocytes (Ismail *et al.* 1996, 1997). In addition, KL promoted granulosa cell proliferation and steroidogenesis (in mice, Reynaud *et al.* 2000, and sows, Brankin *et al.* 2003), and growth and differentiation of theca cells, in bovine (Parrott & Skinner 1997, 2000) and rats (Huang *et al.* 2001). These data imply that, dependent of the species, different follicular compartments may contain the receptor for KL.

Besides in follicles, c-Kit and KL (-1 and -2) mRNA and protein were both also detected in goat corpora lutea, suggesting a possible role of the KL / c-Kit system in luteal activity. Similar immunolocalisation of KL and c-Kit has been described previously in ovine luteal cells (Gentry *et al.* 1996, 1998). KL was, however, undetectable in murine corpora lutea (Manova *et al.* 1993). During their development, maintenance and regression, luteal cells change in composition, size, and function. In tissues other than corpora lutea, such as hematopoietic and muscular tissue, KL has been shown to play a critical role in the regulation of such processes (Broudy 1997, Miyamoto *et al.* 1997).

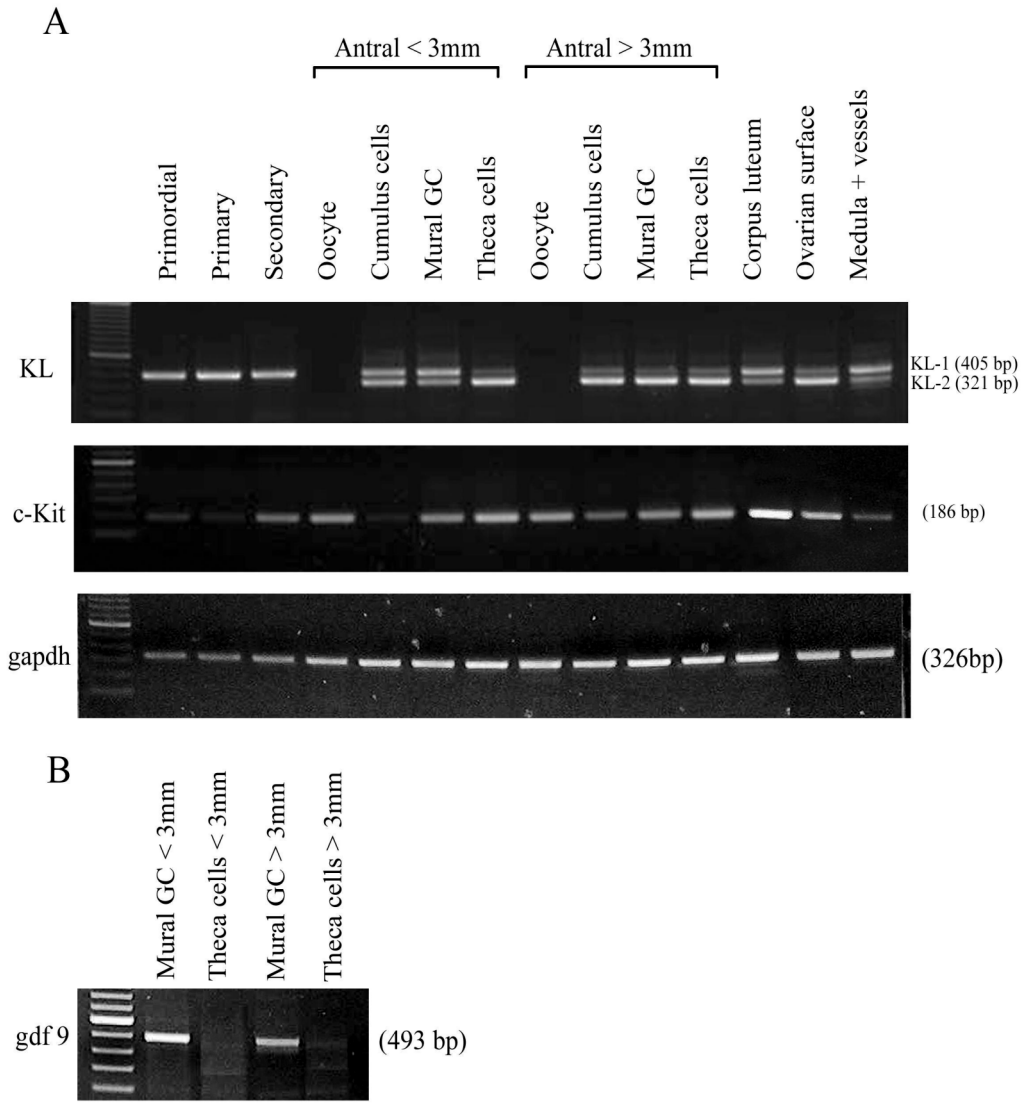

Figure 5. Expression of (A) KL, c-Kit and GAPDH mRNA in different follicle and cell types in goat ovaries and (B) GDF-9 in mural granulosa and theca cells. Follicle and cell types are indicated at the top. One-hundred base pair ladders are included as markers for fragment size.

In sheep, the level of KL mRNA expression within corpora lutea does not change during the luteal phase (Gentry *et al.* 1996), which could mean that KL has a continuous role during development, maintenance and regression of a corpus luteum.

The strong expression of both mRNA (KL-1 and KL-2) and proteins for KL and c-Kit in goat ovarian surface epithelium also suggests a role for KL / c-Kit at this site. Expression of KL and its receptor c-Kit in ovarian surface epithelium has been described

previously in human, cattle and sheep (Tisdall *et al.* 1997, Parrott *et al.* 2000). In the rat, ovarian surface epithelium cells expressed predominantly KL-1 mRNA (Ismail *et al.* 1999). In vitro, KL has been shown to stimulate growth of ovarian surface epithelium (Parrott *et al.* 2000).

In the present study with goats, KL-1, KL-2 and c-Kit mRNA were also expressed in ovarian medullary tissue. These mRNAs could be derived from blood vessel walls, since the corresponding KL and c-Kit proteins were demonstrated at these sites. Such demonstration is supported by similar findings that were obtained with sheep ovaries, using in situ hybridization (Tisdall *et al.* 1997, 1999). The presence of KL and c-Kit in blood vessel walls probably reflects a local function within the circulatory system (Miyamoto *et al.* 1997), although a paracrine influence on folliculogenesis and/or luteogenesis cannot be excluded.

In summary, the present study demonstrates a KL / c-Kit system in goat ovarian follicles at all stages of follicle development, corpora lutea, ovarian surface epithelium, and ovarian medulla. This widespread distribution of the KL / c-Kit system shows that, in goat ovaries, it may play an important role in various processes, including folliculogenesis and luteal activity.

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Chapter 6

Summarizing discussion

Ovarian folliculogenesis is a complex process, consisting of follicular development through primordial, primary, secondary and antral stages, after which ovulation may occur and the residual follicle cells luteinize to form corpora lutea. In mammalian species, folliculogenesis is a highly selective process since only a very small proportion of follicles (~0.1%) survive atresia and reach ovulatory stages. Progression through successive stages of folliculogenesis is dependent on effective two-way communication between oocyte and granulosa cells, between granulosa and theca cells and between theca cells and the surrounding stroma. It also requires appropriately timed endocrine signals, notably gonadotrophins FSH and LH, which act on receptors localized on granulosa and theca cells and interact with local autocrine/paracrine signaling pathways.

In the last decade, an increasing body of evidence from sheep and rodents indicates that various peptide growth factors are expressed by oocytes, granulosa cells and theca cells in a developmental-stage related manner, which may function as intraovarian regulatory molecules. These peptides may have a role in follicle growth, granulosa and theca cells proliferation, steroidogenesis, oocyte maturation and/or follicular atresia. This thesis focused on the expression of activin-A, follistatin, GDF-9, BMP-15, Kit Ligand and their receptors in goat unilaminar (i.e., primordial and primary), multilaminar (secondary) and antral follicles to find arguments for their possible importance in the control of folliculogenesis in this species. A possible role of activin-A in the development of early-staged follicles was further studied by using an in vitro culture system for these follicles, to which activin-A and (or) its binding protein follistatin were (was) added. This final chapter contains a brief summary of the immunohistochemical, PCR and in vitro culture data obtained with goat follicles and compares these findings, where possible, with those obtained with follicles from other ruminants, i.e. the sheep, a species in which folliculogenesis has been thoroughly studied by McNatty´s research group in New Zealand, and the cow, which has been and still is an important species under study in the reproductive research group at the Faculty of Veterinary Medicine of the Utrecht University.

Activin-A, follistatin and folliculogenesis in goats

C*hapter 2* described the presence of protein and mRNA for both inhibin/activin βA subunit and follistatin in primordial, primary and secondary goat follicles. Activin-A and follistatin proteins were found in goat oocytes of all early follicle classes and in granulosa cells from the primary follicle stage onwards. The mRNAs for activin-A (βA subunit) and follistatin were detected in unilaminar and multilaminar follicles. Activin-A protein was previously detected in oocyte and granulosa cells from early follicles in sheep (McNatty *et al*. 1999) and cow (Hulshof *et al*. 1997). Inhibin/activin βA mRNA was demonstrated in granulosa cells and oocytes from intermediate follicles onward in sheep (McNatty *et al*. 1999), but not in primordial follicles, which is different from goat. These differences are probably due to species specificity or differences in the technique used to detect mRNA expression, since we used PCR instead of in situ hibridization that was used in sheep ovaries (McNatty *et al*. 1999). The follistatin protein was previously demonstrated in sheep oocytes of primary follicles and in oocytes and granulosa cells of

secondary follicles (McNatty *et al*. 1999), but its mRNA was only detected in granulosa cells of secondary follicles (Braw-Tal 1994, Tisdall *et al*. 1994). Table 1 gives a survey of the protein and mRNA expression of activin-A, its receptors and its binding protein follistatin in the various types of goat, sheep and cow ovarian follicles.

ActR-IIA/B protein was found in goat oocytes of unilaminar and multilaminar follicles, and in granulosa cells from the primordial follicle stage onwards. The localisation of ActR-IA protein corresponded with that of ActR-IIA/B. The mRNAs for ActR-IIA, -IIB, -IA and -IB were detected at all early follicle types studied, except that ActR-IIB was not found in follicles that had not developed an antrum yet. Previous immunohistochemical studies, carried out in our laboratory, have shown the ActR-IIA/B protein in the oocyte and granulosa cells of bovine primordial, primary and secondary follicles, and also in the theca cells of the latter type of follicles (Hulshof *et al*. 1997). All these data, including the ones presented in chapter 2, point an autocrine action of activin-A on ovarian follicles.

After localizing an activin-follistatin system in goat ovaries, in vitro studies were carried out to further investigate the effect of this system on goat early folliculogenesis. *Chapter 3* described that, in ovarian tissue cultures, activin-A increases the diameter and the number of granulosa cells of unilaminar follicles, whereas it reduces atresia of these follicles. Correspondingly, activin-A stimulated the growth of cultured goat primary follicles that had been isolated from their ovarian environment and this effect could be counteracted by follistatin. These results are indicative for a functional activin-activin receptor complex and its control by follistatin in goat ovaries. In sheep, activin-A also stimulated oocyte growth 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) and murine primary and/or secondary follicles (Smitz *et al.* 1998, Zhao *et al.* 2001). The effect of activin-A in vitro probably occurs through granulosa cell proliferation, since targeted deletion of the inhibin-α subunit in mice, leading to overproduction of activin, is associated with uncontrolled proliferation of granulosa cells and ovarian tumor development (Matzuk *et al.* 1992). In null mutant mice, lacking activin type IIB receptor, follicle development was arrested at an early antral stage, which further supports a role for activin in promoting granulosa cell proliferation (Matzuk *et al.* 1996). In addition, activin-A was able to up-regulate FSH-R expression in undifferentiated murine granulosa cells (Xiao *et al.* 1992). Thus far, however, there is no evidence that activin has such an effect on preantral / early antral follicles in small ruminants.

Chapter 2 further described the presence of proteins and mRNAs for activin-A and follistatin in the oocyte, cumulus cells, mural granulosa cells and theca cells of caprine antral follicles. Thus far, expression of activin-A mRNA in antral follicles appeared restricted to the granulosa cells of sheep (Braw-Tal 1994, Tisdall *et al*. 1994), while in the bovine it was also detected in oocytes (Izadyar *et al*. 1998). Activin protein has been detected in oocytes and granulosa cells of antral follicles in sheep (McNatty *et al.* 1999) and cows (Izadyar *et al*. 1998, Silva *et al*. 2003). Expression of follistatin mRNA in antral follicles has also been reported in sheep (Braw-Tal 1994,Tisdall *et al*. 1994) and appeared to be confined to granulosa cells. As described above, follistatin is often produced in the same cells that produce activin or in cell types adjacent to those that produce activin. These sites of production apparently serve to regulate the local actions of

activin and may limit, through concentration gradients, the capacity of this multi-potent growth factor to diffuse into the circulation and exert their action at more distant sites (Phillips & de Kretser 1998)

We furthermore localized in *Chapter 2* ActR-IIA/B and ActR-IA proteins and the mRNAs for ActR-IIA, -IIB, -IA and -IB in the oocyte, cumulus cells, mural granulosa cells and theca cells of goat antral follicles. Previously, our group reported the expression of ActR-IIA mRNA and protein in oocyte and granulosa cells of bovine (Izadyar *et al*. 1998) antral follicles. Evidence implicating activin as intrafollicular regulators in ruminants has been produced predominantly by in vitro studies with cells isolated from antral follicles or from in vivo studies in which antral follicle function has been monitored. For instance, experiments with bovine granulosa cells have shown that activin enhances P450 aromatase activity and oestradiol production, while it inhibits progesterone secretion (Hutchinson *et al.* 1987). These observations suggest a role for activin in delaying the onset of atresia and/or luteinization (Knight & Glister 2003). Indeed, FSH promotes activin-A secretion by bovine granulosa cells in vitro and there is evidence that activin may mediate the stimulatory effect of FSH on secretion of oestradiol (Glister *et al.* 2001). Additionally, activin suppressed androgen production in bovine (Wrathall & Knight 1995) and ovine theca cells (Campbell & Baird 2001). Consequently, activin is thought to play a crucial role in basal growth, recruitment and selection of antral follicles through stimulation of proliferation and FSH receptor expression in granulosa cells and modulation of steroidogenesis in granulosa and theca cells. These actions are time and concentration dependent and are regulated by follistatin (reviewed by Findlay *et al*. 2002, Driancourt 2001, Knight & Glister 2001). In several species, activin-A also influenced in vitro oocyte maturation (bovine: Silva & Knight 1998, human: Alak *et al*. 1998, mouse: Sidis *et al*. 1998).

The distribution pattern of activin-A, its binding protein follistatin and activin receptors in the current study on goat antral follicles point to an important role of these proteins in the development of antral follicles also in this species.

Because of the presence of both mRNA and protein for activin-A, follistatin and all four types of activin receptors, we additionally produced evidence for the presence of an activin-follistatin-activin receptor system in corpora lutea and ovarian surface epithelium. With regard to corpora lutea, expression of protein and mRNA for activin-A and follistatin were previously demonstrated in the cow (Singh & Adams 1998). Expression of activin-A protein and mRNA in the ovarian surface epithelium of the cow has been found before by Hulshof and co-workers (1997). Choi *et al.* (2001) demonstrated that activin induces apoptosis in cultured ovarian surface epithelial cells and inhibits their growth. Therefore, the functional significance of the activin-follistatin-activin receptor system in goat corpora lutea and surface epithelium remains to be established.

BMP-15, GDF-9 and folliculogenesis in goats

In C*hapter 4,* we demonstrated that the mRNAs and proteins of GDF-9 and BMP-15, as well as BMP receptor mRNAs are expressed in oocytes of goat follicles at all stages of their development and in granulosa cells of antral follicles. In sheep, GDF-9 and

BMP-15 mRNA were also found as early as in oocytes of primordial follicles (Bodensteiner *et al.* 1999, 2000). Recently, the protein and mRNA for GDF-9, but not for BMP-15, were found in sheep ovaries even before follicle formation, i.e., on day 56 of foetal life (Mandon-Pepin *et al.* 2003, Juengel *et al*. 2004). Although expression of GDF-9 and BMP-15 in early-staged, non-growing, follicles is indicative of a possible role in the maintenance of primordial follicles in small ruminants, it cannot be excluded, that their biological activity is blocked by a factor like follistatin (Otsuka *et al.* 2001). The currently demonstrated expression pattern of GDF-9 in the oocyte and granulosa cells of goat antral follicles is similar to that described for primates (Sidis *et al.* 1998, Yamamoto *et al.* 2002, Duffy 2003). In contrast, GDF-9 was found exclusively in oocytes of bovine and sheep follicles, including oocytes of the antral stages (Bodensteiner *et al.* 1999), while BMP-15 has thus far not been demonstrated in granulosa cells of antral follicles of other species, but only in their oocytes (mice: Dube *et al.* 1998, sheep: Galloway *et al.* 2000, human: Aaltonen *et al.* 1999). Protein and mRNA expression of BMP-15, GDF-9 and their receptors in ruminant follicles are shown in Table 1.

Recently, BMPR-II was identified as a receptor involved in both BMP-15 and GDF-9 signaling (Vitt *et al.* 2002, Moore *et al.* 2003). In addition, BMP-15 was shown to signal through the BMPR-IB receptor (Moore *et al.* 2003), while GDF-9 signals through a separate type I receptor, namely TGFßR-I (Mazerbourg *et al.* 2004). BMP-15 first binds to a type I receptor, whereafter the complex binds to a type II receptor. In goats, the mRNA of BMPR-IA, BMPR-IB and BMPR-II are expressed in early follicles (primordial, primary and secondary), but we could not precisely establish whether the oocyte and/or the granulosa were sites of expression because we were not able to separate them to study mRNA expression by PCR. In the antral follicle stages, the expression of mRNA for BMPR-IA, BMPR-IB and BMPR-II could be detected in the oocyte, granulosa and theca layer. Expression of BMPR-II protein was recently demonstrated in bovine primordial, primary and secondary follicles both in their granulosa cells and oocytes (Fatehi *et al.* 2005). In bovine antral follicles, proteins for both BMPR-II and BMPR-IB were found in granulosa and theca cells (Glister *et al.* 2004). In sheep ovaries, mRNAs for BMPR-II and BMPR-IB were observed in both granulosa cells and the oocytes of early-staged follicles using in situ hibridization (Wilson *et al.* 2001, Souza *et al.* 2002), suggesting that BMP-15 and GDF-9 are potential regulators of both oocyte and granulosa cell function at very early stages of follicular development. BMPR-II and BMPR-IB were also detected in the theca of sheep follicles (Souza *et al.* 2002), which indicates that BMP-15 and GDF-9 may also influence theca cell function. In contrast, in the rat, expression of BMPR-II was primarily restricted to the granulosa cells (Shimasaki *et al.* 1999, Erickson & Shimasaki 2003), whereas expression of BMPR-IB was observed in the oocyte, granulosa and theca layers. These findings suggest that differences exist between species regarding the actions of GDF-9 and BMP-15.

The importance of BMP-15 and GDF-9 has been extensively demonstrated in sheep, by studying animals with naturally occurring mutations in BMP-15 and GDF-9. Ewes with a single inactive BMP-15 gene are fertile and have an increased ovulation rate and higher incidence of twin or triplet births (Galloway *et al.* 2000, Hanrahan *et al.* 2004). On the other hand, ewes homozygous for inactivating mutations in BMP-15 are sterile, because of the arrest of follicular development at the primary stage (Juengel *et al.* 2004).

Similar to BMP-15, inactivating mutations in GDF-9 appear to affect fertility, but the physiological characteristics of sheep with this mutation have not been well characterized. Some studies show that ewes with a single copy of the mutated GDF-9 gene are fertile and have an increased ovulation rate (Hanrahan *et al.* 2004). In contrast, ewes homozygous for this mutation are infertile with primary ovarian failure (i.e., no follicular development). Ewes heterozygous for mutation in both GDF-9 and BMP-15 are fertile and the effects of these mutations on ovulation rate are additive (Hanrahan *et al.* 2004). In mice knocked out for GDF-9, follicular development has been arrested at the primary stage (Dong *et al.* 1996). In contrast, follicular development is not disrupted in BMP-15 knock-out mice, but the animals appear subfertile, because ovulation and fertilization are impaired (Yan *et al.* 2001). These findings show that BMP-15 and GDF-9 are essential for folliculogenesis.

Passive immunization of sheep, using antibodies blocking the biological activities of GDF-9 and BMP-15 has been performed to study the in vivo effects of these growth factors during the later stages of follicular growth. It appeared that neutralization of GDF-9 did not prevent ovulation, but that it did impair luteinization, as secretion of progesterone in the subsequent cycle was suppressed. Neutralization of BMP-15 generally blocked ovulation and sometimes no visible antral follicles were present in the ovary (Juengel *et al.* 2002). Thus, GDF-9 and BMP-15 regulate the later as well as the earlier stages of follicular development, and GDF-9 may be important for normal luteinization of the follicle and thus subsequent luteal function (Juengel *et al.* 2004). In *Chapter 4* we confirmed the possible importance of GDF-9 but not that of BMP-15 in luteal function in goats, since we demonstrated the expression of both its mRNA and protein in corpora lutea, whereas neither mRNA nor protein for BMP-15 were detected at these sites.

In vitro studies with granulosa cells from sheep and cows have shown that BMP-15 and GDF-9 co-operate in regulating their activity (McNatty *et al.* 2005ab). In these studies with sheep, a mixture of BMP-15 and GDF-9 appeared more potent in stimulating proliferation of granulosa cells than each growth factor alone. For bovine granulosa cells, however, there was little or no co-operation between BMP-15 and GDF-9, since BMP-15 alone was as potent as the combination of the two growth factors. BMP-15 and GDF-9 furthermore control the in vitro progesterone and inhibin secretion by ovine and bovine granulosa cells (McNatty *et al.* 2005ab).

Kit Ligand, c-Kit and folliculogenesis in goats

The expression and localization of KL and c-Kit mRNA and protein in goat ovaries was described in *Chapter 5*. With regard to KL in early follicle stages, we demonstrated the presence of KL protein in granulosa cells of primordial, primary and secondary follicles, in particular at their junction with the oocyte. The detection of KL protein in granulosa cells of early-staged caprine follicles is in agreement with previous descriptions of such an expression by sheep, especially in the apical part of granulosa cells from early follicles (Tisdall *et al*. 1997, McNatty *et al*. 1999). Occasionally, we could detect KL protein also in the oocyte of goat primordial follicles, which has been previously described for rodent oocytes (Doneda *et al*. 2002, Kang *et al*. 2003). The

current study furthermore demonstrated the expression of mRNA for the soluble KL-1 in caprine primordial, primary and secondary follicles. In sheep, like its protein, KL mRNA was localised in granulosa cells of early-staged follicles (Tisdall *et al*. 1997, McNatty *et al*. 1999). In this species, KL was already expressed in embryos at day 90 of gestation in mesenchymal cells of the ovarian cortex and, at day 100, in groups of cells around isolated oocytes and primordial follicles. Table 1 shows the protein and mRNA expression of KL and c-Kit thus far detected in primordial follicles and other ovarian follicle types of goats, sheep and cows.

c-Kit mRNA and protein were both detected in the oocytes of early-staged goat follicles, which points to oocytes being a target for granulosa cell-derived KL. A similar KL/c-Kit interaction has been proposed for sheep (Clark *et al*. 1996, Tisdall *et al*. 1999) and mouse (Motro & Bernstein 1993). In sheep, Tisdall *et al*. (1999) reported c-Kit expression during the early stages of oogenesis and its mRNA expression in embryos of day 24 of gestation. Expression is absent when meiosis starts in oocytes but reappears when oocytes have reached the diplotene stage of meiosis and are enclosed in primordial follicles (Tisdall *et al*. 1999). There is also evidence that c-Kit is expressed in all oocytes from primary and secondary ovine follicles, present at day 100-135 of gestation (Clark *et al*. 1996). The same is true after birth for all oocytes throughout early folliculogenesis in murine and ovine (Driancourt *et al*. 2000). Studies to investigate the functional significance of KL/c-Kit system in vivo and in vitro have been performed mainly in rodents. In vivo studies demonstrated that in ovaries of mice carrying a natural mutation in the *steel* gene that encodes KL (Bedell *et al*. 1995), follicular growth is initiated but follicles rarely grow beyond the primary stage. Similarly, injection of a KL antibody into the ovaries of mice severely retarded early folliculogenesis (Yoshida *et al*. 1997). In vitro, KL was shown to be essential for mouse primordial follicle activation (Parrott & Skinner 1999) and it also appeared to promote the formation of theca cell layers around mouse primary and secondary follicles (Parrott & Skinner 1997, 2000).

In caprine antral follicles, KL protein was found in cumulus and mural granulosa cells, and occasionally in theca cells. At these sites, mRNA for both the soluble and membrane-bound KL subtypes were also detectable. Apart from the thecal and the different granulosal compartments, c-Kit was also expressed in oocytes of goat antral follicles. In sheep, however, the presence of KL was restricted to mural granulosa cells (Tisdall *et al.* 1997), while patterns of c-Kit expression were similar as currently found in our studies with caprine follicles (Clark *et al*. 1996, Tisdall *et al*. 1999, Juengel *et al*. 2000). In mice, c-Kit was not expressed by granulosa cells, while KL was (Motro & Bernstein 1993).

In vitro studies with antral follicles have been performed mainly in rodents and cows and demonstrated that KL can promote granulosa cell proliferation (mice: Reynaud *et al*. 2000), oocyte growth and meiotic maturation (mice: Nilsson & Skinner 2001). KL also promoted growth and differentiation of bovine theca cells (Parrott & Skinner 1997, 2000).

Apart from their presence in follicles, we also detected mRNA and protein of c-Kit and KL in corpora lutea, ovarian surface epithelium and blood vessels of caprine ovaries. Similar immunolocalisations of KL and c-Kit have been previously described

Table 1: Protein and mRNA expression of Activin-A, GDF-9, BMP-15 and KL, their receptors and the activin binding protein follistatin in goat, sheep and cow ovarian follicles.

Ligands / receptors		Goat				Sheep			Cow		
		WF	OO	GC	TC	OO	GC	TC	OO	GC	TC
Activin-A	Primordial:	■	●	●	nd	□○	□○	nd	●	●	nd
	Primary:	■	●	●	nd	□●	■●	nd	●	●	nd
	Secondary:	■	●	●	○	□●	■●	□○	●	●	○
	Antral:		■●	■●	■●	□●	■●	□○	●	●	□○
Follistatin	Primordial:	■	●	○	nd	□○	□○	nd	ns	ns	nd
	Primary:	■	●	●	nd	□●	□○	nd	ns	ns	nd
	Secondary:	■	●	●	○	□●	■●	□○	ns	ns	ns
	Antral:		■●	■●	■●	□●	■●	□○	■●	■●	□○
ActR-IIA	Primordial:	■	●	●	nd	ns	ns	nd	●	●	nd
	Primary:	■	●	●	nd	ns	ns	nd	●	●	nd
	Secondary:	■	●	●	○	ns	ns	ns	●	●	○
	Antral:		■●	■●	■●	ns	●	ns	■●	■●	ns
ActR-IIB	Primordial:	□	●	●	nd	ns	ns	nd	●	●	nd
	Primary:	□	●	●	nd	ns	ns	nd	●	●	nd
	Secondary:	□	●	●	○	ns	ns	ns	●	●	○
	Antral:		■●	■●	■●	ns	●	ns	■●	■●	ns
ActR-IA	Primordial:	■	●	●	nd	ns	ns	nd	ns	ns	nd
	Primary:	■	●	●	nd	ns	ns	nd	ns	ns	nd
	Secondary:	■	●	●	○	ns	ns	ns	ns	ns	ns
	Antral:		■●	■●	■●	ns	●	ns	ns	ns	ns
ActR-IB	Primordial:	■	ns	ns	nd	ns	ns	nd	ns	ns	nd
	Primary:	■	ns	ns	nd	ns	ns	nd	ns	ns	nd
	Secondary:	■	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Antral:		■	■	■	ns	ns	ns	ns	ns	ns
GDF-9	Primordial:	■	●	○	nd	●	○	nd	●	○	nd
	Primary:	■	●	●	nd	●	○	nd	●	○	nd
	Secondary:	■	●	●	○	●	○	○	●	○	○
	Antral:		■●	■●	□○	■●	□○	□○	■●	□○	□○
BMP-15	Primordial:	■	●	○	nd	●	○	nd	●	○	nd
	Primary:	■	●	●	nd	●	○	nd	●	○	nd
	Secondary:	■	●	●	○	●	○	○	●	○	○
	Antral:		■●	■●	□○	■●	□○	□○	■●	□○	□○

Table 1: Continuing.

Ligands / receptors		WF	Goat OO	Goat GC	Goat TC	Sheep OO	Sheep GC	Sheep TC	Cow OO	Cow GC	Cow TC
BMPR-II	Primordial:	■	ns	ns	nd	●	●	nd	●	●	nd
	Primary:	■	ns	ns	nd	●	●	nd	●	●	nd
	Secondary:	■	ns	ns	ns	●	●	○	●	●	○
	Antral:		■	■	■	●	●	●	●	●	●
BMPR-IA	Primordial:	■	ns	ns	nd	●	●	nd	ns	ns	nd
	Primary:	■	ns	ns	nd	●	●	nd	ns	ns	nd
	Secondary:	■	ns	ns	ns	●	●	○	ns	ns	ns
	Antral:		■	■	■	●	●	●	ns	ns	ns
BMPR-IB	Primordial:	■	ns	ns	nd	●	●	nd	ns	ns	nd
	Primary:	■	ns	ns	nd	●	●	nd	ns	ns	nd
	Secondary:	■	ns	ns	ns	●	●	○	ns	ns	ns
	Antral:		■	■	■	●	●	●	ns	●	●
Kit Ligand	Primordial:	■	●	●	nd	●	■●	nd	ns	ns	nd
	Primary:	■	○	●	nd	●	■●	nd	ns	ns	nd
	Secondary:	■	○	●	●	●	■●	□○	ns	ns	ns
	Antral:		□○	■●	■●	●	■●	□○	ns	ns	ns
c-Kit	Primordial:	■	●	○	nd	■●	□○	nd	ns	ns	nd
	Primary:	■	●	●	nd	■●	□○	nd	ns	ns	nd
	Secondary:	■	●	●	●	■●	■●	□○	ns	ns	ns
	Antral:		■●	■●	■●	■●	■●	■●	ns	ns	ns

● protein (+), ○ protein (-), ■ mRNA (+) □ mRNA (-)
WF: whole follicles, OO: oocyte, GC: granulosa cells, TC: theca cells, nd: not developed, ns: not studied.

in sheep (Gentry *et al*. 1996, 1998, Tisdall *et al*. 1997). The presence of KL and c-Kit at these sites probably reflects a paracrine influence on luteogenesis. The KL/c-Kit system might also be involved in the control of surface epithelium cells proliferation to repair injuries of ovulation.

In general, oocyte, granulosa or theca cells express activin-A, follistatin, GDF-9, BMP-15, KL and their receptors in all follicular categories in goats, sheep and cows, which shows the importance of these factors for folliculogenesis. The only exception was the expression pattern of GDF-9 and BMP-15, since the expression of these factors was different between goat, sheep and cow (Table 1).

Concluding remarks and future perspectives

The past ten years has been an exciting period for reproductive biologists, especially due to the discoveries and rapid advancement of research on the roles of several growth factors in mammalian folliculogenesis. In goats, the findings presented in this thesis provide insight into the functional involvement of activin-A, follistatin, GDF-9, BMP-15 and KL throughout follicular development. Apart from a role of KL and TGF-β family members, findings with follicles from species other than goats indicate that also several other growth factor systems, like an IGF/ IGF-binding protein/ IGF receptor system, an EGF/TGF-α receptor system, a fibroblast growth factor (receptor) system, and a vascular endothelium-derived growth factor (receptor) system, appear to be operational in ovarian follicles (reviewed by van den Hurk & Zhao 2005). It remains a real challenge to fully define the nature of the multiplex interactions taking place at different stages of follicle development and to discriminate between obligatory and non-essential intercellular signaling, which influence theca cells, granulosa cells and oocytes. An understanding of how these different signals are integrated to bring about a coordinated response in terms of ordered follicular development to ovulatory status or to selective atresia, remains a major goal of reproductive biologists.

An important challenge for the future will be to quantify, from primordial to late antral follicles, the mRNAs and proteins of the identified growth factors. This will not be a simple task, as these regulators are produced in minute quantities, and a variety of molecular technologies will be required for their detection. Moreover, identification and quantification of these factors, in each follicular class, are only at the beginning. Elucidation of their function will require isolation of specific cell types from ovarian structures, implementation of follicle culture techniques and use of specific markers to evaluate the effect of each growth factor alone or in combination, at different concentrations, on several steps of folliculogenesis. Success in theses tasks will generate new knowledge of the fundamental mechanisms that govern the follicle development in general, and that of goats in particular, as well as a plethora of new targets for fertility control.

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Samenvatting

Onder ovariële follikelontwikkeling bij zoogdieren verstaan we het proces waarbij een kleine primordiale follikel, bestaande uit een oöcyt omgeven door een enkele laag van granulosacellen, zich ontwikkelt tot een grote preovulatoire follikel met meerdere lagen murale granulosacellen die een cumulus-eicel complex omgeven. Gedurende dit proces, groeien en differentiëren de oöcyt en granulosacellen, terwijl er thecacellen worden gevormd uit stromacellen. Na de eisprong (ovulatie), differentiëren de granulosa- en thecacellen tot luteale cellen. Dit hele proces wordt op een endocriene wijze gereguleerd en gecoördineerd door hormonen, zoals de gonadotropines, en op een autocriene of paracriene manier door lokale groeifactoren. Onze meeste kennis over de locale regulatie van de follikelontwikkeling beperkt zich tot die van knaagdieren, de mens en andere herkauwers dan geiten. Dit proefschrift richt zich op de eiwit- en mRNA-expressie van groeifactoren in geitenovaria en op de in-vitro kweek van primordiale en primaire follikels.

In Hoofdstuk 1 wordt algemene informatie gegeven over de verschillende fases van de follikelontwikkeling, waarbij de nadruk is gelegd op de follikelontwikkeling bij de geit. Vervolgens worden de structuur en het belang van locale groeifactoren, te weten activine-A, follistatine, growth differentiation factor-9 (GDF-9), bone morphogenetic protein-15 (BMP-15), Kit Ligand (KL) en hun receptoren beschreven. Tevens worden in vitro modellen besproken, waarmee in ovariëel schorsweefsel aanwezige primordiale follikels en geïsoleerde primaire en secundaire follikels kunnen worden gekweekt.

In Hoofdstuk 2, is de eiwit- en mRNA-expressie van activine-A, follistatine, en activine receptoren in geitenovaria bestudeerd om aanwijzingen te vinden voor hun mogelijke rol in de verschillende fases van de follikelontwikkeling. Ovaria van cyclische geiten werden verzameld en vervolgens gefixeerd in paraformaldehyde t.b.v. de immunohistochemische localisatie van activine-A, follistatine, de activine receptoren IIA/B (ActR-IIA/B) en IA (ActR-IA) eiwitten of ovaria werden, zonder fixatie, gebruikt om mRNA expressie van activine-A, follistatine, ActR-IIA, -IIB, -IA and IB aan te tonen m.b.v. een omgekeerde transcriptase-polymerase kettingreactie (reverse transcriptase polymerase chain reaction; RT-PCR). Voor dit RT-PCR onderzoek werden primordiale, primaire en secundaire follikels mechanisch geïsoleerd, waarna eventuele stromacellen werden verwijderd d.m.v. een spoelingsprocedure. Tevens werden oöcyten, cumuluscellen, murale granulosacellen en thecacellen van kleine (<3 mm) en grote (>6 mm) antrale follikels, alsmede luteale cellen en ovariëel oppervlakte-epitheel verzameld om de genoemde mRNA expressie te bestuderen.

De eiwitten activine-A en follistatine werden gevonden in oöcyten van alle follikelklassen, in granulosacellen vanaf het primaire follikelstadium, in thecacellen van antrale follikels, in corpora lutea en in het oppervlakte-epitheel van het ovarium. In antrale follikels werden deze eiwitten aangetoond in zowel de cumuluscellen als de murale granulosacellen. Op dezelfde plaatsen in deze follikels werd het ActR-IIA/B eiwit aangetroffen en daarnaast in granulosacellen vanaf het primordiale follikelstadium. De localisatie van het ActR-IA eiwit kwam overeen met die van het ActR-IIA/B eiwit, maar het eerste eiwit ontbrak in de theca van grote antrale follikels. De mRNA's van

activine-A (βA-subunit), follistatine en ActR-IIA, -IIB, -IA en -IB werden in alle bestudeerde folliculaire en cellulaire celtypes aangetoond met uitzondering van ActR-IIB mRNA, dat niet werd aangetroffen in follikels die nog geen antrum hadden ontwikkeld.

In Hoofdstuk 3, werden de effecten van activine-A en follistatine op de ontwikkeling van in vitro gekweekte primordiale en primaire follikels onderzocht om aanvullend bewijs te verkrijgen voor hun rol in de vroege follikelontwikkeling. Voor de studie van de primordiale follikelontwikkeling werden stukjes ovariëel schorsweefsel gedurende 5 dagen in vitro gekweekt in 'Minimum Essential Medium' (MEM), dat werd aangevuld met activine-A (0, 10 of 100 ng/mL), follistatine (0, 10 of 100 ng/mL) of combinaties daarvan. Na de kweek werden het aantal primordiale follikels en dat van verder ontwikkelde follikelstadia vastgesteld en vergeleken met die in weefsel dat niet was gekweekt. De eiwit- en mRNA-expressie van activine-A, follistatine, KL, GDF-9 en BMP-15 in ongekweekte en gekweekte follikels werd bestudeerd m.b.v. immunohistochemie en RT-PCR. Ter evaluatie van de groei van primaire follikels werden deze follikels vers geïsoleerd en vervolgens gedurende 6 dagen gekweekt in o.a. MEM plus 100 ng/mL activine-A, MEM plus 100 ng/mL follistatine of MEM plus 100 ng/mL activine-A en 100 ng/mL follistatine. Vóór en na de kweekperiode werd de morfologie van follikels en oöcyten in weefselstukjes en van geïsoleerde follikels beoordeeld en werden de diameters ervan gemeten. TUNEL-reacties werden uitgevoerd om de DNA-fragmentatie in follikels te bestuderen. De resultaten toonden aan dat primordiale follikels van de geit gedurende de in vitro kweek van schorsweefsel werden geactiveerd en dat ze zich ontwikkelden tot verder gevorderde stadia, d.w.z. intermediaire- en primaire follikels, maar dat nóch activine-A, nóch follistatine het aantal groeiende primordiale follikels significant beïnvloedde. Na het kweken van ovariëel schorsweefsel met een activine-A behandeling bleek het aantal morfologisch normale follikels te zijn verhoogd en hun groei te zijn gestimuleerd. Deze effecten werden echter niet geneutraliseerd door follistatine. De gekweekte geitenfollikels behielden hun vermogen tot expressie van zowel het eiwit als het mRNA voor respectievelijk activine-A, follistatine, KL, GDF-9 en BMP-15. Ongeveer 30% van de atretische follikels in gekweekt schorsweefsel had een TUNEL-positieve oöcyt of TUNEL-positieve granulosacellen. Activine-A had geen invloed op het percentage atretische follikels en de aanwezigheid van TUNEL-positieve cellen in follikels. De toevoeging van activine-A aan gekweekte geïsoleerde primaire follikels stimuleerde hun groei significant, terwijl het effect door follistatine werd voorkomen. De afwezigheid van een dergelijk neutraliserend effect van follistatine tijdens het kweken met ovariëel schorsweefsel wijst op een onvolledige blokkering van activine in deze experimenten. De atretische follikels die zijn ontstaan in kweken met geïsoleerde primordiale follikels hadden, i.t.t. de atretische follikels in schorsweefsel, allen TUNEL-positieve cellen, hetgeen wijst op verschillen tussen geïsoleerde follikels en follikels in schorsweefsel voor wat betreft de cellulaire degeneratieweg die zij doorlopen.

Studies met follikels van knaagdieren en schapen hebben aangetoond, dat de TGF-ß familieleden GDF-9 en BMP-15 cruciaal zijn voor een normale follikelontwikkeling. Muizen waarin BMP-15 ontbreekt zijn subfertiel, terwijl GDF-9 knockout-muizen infertiel zijn. Ooien met natuurlijk voorkomende inactiverende BMP-15 genmutaties tonen een follikelontwikkeling die niet verder gaat dan het primaire follikelstadium, en deze dieren zijn dus infertiel. Gezien het belang van GDF-9 en BMP-

15 voor een normaal verloop van de follikelontwikkeling, bestudeerden we in Hoofdstuk 4 de aanwezigheid en verspreiding van deze groeifactoren en hun receptoren, te weten de BMP receptortypes II (BMPR-II), IA (BMPR-IA) en IB (BMPR-IB), in ovaria van geiten. Ovaria van cyclische geiten werden verzameld en gefixeerd in paraformaldehyde voor de immunohistochemiche detectie en localisatie van GDF-9 en BMP-15 eiwitten of zij werden verzameld voor de isolatie van follikels en luteaal weefsel ter bestudering van de mRNA-expressie van GDF-9, BMP-15 en BMP receptoren m.b.v. RT-PCR. GDF-9 en BMP-15 eiwitten werden waargenomen in de oöcyten van alle soorten follikels en de granulosacellen van primaire, secondaire en antrale follikels, maar niet in die van primordiale follikels. De mRNA's voor GDF-9, BMP-15, BMPR-II, BMPR-IA en BMPR-IB werden aangetoond in primordiale, primaire en secondaire follikels, alsmede in de oöcyten en de granulosacellen van antrale follikels. Transcripten voor BMPR-II, BMPR-IA, BMPR-IB en GDF-9 en het GDF-9 eiwit werden verder ook in corpora lutea aangetroffen.

Om meer informatie te verkrijgen over de lokaal geproduceerde groeifactoren, die de follikelontwikkeling in de geit kunnen reguleren, werden in Hoofdstuk 5 de intraovariële aanwezigheid en verspreiding van KL en zijn receptor c-Kit bestudeerd. Het eiwit en het mRNA van KL werden in alle follikelstadia in de follikels aangetroffen, d.w.z. in primordiale, primaire, secondaire, kleine en grote antrale follikels, en daarnaast in corpora lutea, ovariëel oppervlakte-epitheel en ovariëel mergweefsel. Antrale follikels brachten zowel KL-1 als KL-2 mRNA's tot expressie, terwijl vroegere follikelstadia alleen over het KL-1 transcript beschikten. Vanaf het primordiale follikelstadium kon het KL eiwit worden aangetoond in granulosacellen. KL mRNA kon in de granulosacellen worden dedetecteerd, die uit antrale follikels waren geïsoleerd, en soms ook in hun thecacellen. c-Kit mRNA werd op alle stadia van de follikelontwikkeling en in alle compartimenten van antrale follikels tot expressie gebracht. Het c-Kit eiwit werd vooral gevonden in oöcyten vanaf het primordiale follikelstadium, in thecacellen van antrale follikels, alsmede in corpora lutea, ovariëel oppervlakte-epitheel en ovariëel mergweefsel (in het bijzonder in de wand van bloedvaten), hetgeen erop kan wijzen dat deze cellen en weefsels de belangrijkste structuren zijn waarop KL zijn werking uitoefent.

Tenslotte geeft Hoofdstuk 6 een overzicht van de belangrijkste resultaten en worden deze resultaten hierin bediscussieerd. Geconcludeerd werd dat de eiwitten en mRNA's van activine-A, zijn bindingseiwit follistatine en zijn receptoren op alle follikelstadia tot expressie worden gebracht, behalve het mRNA van ActR-IIB, dat niet kon worden aangetoond in follikels die nog geen antrum hadden ontwikkeld. Activine-A bevordert de overleving en groei van geactiveerde follikels in in-vitro gekweekt ovariëel schorsweefsel, maar alleen de groei en niet de overleving van gekweekte geïsoleerde primaire follikels. KL, c-Kit, GDF-9, BMP-15 en BMP receptoren komen op alle stadia van de follikelontwikkeling tot expressie in ovariële follikels van de geit. De bestudeerde groeifactoren maken tezamen met hun receptoren deel uit van een complex intrafolliculair systeem, dat de follikelontwikkeling reguleert.

Summary

Mammalian folliculogenesis involves the developmental progression from a primordial follicle, containing a single layer of granulosa cells around the oocyte, to a large preovulatory follicle consisting of multiple layers of mural granulosa cells enclosing a cumulus-oocyte complex. During this process, the oocyte and granulosa cells grow and differentiate, while theca cells are recruited from stromal tissue. After ovulation, granulosa and theca cells differentiate into luteal cells. This entire process is regulated and coordinated by endocrine hormones such as the gonadotropins, and by local growth factors in an autocrine or paracrine manner. Most of our present knowledge on local control of folliculogenesis is restricted to rodents, human and ruminants different from goats. This thesis focuses on the protein and mRNA expression of growth factors in goat ovaries and on in vitro culture of primordial and primary follicles.

In *Chapter 1*, general information about different developmental phases of folliculogenesis was given, with emphasis on goats. Then, the structure and importance of local growth factors, including activin-A, follistatin, growth differentiation factor-9 (GDF-9), bone morphogenetic protein-15 (BMP-15), Kit Ligand (KL) and their receptors was described. Additionally, in vitro models to culture primordial follicles enclosed in ovarian cortical tissue as well as to culture isolated primary and secondary follicles were discussed.

In Chapter 2, the protein and mRNA expression for activin-A, follistatin and activin receptors in goat ovaries was studied to find indications for their possible role in the various stages of follicle development. Ovaries of cyclic goats were collected and then either fixed in paraformaldehyde for immunohistochemical localisation of activin-A, follistatin, activin receptors IIA/B (ActR-IIA/B) and IA (ActR-IA) proteins or, without fixation, used to demonstrate mRNA expression of activin-A, follistatin, ActR-IIA, -IIB, -IA and -IB, using reverse transcriptase polymerase chain reaction (RT-PCR). At this purpose, primordial, primary and secondary follicles were isolated mechanically, washed to remove the stromal cells and then used for RT-PCR. In addition, oocytes, cumulus, mural granulosa and theca cells from small (< 3 mm) and large (3-6 mm) antral follicles and luteal cells were collected to study mRNA expression. Activin-A and follistatin proteins were found in oocytes of all follicle classes, granulosa cells from the primary follicle stage onwards, theca cells of antral follicles, corpora lutea and ovarian surface epithelium. In antral follicles, these proteins were detected both in cumulus and mural granulosa cells. ActR-IIA/B protein was found at the same follicular sites, and additionally in granulosa cells of primordial follicles onwards. The localization of ActR-IA corresponded with that of ActR-IIA/B, but the former protein was absent in the theca of large antral follicles. The mRNAs for activin-A (βA subunit), follistatin, and ActR-IIA, -IIB, -IA and -IB were detected at all follicular and cellular types studied, except that ActR-IIB was not found in follicles that had not developed an antrum yet.

In *Chapter 3,* the effects of activin-A and follistatin on the development of primordial and primary follicles cultured in vitro were investigated to find additional evidence for their role in early folliculogenesis. To study primordial follicle development, pieces of ovarian cortex were cultured in-vitro for 5 days in Minimum Essential Medium (MEM) supplemented with activin-A (0, 10 or 100 ng/mL), follistatin (0, 10 or 100 ng/mL) and their combinations. After culture, the number of primordial follicles and that of more advanced follicle stages were assessed and compared with those in non-cultured tissue. Protein and mRNA expression of activin-A, follistatin, KL, GDF-9 and BMP-15 in non-cultured and cultured follicles were studied by immunohistochemistry and PCR. To evaluate primary follicle growth, freshly isolated follicles were cultured for 6 days in MEM plus 100 ng/mL activin-A, MEM plus 100 ng/mL follistatin, or MEM plus 100 ng/mL activin-A and 200 ng/mL follistatin. Morphology, follicle and oocyte diameters in cultured tissue and in isolated follicles were assessed before and after culture. TUNEL reactions were performed to study DNA fragmentation in follicles. Results demonstrated that during the in vitro culture of cortical tissue, goat primordial follicles were activated to develop into more advanced stages, i.e., intermediate and primary follicles, but neither activin-A nor follistatin significantly 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. These effects were, however, not counteracted by follistatin. The cultured goat follicles maintained their expression of proteins and mRNA for activin-A, follistatin, KL, GDF-9 and BMP-15. From the atretic follicles in cultured cortical tissue about 30% had an oocyte or granulosa cells that were TUNEL-positive. Activin-A neither affected the percentage of atretic follicles nor the occurrence of TUNEL-positive cells in follicles. 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 indicates incomplete blockage of activin in these experiments. In contrast to cortical enclosed atretic follicles, all atretic follicles that had arisen in cultures with isolated primary follicles had TUNEL-positive cells, which points to differences between isolated and ovarian tissue enclosed follicles with regard to the cellular pathways leading to their degeneration.

Studies in rodents and sheep have demonstrated that the TGF-ß family members GDF-9 and BMP-15 are crucial for normal follicle development. Mice lacking BMP-15 are subfertile, while GDF-9 knockout mice are infertile. Ewes that have naturally occurring inactivating mutations in the BMP-15 gene showed follicular development arrested at the primary follicle stage and are infertile. Considering the importance of GDF-9 and BMP-15 for a normal progress of folliculogenesis, we studied in *Chapter 4* the presence and distribution of these growth factors and their receptors, the BMP receptors types II (BMPR-II), IA (BMPR-IA) and IB (BMPR-IB), in goat ovaries. Ovaries of cyclic goats were collected and fixed in paraformaldehyde for immunohistochemical detection and localization of GDF-9 and BMP-15 proteins or were collected to isolate follicles and luteal tissue for studying the mRNA expression of GDF-9, BMP-15 and BMP receptors using RT-PCR. GDF-9 and BMP-15 proteins were found in oocytes of all types of follicles and granulosa cells of primary, secondary and antral follicles but not in those of primordial follicles. The mRNAs for GDF-9, BMP-15, BMPR-II, BMPR-IA and BMPR-IB were detected in primordial, primary and secondary

follicles as well as in oocytes and granulosa cells of antral follicles. Transcripts for BMPR-II, BMPR-IA, BMPR-IB and GDF-9, and GDF-9 protein were furthermore found in corpora lutea.

To obtain more information about the locally produced growth factors that may control folliculogenesis in goats, the intraovarian presence and distribution of KL and its receptor, c-Kit, was studied in *Chapter 5*. KL protein and mRNA was found in follicles at all stages of development, i.e., primordial, primary, secondary, small and large antral follicles, as well as in corpora lutea, ovarian surface epithelium and ovarian medullary tissue. Antral follicles expressed both KL-1 and KL-2 mRNAs, while earlier staged follicles expressed KL-1 transcript only. KL protein was demonstrated in granulosa cells from the primordial follicle stage onward. Its mRNA could be detected in granulosa cells isolated from antral follicles and occasionally in their theca cells. c-Kit mRNA was expressed at all stages of follicular development and in all antral follicular compartments. c-Kit protein was predominantly found in oocytes from the primordial follicle stage onwards, in theca cells of antral follicles, as well as in corpora lutea, ovarian surface epithelium and ovarian medullary tissue (particularly in the wall of blood vessels), which may indicate these cells and tissues as main action sites of KL.

Finally, *Chapter 6* contained an overview and a discussion of the main results of this thesis. It was concluded that the mRNAs of activin-A, its receptors and its binding protein follistatin are expressed and their proteins formed at all follicular stages, except the mRNA for ActR-IIB, which could not be demonstrated in early-staged follicles without an antrum. Activin-A promotes in vitro survival and growth of activated follicles in cortical tissue, but in cultured isolated primary follicles it only promotes their growth and not the survival. KL, c-Kit, GDF-9, BMP-15 and BMP receptors are expressed in goat ovarian follicles at all stages of their development. Together with their receptors, the studied growth factors form part of a complex intrafollicular system that regulates folliculogenesis.

Resumo

A foliculogênese em mamíferos envolve o desenvolvimento do folículo primordial, contendo uma única camada de células da granulosa ao redor do oócito, para o estágio de folículo pré-ovultório, que consiste de múltiplas camadas de células da granulosa murais que circundam o complexo cúmulus-oócito. Durante este processo, o oócito e as células da granulosa crescem e se diferenciam, enquanto as células da teca são recrutadas do estroma tecidual. Após a ovulação, as células da granulosa e as células da teca se diferenciam em células luteais. Este processo é regulado e coordenado por hormônio endócrinos, como as gonadotrofinas, e por fatores de crescimento locais que atuam de forma autócrina ou parácrina. A grande parte dos conhecimentos sobre o controle local da foliculogênese é restrita a roedores, humanos e ruminantes, exceto caprinos. Esta tese aborda a expressão das proteínas e dos RNA mensageiros de vários fatores de crescimento em ovários caprinos, bem como estudos in vitro que visam o cultivo de folículos primordiais e primários.

No capítulo 1 são apresentadas informações gerais sobre os diferentes estágios de desenvolvimento da foliculogênese, com ênfase em caprinos. Em seguida, foram descritas a estrutura e a importância dos fatores de crescimento locais, incluindo ativina-A, folistatina, fator de crescimento de diferenciação-9 (GDF-9), proteína morfogenética do osso-15 (BMP-15), Kit Ligante (KL) e seus receptores. Além disso, foram discutidos os modelos in vitro para o cultivo de folículos primordiais inclusos em córtex ovariano, bem como para o cultivo de folículos primários e secundários após o isolamento.

No capítulo 2 foi estudado a expressão das proteínas e dos RNAs mensageiros para ativina-A, folistatina e receptores de ativina em ovários caprinos para encontrar evidências de uma possível função destes fatores nos vários estágios do desenvolvimento folicular. Para isto, ovários de cabras cíclicas foram coletados e fixados em paraformaldeído para localização imunohistoquímica das proteínas da ativina-A, da folistatina e dos receptors de ativina dos tipos IIA/B (ActR-IIA/B) e IA(ActR-IA). Alguns ovários não fixados foram usados para demonstrar a expressão dos RNAs mensgeiros da ativina-A, da folistatina e dos receptores de ativina dos tipos IIA, II-B, IA e IB, utilizando a técnica de transcriptase reversa da reação em cadeia da polimerase (RT-PCR). Para esta finalidade, folículos primordiais, primários e secundários foram isolados mecanicamente, lavados diversas vezes para remoção de células do estroma, e em seguida utilizados para RT-PCR. Além disso, oócitos, células do cúmulus, células da granulosa murais e da teca de pequenos (< 3mm) e grandes (3-6mm) folículos antrais, bem como células luteais foram coletadas para estudar a expressão do RNA mensageiro. As proteínas da ativina-A e folistatina foram encontradas nos oócitos de todas as categorias foliculares, nas células da granulosa de folículos primários e das categorias subseqüentes, nas células da teca de folículos antrais, nos corpos lúteos e nas células do epitélio ovariano. Em folículos antrais, estas proteínas foram detectadas tanto nas células do cúmulus como nas células da granulosa murais. As proteínas dos receptores de ativina dos tipos IIA/B foram demonstradas nos mesmos compartimentos foliculares descritos e também nas células da granulosa, a partir de folículos primordias. A localização do ActR-

IA foi correspondente à localização do ActR-IIA/B, mas o receptor de activina IA não foi encontrado nas células da teca de grandes folículos antrais. O RNA mensageiro para ativina-A (subunidade βA), folistatina e ActR-IIA, -IIB, -IA e -IB foram detectadas em todas as categorias foliculares e tipos de células estudados, exceto ActR-IIB que não foi encontrado em folículos que não apresentavam formação de antro.

No capítulo 3 foram investigados os efeitos da ativina-A e da folistatina sobre o desenvolvimento de folículos primordiais e primários durante o cultivo in vitro, visando encontrar evidências adicionais do papel destes fatores no controle da foliculogênese inicial. Para estudar o desenvolvimento de folículos primordiais, fragmentos de córtex ovariano foram cultivados in vitro, por 5 dias, em Meio Essencial Mínimo (MEM) suplementado com ativina-A (0, 10 or 100 ng/mL), folistatina (0, 10 or 100 ng/mL) e suas combinações. Após o cultivo, o número de folículos primordiais e de folículos em estágios mais avançados de desenvolvimento foram avaliados e comparados com os valores observados em tecido não cultivado. A expressão da proteína e dos RNAs mensageiros para ativina-A, folistatina, KL, GDF-9 e BMP-15 em folículos não cultivados ou culivados in vitro foi estudada por imunohistoquímica e PCR. Para avaliar o crescimento de folículos primários, após o isolamento, os folículos foram cultivados por 6 dias em MEM adicionado de 100 ng/mL de ativina-A, de 100 ng/mL de folistatina ou em MEM suplementado com ativina-A (100 ng/mL) e follistatina (200 ng/mL). A morfologia e o diâmetro folicular em tecido cultivado e de folículos isolados foram avaliados antes e após o cultivo. A reação de TUNEL foi realizada para estudar a fragmentação do DNA em folículos antes ou após cultivo in vitro. Os resultados demonstraram que, durante o cultivo in vitro de córtex ovariano, os folículos primordiais caprinos foram ativados e se desenvolveram para estágios mais avançados de crescimento, ou seja, folículos intermediários e primários. No entanto, a adição de ativina-A ou de folistatina não influenciou significativamente o número de folículos primordiais que iniciaram o crescimento. Por outro lado, quando comparado ao meio controle, o tratamento com ativina-A aumentou o número de folículos morfologicamente normais e estimulou o seu crecimento durante o cultivo de córtex ovariano. Estes efeitos não foram, no entanto, bloqueados pela adição de folistatina. O cultivo de folículos caprinos manteve a expressão das proteínas e dos RNAs mensageiros para ativina-A, folistatina, KL, GDF-9 e BMP-15. Cerca de 30% dos folículos atrésicos após o cultivo continham oócito ou células da granulosa positivas para a reação de TUNEL. A ativina-A não influenciou a percentagem de folículos atrésicos ou a ocorrência de células positivas para TUNEL em folículos ovarianos após o cultivo. Já a adição de ativina-A aumentou significativamente o diâmetro de folículos isolados durante o cultivo, sendo este efeito bloqueado pela adição de folistatina. A ausência de um efeito neutralizante da folistatina, durante o cultivo de cortex ovariano, indica que a concentração de folistatina não foi suficiente para bloquear completamente o efeito da ativina-A, naqueles experimentos. Ao contrário dos folículos atrésicos presentes no interior de córtex ovariano cultivado, todos os folículos atrésicos após o cultivo de folículos primários isolados tinham células positivas para o TUNEL, o que indica diferenças entre folículos isolados e folículos no interior do córtex ovariano no tocante aos mecanismos celulares que levam a degeneração.

Estudos com roedores e ovinos têm demonstrado que os membros da família TGF-ß, GDF-9 e BMP-15, são essenciais para o desenvolvimento folicular normal. Camundongos que não produzem BMP-15 são subférteis enquanto aqueles que não sintetizam GDF-9 são inférteis. Ovelhas que apresentam mutações naturais que inativam o gene responsável pela síntese de BMP-15 apresentam uma interrupção do desenvolvimento follicular no estágio de folículo primário e são inférteis. Considerando a importânica do GDF-9 e do BMP-15 para a progressão normal da foliculogênese, no capítulo 4 foram estudados a presença e distribuição destes fatores e de seus receptors, ou seja, os receptores de BMP dos tipos II (BMPR-II), IA (BMPR-IA) e IB (BMPR-IB) em ovários caprinos. Para isto, ovários de cabras cíclicas foram coletados e fixados em paraformaldeído para detecção imunohistoquímica e localização das proteínas para GDF-9 e BMP-15. Um outro grupo de ovários foi coletado e destinado ao isolamento de folículos e de células luteais para estudar a expressão dos RNAs mensageiros para GDF-9, BMP-15 e receptores de BMP utilizando RT-PCR. As proteínas GDF-9 e BMP-15 foram localizadas em oócitos de todos os tipos foliculares e em células da granulosa de folículos primários, secundários e antrais, exceto nas céluas da granulosa de folículos primordiais. Os RNAs mensageiros para GDF-9, BMP-15, BMPR-II, BMPR-IA e BMPR-IB foram detectados em folículos primordiais, primários e secundários, bem como em oócitos e células da granulosa de folículos antrais. Os RNAs mensageiros para GDF-9, BMPR-II, BMPR-IA e BMPR-IB, bem como a proteína GDF-9 também foram demonstrados em corpo lúteo.

Para obter mais informações sobre os fatores de crescimento localmente produzidos no ovário que podem controlar a foliculogênese em caprinos, a presença e distribuição de KL e seu receptor, c-Kit, foram estudados no capítulo 5. A proteína e o RNA mensageiro para o KL foram encontrados em folículos de todos os estágios de desenvolvimento, ou seja, folículos primordiais, primários, secundários e antrais, bem como em corpo lúteo, epitélio ovariano e tecido medular. Os folículos antrais expressaram os RNAs mensageiros para KL-1 e KL-2, enquanto folículos pré-antrais expressaram apenas KL-1. A proteína KL foi demonstrada nas células da granulosa a partir de folículos primordiais. O RNA mensageiro para KL foi demonstrado em células da granulosa isoladas de folículos antrais e também nas células da teca. O RNA mensageiro para c-Kit foi expresso em todos os estágios do desenvolvimento folicular e em todos os compartimentos foliculares. A proteína c-Kit foi predominantemente expressa em oócitos, a partir de folículos primordiais, nas células da teca de folículos antrais, bem como em corpo lúteo, epitélio ovariano e tecido medular (particularmente na parede dos vasos sangüíneos), o que pode indicar que estas células e tecidos são os principais sítios de ação do KL.

Finalmente, o capítulo 6 contém um resumo e uma discussão dos principais resultados apresentados nesta these. Em conclusão, esta tese mostra que os RNAs mensageiros, seus receptores e sua proteína de ligação folistatina são expressas e suas proteínas são sintetizadas em todos os estágios foliculares, exceto o RNA mensageiro para ActR-IIB, que não foi demonstrado em folículos antes da formação do antro. A ativina-A promove a sobrevivência e crescimento de folículos ativados in vitro no interior de córtex ovariano. Em folículos primários isolados, a ativina-A promove o crescimento, mas não aumenta as taxas de sobrevivência. KL, c-Kit, GDF-9, BMP-15 e os receptores

de BMP são expressos em folículos ovarianos em todos os estágios de desenvolvimento. Juntos com os seus receptores, os fatores de crescimento estudados formam parte de um complexo sistema intrafolicular que regula a foliculogênese.

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Curriculum Vitae

José Roberto Viana Silva was born on the 8th of March 1973 in Itatira, Ceará, Brazil. He studied Veterinary Medicine at the State University of Ceará, Brazil and received his D.V.M. in 1999. In 1996, he started working in the Laboratory of Manipulation of Oocytes and Preantral Follicles, at State University of Ceará, as a trainee. In 2000, he obtained his Master of Science degree, in Animal Reproduction, under supervision of Prof. Dr. José Ricardo de Figueiredo, at the State University of Ceará. The research project of his Master's study focused on ultrastructural evaluation of goat preantral follicles after preservation in vitro. In March 2001 he started working as a PhD student at the Faculty of Veterinary Medicine - State University of Ceará. The research project of his PhD focused on in vitro culture of ovarian cortical tissue to evaluate primordial follicle activation in goats. In August 2004, he obtained his first PhD degree, in Animal Reproduction, under supervision of Prof. Dr. José Ricardo de Figueiredo, at the State University of Ceará. After performing the experiments for his thesis in Brazil, from March 2003 onwards, he has been working as a PhD student at the Department of Farm Animal Health - Utrecht University, where he started his second PhD project under the supervision of Dr. Rob van den Hurk, resulting in this thesis. After returning to Brazil he got a postdoc position at the Faculty of Veterinary Medicine at the State University of Ceará.

List of publications

Santos RR, Rodrigues APR, Costa SHF, Silva JRV, Matos MHT, Lucci CM, Báo SN, van den Hurk R & Figueiredo JR 2005 Histological and ultrastructural analysis of cryopreserved sheep preantral follicles. *Animal Reproduction Science, in press.*

Costa SHF, Andrade ER, Silva JRV, Rodrigues APR, Amorim CA, Lôbo RNB, Ohashi OM & Figueiredo JR 2005 Preservation of goat preantral follicles enclosed in ovarian tissue in saline or TCM 199 solutions. *Small Ruminant Research* **58** 189-193**.**

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Silva JRV, van den Hurk R, Van Tol HTA, Roelen BAJ & Figueiredo JR 2004 Expression of growth differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15) and BMP receptors in goat ovaries. *Molecular Reproduction and Development* **70** 11-19.

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Matos MHT, Andrade ER, Lucci CM, Báo SN, Silva JRV, Santos RR, Ferreira MAL, Costa SHF, Celestino JJH & Figueiredo JR 2004 Morphological and ultrastructural analysis of sheep primordial follicles preserved in 0.9% saline solution and TCM 199. *Theriogenology* **62** 65-80.

Silva JRV, Brasil AF, Santos RR, Costa SHF, Rodrigues APR, Machado VP & Figueiredo JR 2003 Degeneration rate of goat primordial follicles maintained in TCM 199 or PBS at different temperatures and incubation times. *Ciência Rural* **33** 913-919.

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Carvalho FCA, Lucc CM, Silva JRV, Andrade ER, Báo SN & Figueiredo JR 2001 Effect

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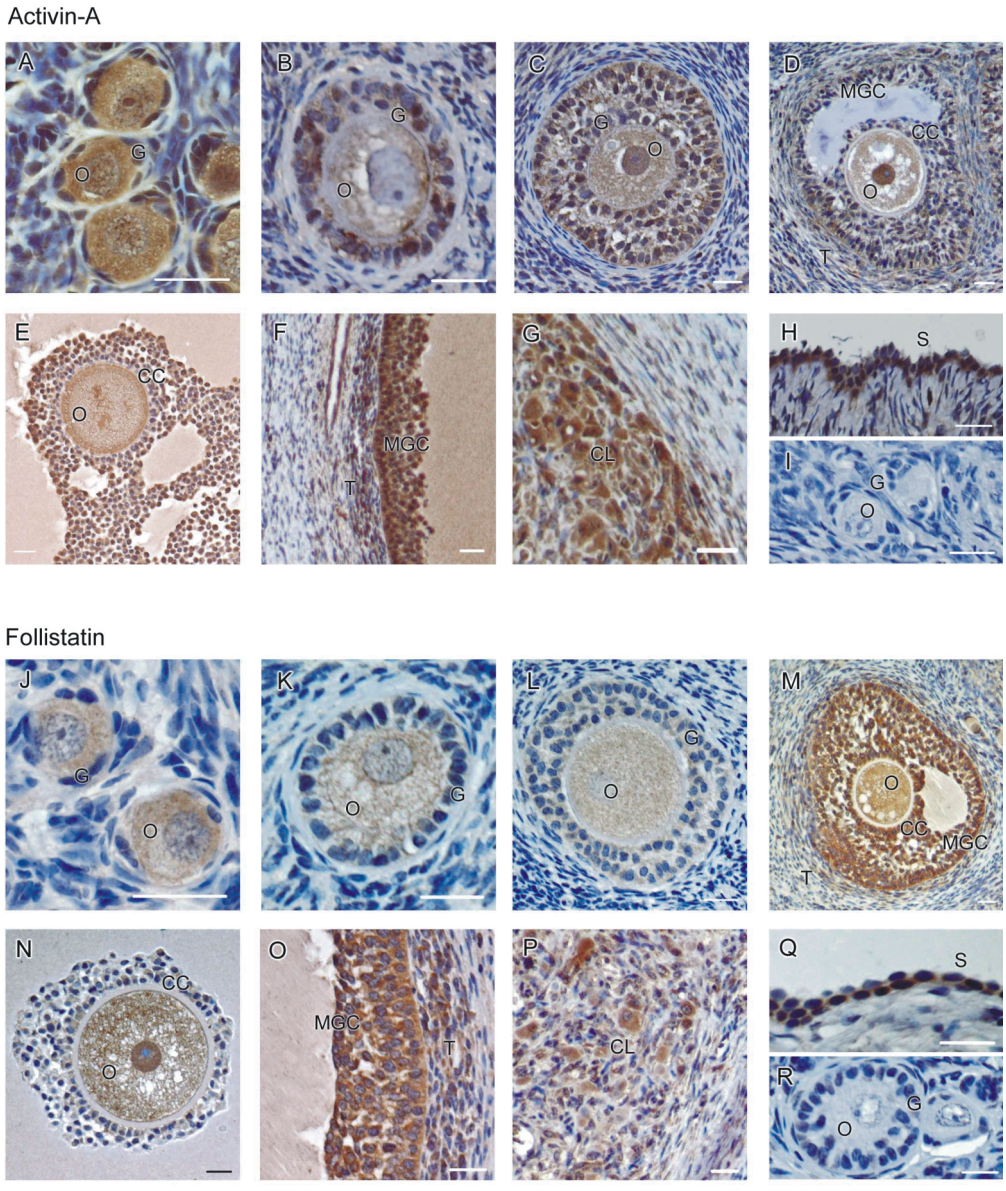
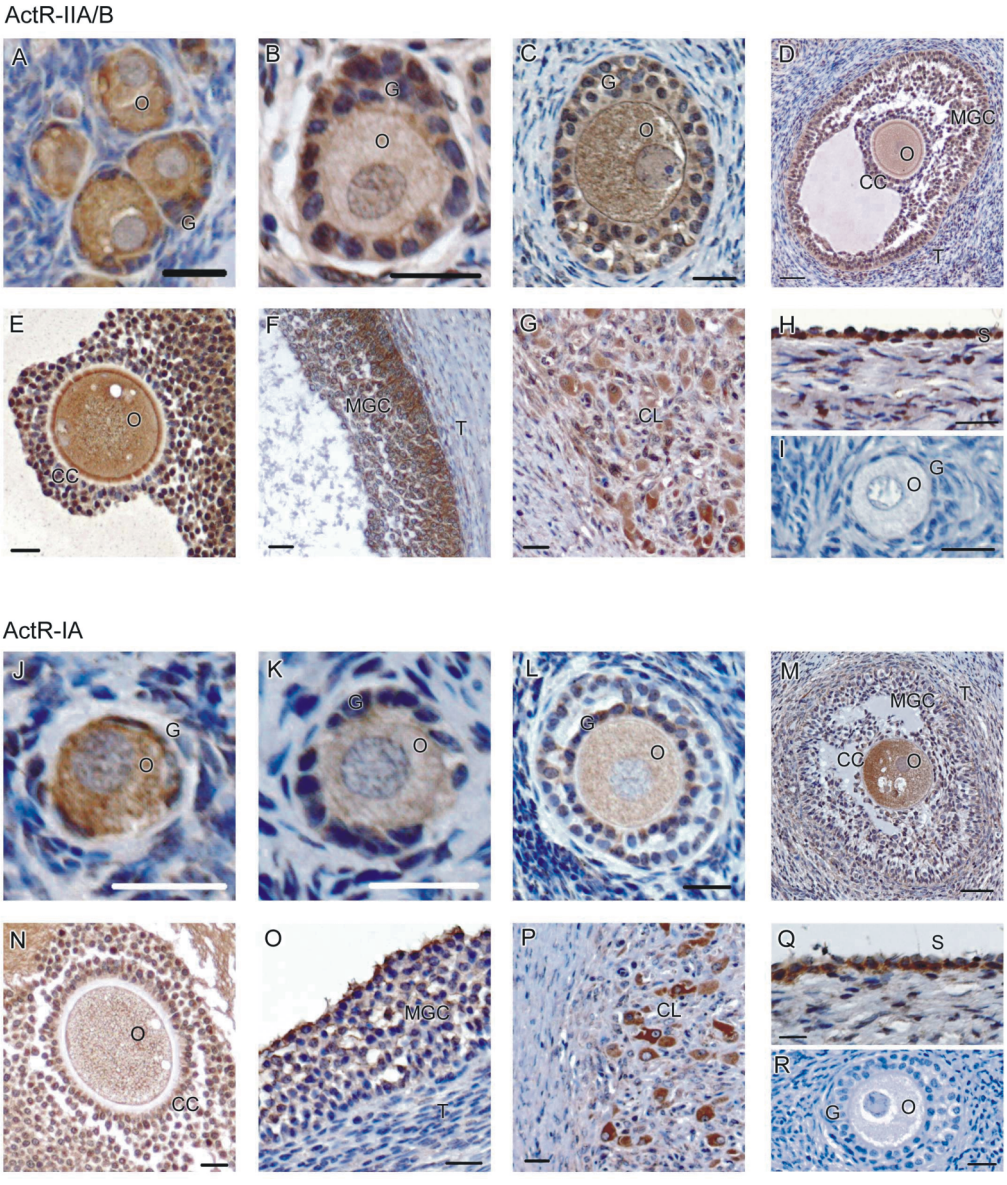
Figueiredo, JR, Silva JRV & Rodrigues APR 1999 *Estado Atual da Biotécnica de Manipulação de Oócitos Inclusos em Folículos Pré-antrais* (State of art of manipulation of oocytes enclosed in preantral follicles). *Ciência Animal* **9** 11-25.

Lucci CM, Amorim CA, Rodrigues APR, Figueiredo JR, Báo SN, Silva JRV & Gonçalves PBD 1999 Study of preantral follicles population in situ and after mechanical isolation from undefined breed type goats at different reproductive stages. *Animal Reproduction Science* **56** 223-236.

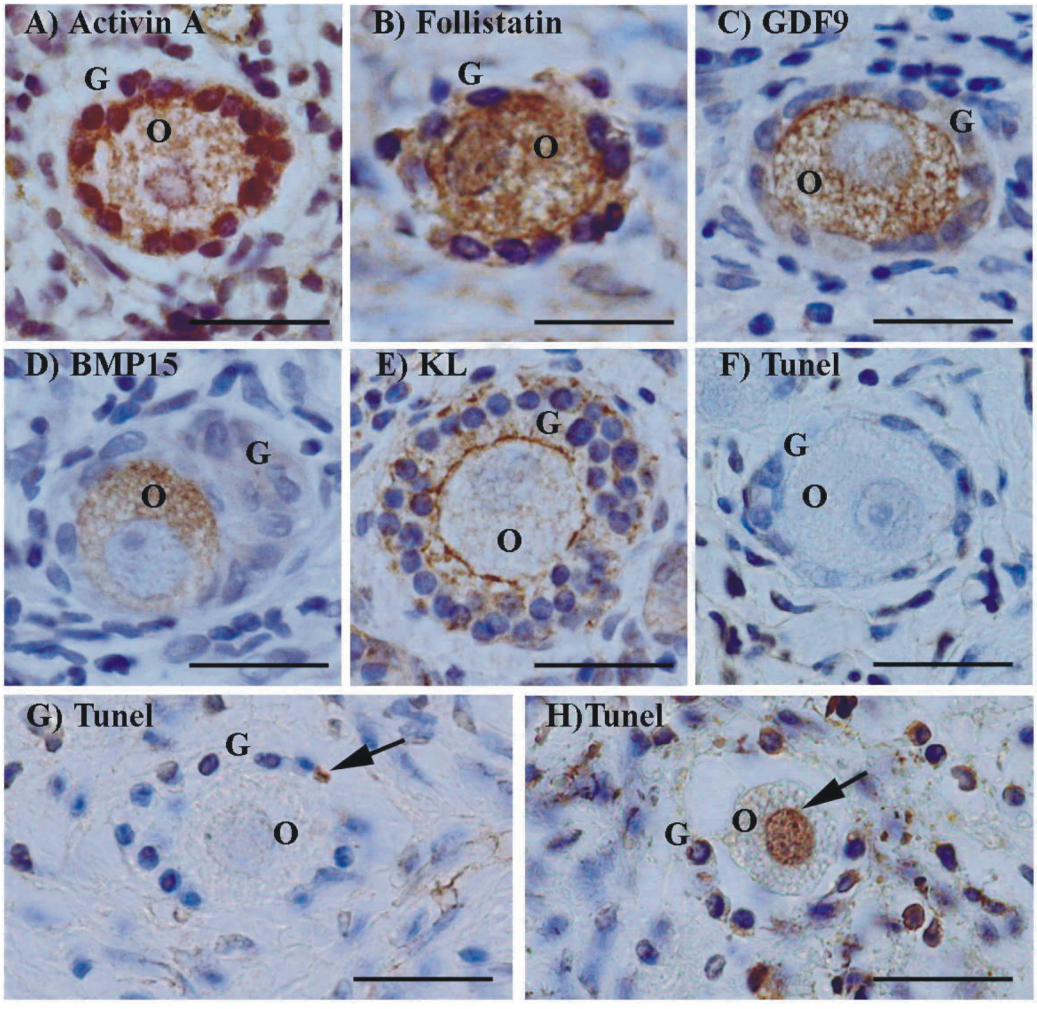
Colour plates



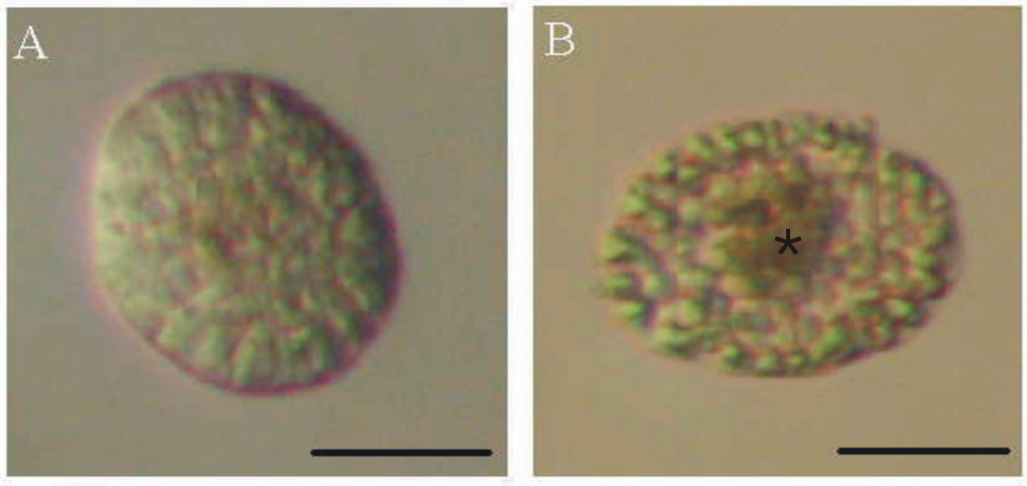
Chapter 2 / Figure 1. Activin-A and follistatin immunoreactivity in the different structures found within goat ovaries. (A, J) Primordial follicle, (B, K) Primary follicle, (C, L) Secondary follicle, (D, M) Small antral follicle, (E, N) COC of a large antral follicle, (F, O) Mural granulosa and theca cells from a large antral follicle, (G, P) Corpus luteum, (H, Q) Ovarian surface epithelium and (I, R) Negative control reaction. O: oocyte, G: granulosa cells, MGC: mural granulosa cells, CC: cumulus cells, T: theca cells, CL: corpus luteum and S: ovarian surface epithelium. Scale bars represent 25 μm.



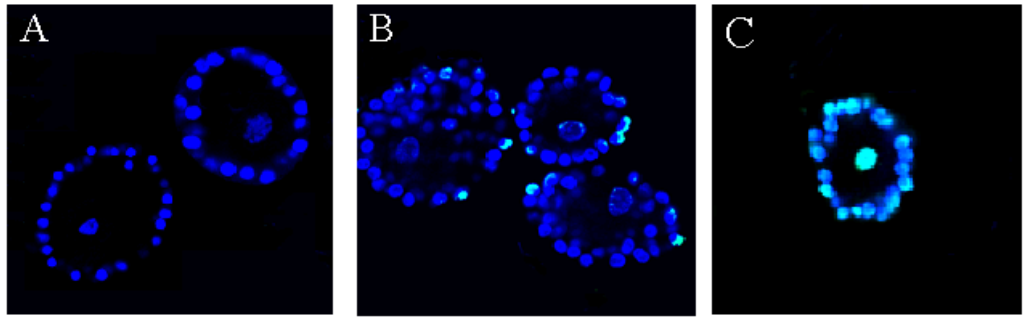
Chapter 2 / Figure 2. ActR-IIA/B, and ActR-IA immunoreactivity in the different structures found within goat ovaries. (A, J) Primordial follicle, (B, K) Primary follicle, (C, L) Secondary follicle, (D, M) Small antral follicle, (E, N) COC of a large antral follicle, (F, O) Mural granulosa and theca cells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



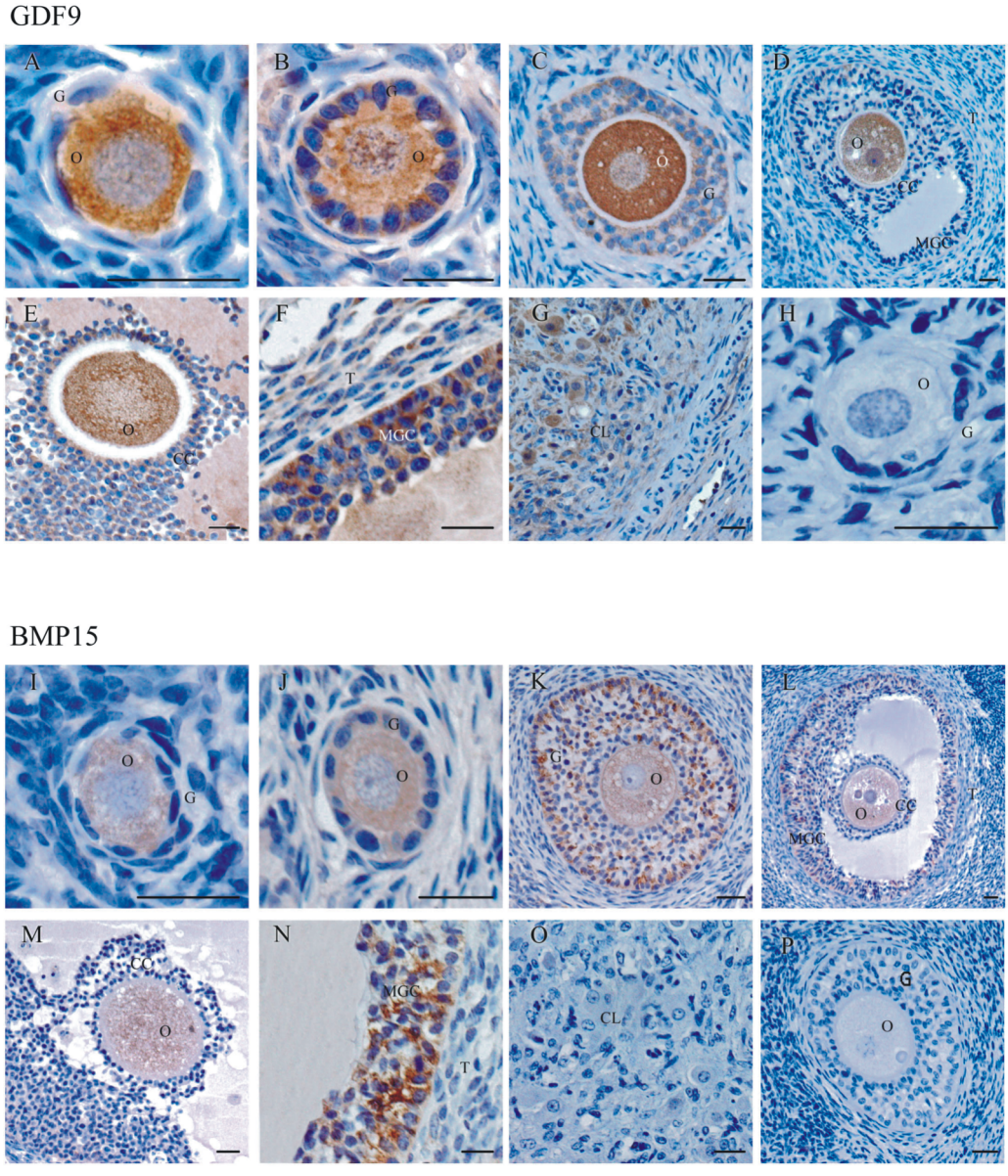
Chapter 3 / Figure 3. Protein expression for activin-A (A), follistatin (B), GDF9 (C), BMP15 (D), KL (E) and TUNEL staining / DNA fragmentation (F, G, H) in goat ovarian follicles after 5 days culture. O: oocyte, G: granulosa cells. Arrow shows DNA fragmentation in granulosa cell (G) and oocyte (H) detected by TUNEL. Bars: 25µm



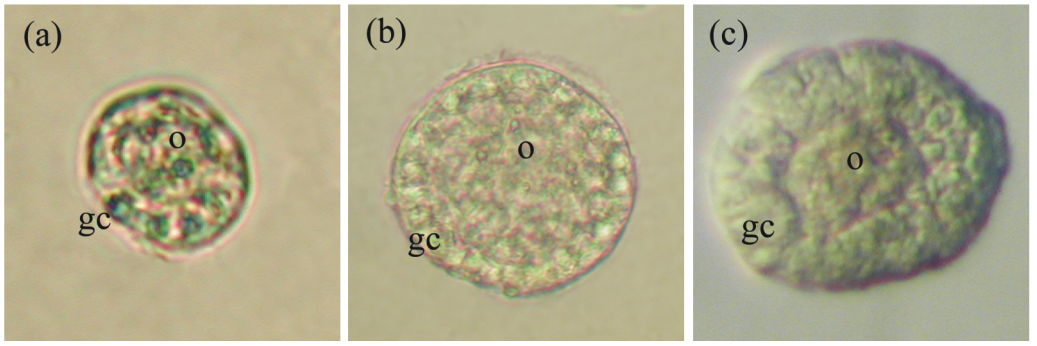
Chapter 3 / Figure 5. A morphologically normal (A) and a degenerated (B) isolated preantral follicle after 6-day culture. Bars: 25µm, (*) shrunken oocyte



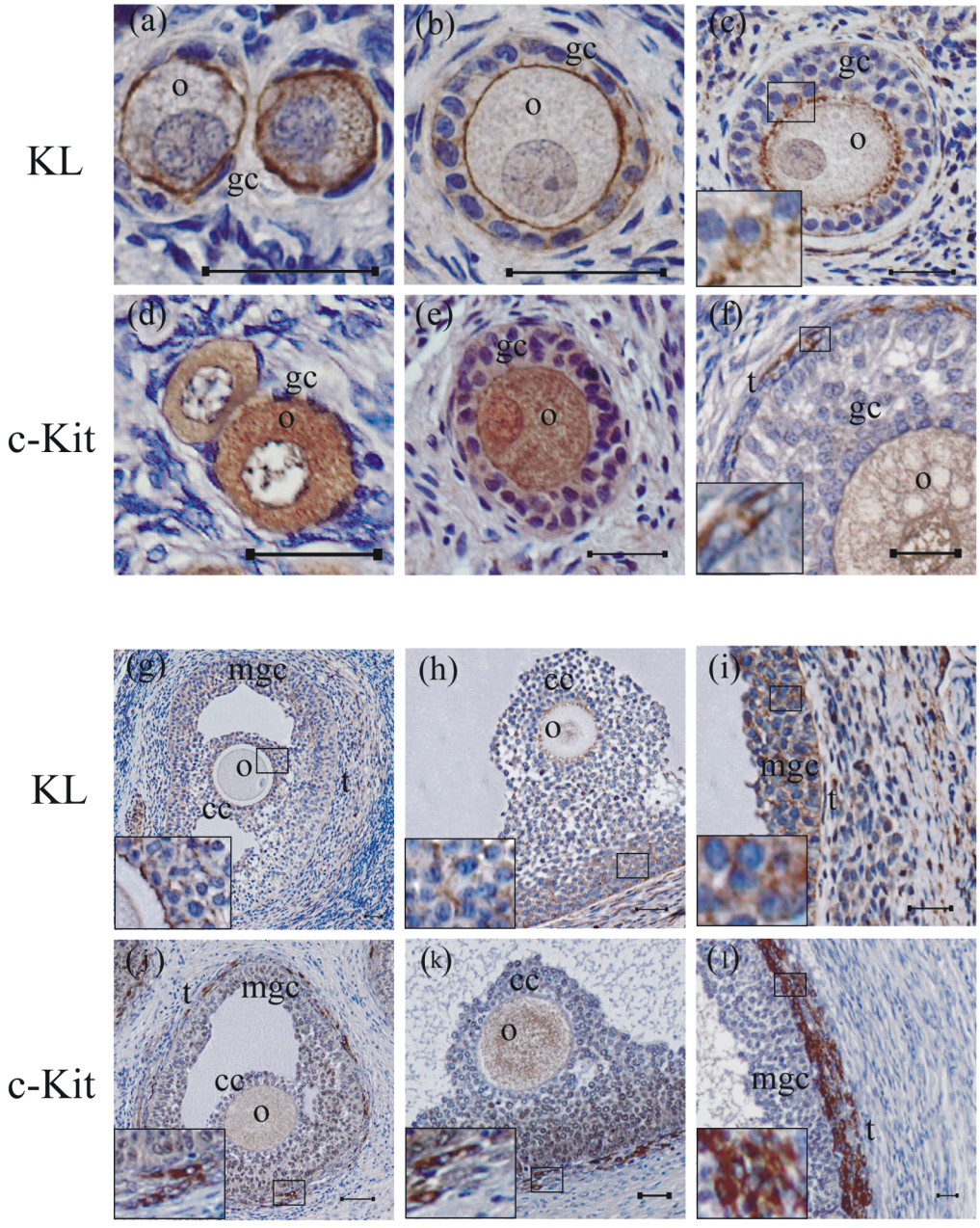
Chapter 3 / Figure 6. Confocal images of morphologically normal follicles before (A) and after 6 days culture (B), and of a follicle that became atretic during in vitro culture (C). Note the green TUNEL-positive fluorescence in granulosa cell nuclei in B and C and oocyte nucleus in C. Normal nuclei are blue (Dapi staining).



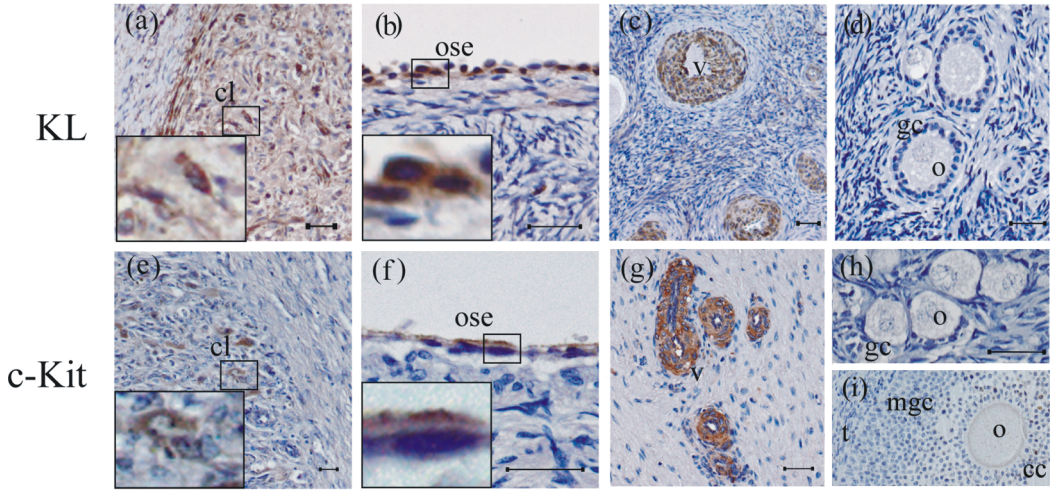
Chapter 4 / Figure 1. GDF-9 and BMP-15 immunoreactivity in the different structures found within goat ovaries. (A, I) Primordial follicle, (B, J) primary follicle, (C, K) secondary follicle, (D, L) small antral follicle, (E, M) COC of a large antral follicle, (F, N) mural granulosa and theca cells from a large antral follicle, (G, O) corpus luteum and (H, P) negative control. O: oocyte, G: granulosa cells, CC: cumulus cells, MGC: mural granulosa cells, T: theca cells, CL: corpus luteum. Scale bars represent 25 μm.



Chapter 5 / Figure 1. Isolated goat ovarian follicles. (a) Primordial follicle, an oocyte surrounded by single layer of flattened / cuboidal granulosa cells, (b) primary follicle with one layer of cuboidal granulosa cells and (c) secondary follicle with more than two layers of granulosa cells. o: oocyte, gc: granulosa cells.



Chapter 5 / Figure 2. KL and c-Kit immunoreactivity in goat ovarian follicles. (a, d) Primordial follicle, (b, e) primary follicle, (c, f) secondary follicle, (g, j) small antral follicle, (h, k) COC of a large antral follicle, (i, l) mural granulosa and theca cells from a large antral follicles. Inserts: higher magnification showing immunoreaction in the cell cytoplasm. o: oocyte, gc: granulosa cells, mgc: mural granulosa cells, cc: cumulus cells, t: theca cells, Scale bars represent 25 µm.



Chapter 5 / Figure 3. KL and c-Kit immunoreactivity in goat (a, e) corpus luteum, (b, f) ovarian surface epithelium, (c, g) blood vessels and (d, h, i) negative control. Inserts: higher magnification showing immunoreaction in the cell cytoplasm. o: oocyte, gc: granulosa cells, mgc: mural granulosa cells, cc: cumulus cells, t: theca cells, cl: corpus luteum, ose: ovarian surface epithelium and v: vessels. Scale bars represent 25 μm.