EXTRACELLULAR MATRIX AND THE DEVELOPMENT AND ATRESIA OF BOVINE OVARIAN FOLLICLES

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Thesis Declaration

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This work is dedicated to the memory of Ruby Doris Martin Irving.

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Abstract

During reproductive life, adult mammalian ovaries contain a reserve of inactive oocytes surrounded by a layer of granulosa cells and separated from the surrounding stroma by the follicular basal lamina. Follicle activation is initially accompanied by growth of the oocyte and replication of granulosa cells. This is followed by differentiation of the adjacent stroma into the vascularised thecal layers, and formation of a fluid-filled antrum within the follicle. Loss of follicles by atresia is an important mechanism ensuring that only an appropriate number of oocytes reach the ovulatory stage. Following ovulation the ruptured follicles develop into corpora lutea.

Studies of the growth and atresia of follicles have primarily focussed on the roles of hormones, growth factors and cell death molecules in these processes. However, the extracellular matrix participates in the regulation of cellular growth both directly and indirectly through interaction with growth factors or their binding proteins. This thesis describes a body of research into changes in the composition of bovine ovarian extracellular matrix during follicular growth and atresia, at ovulation and in the corpus luteum.

The composition of the follicular basal lamina (including collagen type IV α1 to α6, laminin chains, nidogen-1 and -2, perlecan, and versican) was determined by immunohistochemical, biochemical, and gene expression analyses using bovine ovaries collected at an abattoir or in controlled herds, following *in vivo* monitoring of follicle growth by ultrasonography or by regulation with hormonal treatment.

The follicular basal lamina was observed to change in composition as a function of folliclular growth. Culture of granulosa cells showed production of basal lamina and induction by growth factors. A new basal lamina type of matrix (focimatrix) was identified within the membrana granulosa of large antral follicles. Focimatrix is developmentally regulated, although its function is still to be determined. At ovulation, some components of the follicular basal lamina and focimatrix are degraded, while granulosa cells express versican.

Degradation of the follicular basal lamina and focimatrix is completed during the early stages of formation of the corpus luteum, and luteal cells of the bovine and human are not surrounded by a basal lamina. Basal laminas within the bovine and human corpora lutea are only associated with the vasculature. In the bovine there is a reticular network of extracellular matrix throughout the corpus luteum and in the human, luteal parenchymal matrix and subendothelial basal laminas change in composition during development.

The follicular basal lamina is not degraded during the process of atresia. In the definitive characterisation of follicular atresia, a distinctive type of atresia was identified and named basal atresia. Features of basal atresia include precocious expression of cholesterol side-chain cleavage cytochrome P450 and 3β-hydroxysteroid dehydrogenase in granulosa cells, altered steroid concentrations, (increased progesterone and decreased testosterone and androstenedione) in follicular fluid, and increased cell death in the theca interna involving steroidogenic, endothelial and other cells.

The present studies have identified changes in matrix that are associated with folliculogenesis, its lack of change in atresia, destruction at ovulation and the development of new matrix in the corpora lutea.

List of Abbreviations

ADAMTS	A disintegrin and metalloproteinase domain with thrombospondin motifs
AKR1C1	20-alpha-hydroxysteroid dehydrogenase
AMH	Anti-mullerian hormone
Apaf-1	Protease-apoptosis activating factor-1
BMP	Bone morphogenetic protein
CART	Cocaine- and amphetamine-regulated transcript
CL	Corpus luteum / corpora lutea
COX-2	Cyclooxygenase 2
CTGF	Connective tissue growth factor
CYP11A1	Cytochrome P450 cholesterol side-chain cleavage
CYP17	Cytochrome P450 17α-hydroxylase, 17,20 lyase
CYP19	Cytochrome P450 aromatase
EGF	Epidermal growth factor
Erk MAP	Extracellular signal-regulated mitogen-activated protein
ET1	Endothelin-1
ET2	Endothelin-2
FasL	Fas ligand
FGF	Fibroblast growth factor
FSH	Follicle-stimulating hormone
FSHR	Follicle-stimulating hormone receptor
GDF-9	Growth differentiation factor 9
GnRH	Gonadotrophin-releasing hormone
HDL	High-density lipoprotein
HSD3B	3β-hydroxysteroid dehydrogenase, Δ4Δ5 isomerase
IFN	Interferon
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IGF-IR	Insulin-like growth factor-I receptor
IGF-IIR	Insulin-like growth factor-II receptor
LDL	Low-density lipoprotein
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
LLC	Large luteal cell
MCP-1	Monocyte chemoattractant protein-1
MHC	Major histocompatibility gene complex
MIS	Mullerian inhibiting substance
MMP	Metalloproteinase
NGF1B	nerve growth factor inducible protein-B
PAPP-A	Pregnancy-associated protein-A
PG	Prostaglandin
PGRMC1	Progesterone receptor membrane component 1
PK-1	Prokineticin-1
PK-R	Prokineticin-1 receptor
SERBP1	Sterol regulatory element binding protein 1
SLC	Small luteal cell
StAR	Seroidogenic acute regulatory protein
TGFα	Transforming growth factor-α
TGFβ	Transforming growth factor-β
TIMP	Tissue inhibitor of metalloproteinase
TNF	Tumour necrosis factor
VEGF	Vascular endothelial growth factor
XIAP	X-linked inhibitor of apoptosis

Introduction

Contextual Statement

Thesis Structure

Included in this thesis are four published reviews that form part of the literature review. In addition there are eleven first-author, and four co-authored research papers collectively forming a cohesive and cogent body of original research. Copies of all of these papers appear in the appendix of the thesis and are accompanied by signed statements from co-authors granting permission for inclusion in this thesis.

The thesis begins with a brief review of bovine follicular development, including atresia. This forms the physiological context of this dissertation. It is not intended as an exhaustive review, since readers requiring a comprehensive review of the follicle are directed to good recently published reviews (Berisha *et al.*, 2000a; McGee and Hsueh, 2000; Fortune *et al.*, 2001; Hillier, 2001; Richards, 2001; Schams and Berisha, 2002; Campbell *et al.*, 2003; Ginther *et al.*, 2003; Webb *et al.*, 2003; Fortune *et al.*, 2004; Webb *et al.*, 2004; Berisha and Schams, 2005). The extracellular matrix of follicles and corpora lutea has recently been personally reviewed by myself in three first author publications (Irving-Rodgers and Rodgers, 2005;2006; Irving-Rodgers *et al.*, 2006a) and one co-authored publication (Rodgers and Irving-Rodgers, 2002). Together, this introduction and the four reviews I have authored serve as a detailed survey and analysis of the original literature providing the scientific background for experimental studies presented in this thesis.

The studies submitted for this thesis encompass two broad overlapping areas of interest. The **first** is the role of extracellular matrix during folliculogenesis, including ovulation and corpus luteum formation. Despite matrix in the ovary having been poorly studied, it is becoming increasingly likely to regulate key aspects of follicle growth and development. In conducting these studies I identified misconceptions presented in previously published descriptions of follicular atresia in bovine ovaries, and identified two morphological types of small healthy follicles that may be related to the rate of follicular growth. These observations were therefore extended in a **second** series of studies investigating matrix and other parameters of morphologically distinct follicles.

Almost all of the studies submitted for this thesis have used bovine ovaries, but one study describes extracellular matrix changes associated with the development of the human corpus luteum (Irving-Rodgers *et al.*, 2006c). In terms of follicular dynamics (size at ovulation, time for growth from a primordial to ovulatory follicle, number of granulosa cells etc), bovine ovaries are similar to the human. Women also exhibit 2- or 3-wave patterns of follicular development (Fig. 1) (Baerwald *et al.*, 2003). These and other features make the bovine ovary a suitable model for the study of human follicular dynamics (Adams and Pierson, 1995; Malhi *et al.*, 2005). An important functional difference between the two species is that whilst bovine luteal cells do not produce oestrogen, human luteal cells do, albeit in an amount lower than that produced by granulosa cells (Henderson and Moon, 1979).

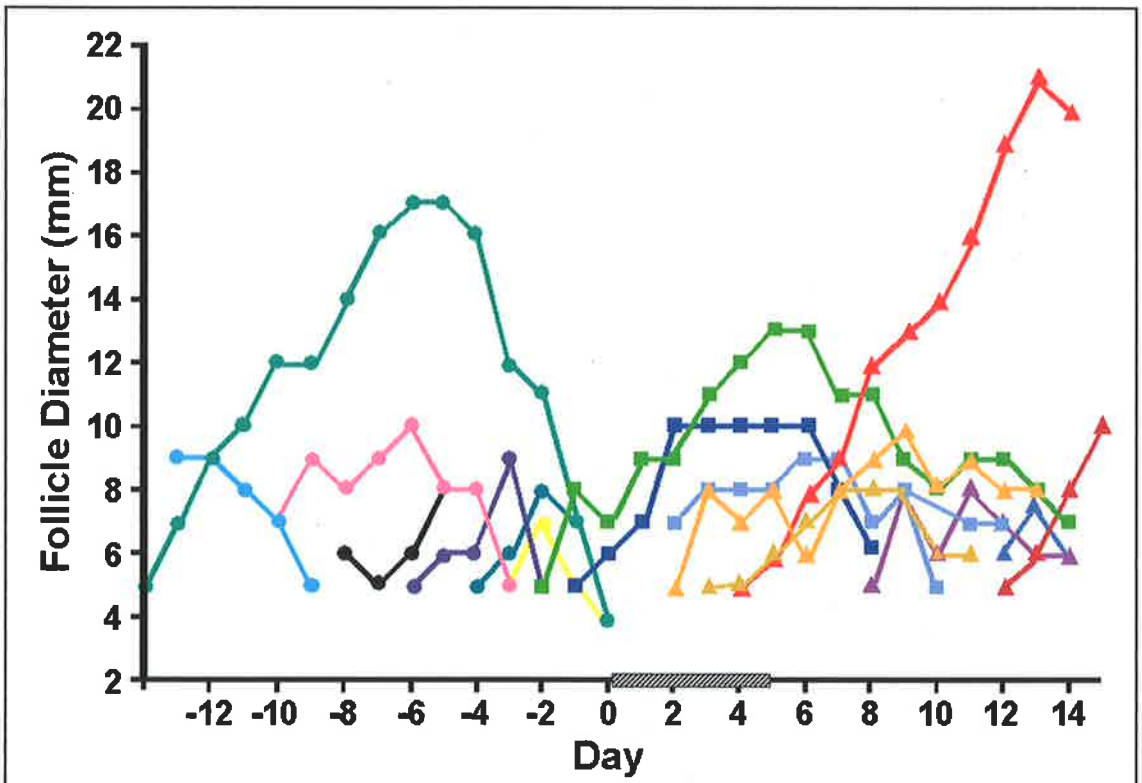

Figure 1. Follicular dynamics for a woman with three follicular waves.
○ Follicles in first follicular wave, □ second wave, △ third wave.
Day 0 is the first day of the menstrual cycle. Hatched area indicates menses.
Redrawn from Baerwald *et al.*, 2003.

Review Articles Contributing to this Thesis

Rodgers RJ, Irving-Rodgers HF 2002 Extracellular matrix of the bovine membrana granulosa. *Molec Cell Endocr* 191, 57-64.

Irving-Rodgers HF, Rodgers RJ 2005 Extracellular matrix in ovarian follicular development and disease *Cell and Tissue Research* 322, 89–98.

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Irving-Rodgers HF, Roger J, Luck MR, Rodgers RJ 2006 Extracellular matrix of the corpus luteum. *Seminars in Reproduction* 24, 242-250.

Articles on the Follicular Basal Lamina Contributing to this Thesis

Rodgers HF, Lavranos TC, Vella CA, Rodgers RJ 1995 Basal lamina and other extracellular matrix production by bovine granulosa cells in anchorage-independent culture. *Cell Tissue Res* 282, 463-471.

Rodgers RJ, Vella CA, Rodgers HF, Scott K, Lavranos TC 1996 Production of extracellular matrix, fibronectin, and steroidogenic enzymes, and growth of bovine granulosa cells in anchorage-independent culture. *Reprod Fert Devel* 8, 249-25.

Rodgers HF, Irvine CM, van Wezel IL, Lavranos TC, Luck M, Sado Y, Nimomiya Y, Rodgers RJ 1998 Distribution of the α1 to α6 chains of type IV collagen in bovine follicles *Biol Reprod* 59, 1334-1341.

van Wezel IL, Rodgers HF, Rodgers RJ 1998 Differential localisation of laminin chains in the bovine follicle. *Biol Reprod* 59, 1334-1341.

Irving-Rodgers HF, Rodgers RJ 2000 Ultrastructure of the follicular basal lamina of bovine antral follicles and its relationship to the membrana granulosa. *J Reprod Fert* 118, 221-228.

McArthur ME, Irving-Rodgers HF, Byers S, Rodgers RJ 2000 Identification and immunolocalisation of decorin, versican, perlecan, nidogen, and chondroitin sulfate proteoglycans in bovine small ovarian follicles. *Biol Reprod* 63, 913-924.

Irving-Rodgers HF, Harland ML, Rodgers RJ 2004 A novel basal lamina matrix of the stratified epithelium of the ovarian follicle. *Matrix Biol* 23, 207-217.

Irving-Rodgers HF, Catanzariti KD, Aspden WJ, D'Occhio MJ, Rodgers RJ 2006 Remodelling of Extracellular matrix at ovulation of the bovine ovarian follicle. *Mol Reprod Dev* 73, 1292-1302

Irving-Rodgers HF, Friden BE, Morris SE, Brannstrom M, Sekiguchi K, Sorokin L, Rodgers RJ 2006 Extracellular matrix of the human corpus luteum *Mol Hum Reprod* 12, 525-534.

Articles on Follicular Atresia Contributing to this Thesis

Irving-Rodgers HF, van Wezel IL, Mussard ML, Kinder JE, Rodgers RJ 2001 Atresia revisited: Two basic patterns of atresia of bovine antral follicles. *Reproduction* 122, 761-775.

Irving-Rodgers HF, Mussard ML, Kinder JE, Rodgers RJ 2002 Composition and morphology of the follicular basal lamina during atresia of bovine antral follicles. *Reproduction* 123, 97-106.

Irving-Rodgers HF, Ivell R, Bathgate RAD, Domagalski R, Rodgers RJ 2002 Dynamic changes in the expression of relaxin-like factor (Insl3), cholesterol side chain cleavage cytochrome P450, and 3β-hydroxysteroid dehydrogenase in bovine ovarian follicles during growth and atresia. *Biol Reprod* 66, 934-943.

Irving-Rodgers HF, Krupa M, Rodgers RJ 2003 Cholesterol side-chain cleavage cytochrome P450 and 3β-hydroxysteroid dehydrogenase expression and the concentrations of steroid hormones in the follicular fluids of different phenotypes of healthy and atretic bovine ovarian follicles. *Biol Reprod* 69, 2022-2028.

Irving-Rodgers HF, Catanzariti KD, Master M, Grant PA, Owens PC, Rodgers RJ 2003 Insulin-like growth factor binding proteins in follicular fluid from morphologically distinct healthy and atretic bovine follicles. *Reproduction* 15, 241-248

Clark LJ, Irving-Rodgers HF, Dharmarajan AM, Rodgers RJ 2004 Differential changes in the theca interna of bovine antral and basal atretic ovarian follicles. *Biol Reprod* 71, 1071-1078.

Research Aims

The research presented in this thesis is designed to address two aims. The **first** is to provide a detailed molecular characterisation of the bovine ovarian follicular basal lamina. Whilst the existence of this structure has been known for a long time, its specific composition and the changes that might occur during follicular growth, atresia and at ovulation, were unknown. The hypothesis underpinning this research is that changes in the composition of the follicular basal lamina are necessary for folliculogenesis. This is described in six publications (Rodgers *et al.*, 1998; van Wezel *et al.*, 1998; Irving-Rodgers and Rodgers, 2000; McArthur *et al.*, 2000; Irving-Rodgers *et al.*, 2006b; Irving-Rodgers *et al.*, 2006c). In addition, the identity of the cells that produce the follicular basal lamina was hitherto unknown, and these findings are described in two additional publications (Rodgers *et al.*, 1995; Rodgers *et al.*, 1996). This research also resulted in the identification of a new type of extracellular matrix (Irving-Rodgers *et al.*, 2004a). Finally, the extracellular matrix composition of the bovine (Irving-Rodgers *et al.*, 2004a) and human corpus luteum is described (Irving-Rodgers *et al.*, 2006c).

A **second** aim is to describe the morphological features of follicular atresia of small bovine antral follicles, including changes in the follicular basal lamina. This is described in four publications (Irving-Rodgers *et al.*, 2001; Irving-Rodgers *et al.*, 2002b; Irving-Rodgers *et al.*, 2002a; Irving-Rodgers *et al.*, 2003b). I hypothesised that different mechanisms of atresia give rise to distinct types of atresia of bovine small antral follicles. Evidence for this is presented in two additional publications (Irving-Rodgers *et al.*, 2003a; Clark *et al.*, 2004).

Literature Review

Oogenesis and Follicular Origins

The ovaries have two important reproductive functions. Ovaries are a reservoir of the female germ cells, the oocytes, and a source of hormones. These hormones are important for the development of reproductive and non-reproductive tissues, and in signalling to other organs the developmental state of the follicle, and hence, the oocyte.

Oocytes are located within follicles in the ovary. In most mammals these are formed during fetal life. The first step in oogenesis involves migration of primordial germ cells from the yolk sac via the hindgut through the dorsal mesentery, and enter the developing gonad early in fetal development (Fig. 2) (Juengel *et al.*, 2002). On day 42 of gestation in the bovine the gonads undergo sex differentiation. In females when primordial germ cells cease to separate after cell division and instead remain in clusters, populations of mitotically active oogonia arise (Russe, 1983). The other cells of the primordial follicles, the pregranulosa cells are derived from invaginating surface epithelial cells and make contact with oogonia to form ovigerous cords, separated from the differentiating stroma by a basal lamina. This process first commences at the extremity of the cord within the medulla (Russe, 1983; Juengel *et al.*, 2002). Meiosis starts at about day 82 of gestation in the bovine and oocytes are clearly distinguishable from oogonia by their being larger and containing more organelles (Russe, 1983).

Apoptosis of the majority of germ cells (Reynaud and Driancourt, 2000) ultimately results in individual oocytes becoming associated with pregranulosa cells and surrounded by the follicular basal lamina, thus forming a primordial follicle (Juengel *et al.*, 2002). Formation of isolated follicles begins at about day 90 in the bovine (170 days before birth) (Russe, 1983). Gap junctions are important portals of communication between the oocyte and surrounding granulosa cells, and begin to be formed as the primordial follicles are established. In gap junctions, connexin 37 from the oocyte communicates with connexin 43 from the adjacent granulosa cells. In addition, granulosa cell coupling via connexin 43 is necessary for continued granulosa cell proliferation and continued folliculogenesis beyond the primary stage (Kidder and Mhawi, 2002).

The peak number of bovine germs cells of about 2.5 million is seen at 110 – 130 days of gestation and declines dramatically to 100,000 by day 170 (Erickson, 1966). Mesonephric derived mesenchymal, mesangial and epithelial cells form masses in the medulla of the ovary (days 25 – 40 in the ovine, where gestation is 145 – 150 days) and extend into the cortex as streams of cells (days 55 – 75 in the ovine) and are the likely source of the ovarian stroma (Fig. 2). Steroidogenic cells in this stream are closely associated with blood vessels and are probably the thecal precursors (Juengel *et al.*, 2002).

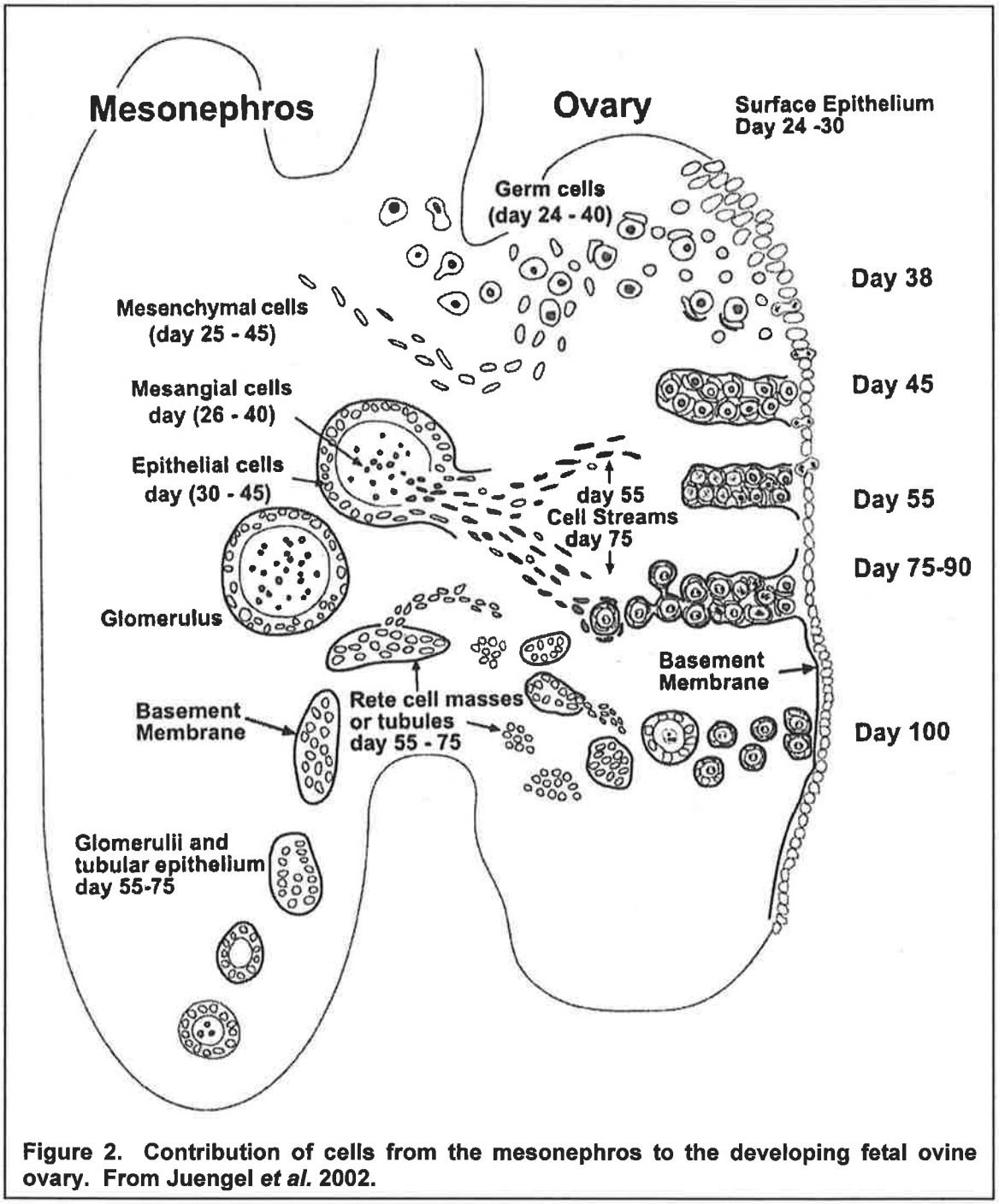


Figure 2. Contribution of cells from the mesonephros to the developing fetal ovine ovary. From Juengel *et al.* 2002.

Once formed, primordial follicles consist of an inactive oocyte, arrested at the diplotene stage of the first meiotic division, surrounded by a single layer of flattened pregranulosa cells. Follicular activation results in the appearance of a primary follicle, with an enlarging oocyte and a single layer of proliferating cuboidal granulosa cells (Fair, 2003). Activation of granulosa cells in primary follicles apparently precedes oocyte activation (Russe, 1983; Fair *et al.*, 1997). Primary follicles appear at 130 - 140 days of gestation in the bovine fetal ovary (Russe, 1983). A glycoprotein layer, the zona pellucida, surrounds the oocyte and is the product of the oocyte and granulosa cells. In some species (including the bovine) the zona pellucida first appears as aggregates in primary follicles but completely surrounds the oocyte only when follicles have progressed to the antral stage (Fair *et al.*, 1997).

The continued replication of granulosa cells results in the formation of a multilayered epithelioid membrana granulosa surrounding the oocyte. Follicles at this stage of development first appear at 210 days of gestation. The numbers of these growing preantral follicles increase significantly in the first 80 days after birth, and remain relatively constant until cows are about 4 years old. Follicles at a later stage of development with a fluid-filled antrum are first seen in the fetal ovary at 230 days of gestation (Russe, 1983). Antral follicles continue to increase in number after birth, particularly during the first 2 months (Erickson, 1966; Evans *et al.*, 1994). The number of antral follicles peaks at 4 months of age, and then declines to a plateau at 8 months (Rawlings *et al.*, 2003). In addition, prepubertal heifers 2 – 36 weeks of age exhibit waves of follicle development similar to adult cows. As prepubertal animals age, follicles increase in size and persist for longer (Evans *et al.*, 1994). Ovulation first occurs in the cow at about 60 weeks of age (Rawlings *et al.*, 2003), although age of puberty can vary greatly due to such factors as genotype, nutritional plane and/or growth rate, social cues and the season of the year (Kinder *et al.*, 1995).

As illustrated in Fig. 3, the elements that constitute an antral follicle include:

- an **oocyte** and associated cumulus cells;
- **mural granulosa cells,** which form an epithelioid layer at the periphery of follicle interior;
- the **follicular basal lamina,** which separates the membrana granulosa from the surrounding stroma;

- the **theca interna,** which is a vascularised layer of connective tissue containing steroidogenic cells;
- the **theca externa,** which is the outer layer of stromal cells and connective tissue containing larger blood and lymphatic vessels.

Extracellular matrix is associated with all these compartments: the follicular fluid, cumulus cells, zona pellucida, focimatrix of the membrana granulosa, the follicular basal lamina and matrix within the thecal layers.

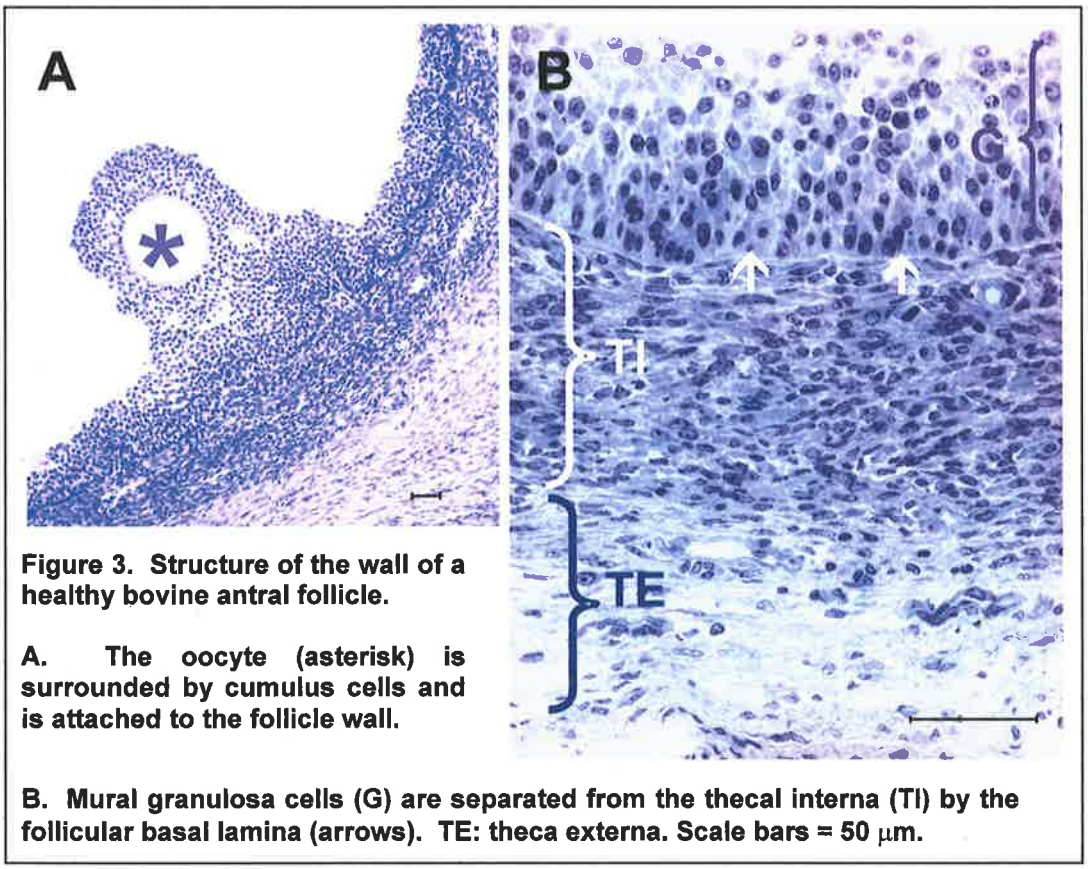


Figure 3. Structure of the wall of a healthy bovine antral follicle.

A. The oocyte (asterisk) is surrounded by cumulus cells and is attached to the follicle wall.

B. Mural granulosa cells (G) are separated from the thecal interna (TI) by the follicular basal lamina (arrows). TE: theca externa. Scale bars = 50 μm.

Folliculogenesis

Gonadotrophin Regulation

Folliculogenesis is under the control of the pituitary gonadotrophic hormones, follicle-stimulating hormone (FSH) and luteinising hormone (LH). Receptors for FSH (FSHR) and LH (LHR) are members of a seven transmembrane domain G-protein associated superfamily. Granulosa cells of growing follicles express FSHR, and the activation of this receptor stimulates production of intracellular cAMP and the activation of genes necessary for proliferation and differentiation. In addition, the actions of FSH are augmented by insulin-like growth factor I (IGF-I). FSH also induces granulosa cell expression of LHR in

follicles at the preovulatory stage, and thecal cells express LHR early in development. At low concentrations of LH (prior to the LH surge), the effects of granulosa cell stimulation via LHR mimics the actions of FSH. Higher concentrations of LH (stimulated by high circulating concentrations of oestradiol from the granulosa cells of the preovulatory follicle) result in the expression of cAMP responsive genes involved in the final stages of granulosa cell maturation (including suppression of granulosa cell division), and ovulation (Hillier, 2001).

Features of bovine follicle development are described in the following terms:

- recruitment
- selection
- dominance
- ovulation

The processes of cyclic recruitment, selection and dominance are regulated by IGFs and members of the transforming growth factor-β (TGFβ) family [inhibins, activins, and bone morphogenetic proteins (BMP)] (Fig. 4). FSHR expression is up-regulated by activins and BMPs, as is oestrogen synthesis. Meanwhile, activin suppresses LH-induced androgen production by thecal cells, while the antagonist inhibin enhances thecal androgen production (Glister *et al.*, 2006). Follistatin, a high affinity activin-binding protein, is present in excess in the follicular fluid of small antral follicles, limiting activin availability until follicles reach about 6 mm in diameter (Glister *et al.*, 2006).

Recruitment

Follicular recruitment is divided into two continuous but distinct phases: initial and cyclic recruitment (Fig. 4). Initial recruitment is a continuous process beginning with the activation of primordial follicles, while cyclic recruitment is initiated by an increase in circulating FSH (McGee and Hsueh, 2000). Initial recruitment is a slow growth phase in comparison to cyclic recruitment. It has been estimated to take 27 days for a bovine follicle to increase in diameter from 0.13 mm to 0.67 mm, a further 7 days to increase to 3.7 mm, and 8 more days to double in size to 8.6 mm (Lussier *et al.*, 1987). Once cyclic recruitment has been initiated, follicles will undergo destruction via the process of atresia, unless ovulation occurs.

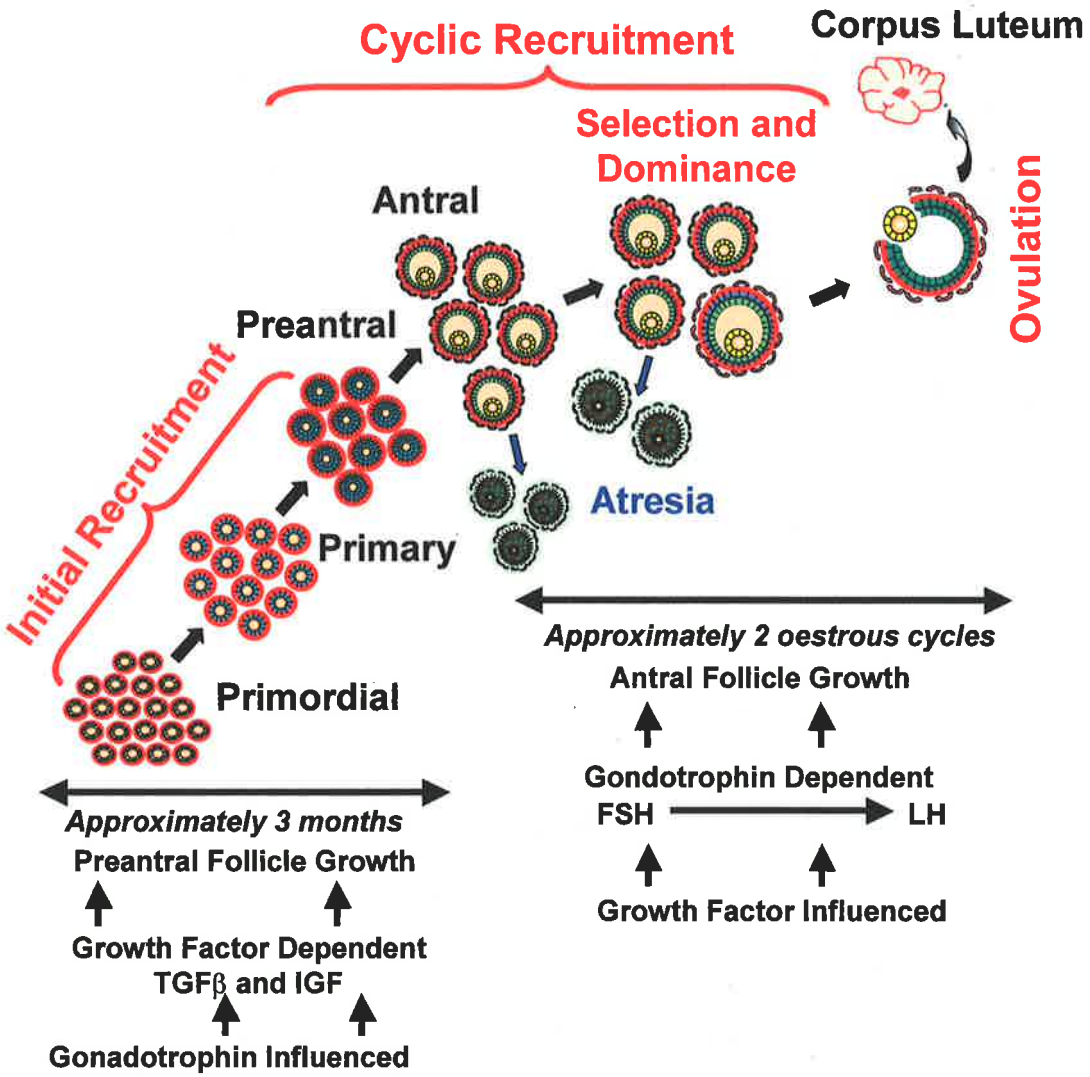


Figure 4. Stages of folliculogenesis and requirements for gonadotrophins and growth factors. Redrawn from McGee and Hsueh (2000) and Webb et al. (2004).

Small Antral Follicle Development

Granulosa cells of primary or secondary follicles express mRNA for the FSHR (Xu *et al.*, 1995) but binding of FSH to granulosa cells of primary and secondary follicles is significantly lower than binding to antral follicles (Wandji *et al.*, 1992). When FSH secretion is inhibited by treatment with a gonadotrophin-releasing hormone (GnRH) agonist, ovarian follicle development arrests at 5 mm in diameter (Gong *et al.*, 1996), indicating that follicle growth up to this size is independent of FSH. Daily subsurges in FSH occur 5.6 hours prior to an increase in follicle diameter (Jaiswal *et al.*, 2004). During the oestrous cycle, the number of small antral follicles (1-3 mm in diameter) is inversely proportional to the number of large antral follicles (>4 mm), suggesting that a wave-like pattern of development of small antral follicles also occurs (Jaiswal *et al.*, 2004).

Diameters of the dominant and first and second subordinate follicles are correlated with rising plasma FSH concentration until FSH levels peak; when the dominant follicle is about 4 mm in diameter. Thereafter FSH correlates negatively with follicle size (Haughian *et al.*, 2004; Jaiswal *et al.*, 2004). A periovulatory surge in FSH is responsible for the initiation of a follicular wave (Adams *et al.*, 1992; Haughian *et al.*, 2004) and increasing numbers of follicles >5 mm in diameter is hypothesised to have an inhibitory effect on FSH secretion (Haughian *et al.*, 2004).

Selection

Selection occurs during cyclic recruitment, and culminates in the establishment of a dominant follicle. However in cows and humans (Baerwald *et al.*, 2003), development of large follicles does not occur at random and two or three waves of growing follicles, >5 mm in diameter occur during the oestrous cycle in response to increased plasma FSH concentration. The first follicular wave (defined by the detection by ultrasound of follicles >4 mm in diameter) is initiated 1-2 days after ovulation (Valdez *et al.*, 2005). The number of follicular waves is determined by the duration of the luteal phase (Fortune, 1994). Large variation exists between animals in terms of number of follicles per wave (range 11-54), principally due to differences in the numbers of small follicles (those approximately 3 mm in diameter). The number of follicles per wave is relatively consistent within individual cows and does not correlate with either two or three wave patterns per oestrous cycle, or with dominant follicle development (Burns *et al.*, 2005).

In the bovine, follicular waves include an initial common growth phase of about 3 days. The future dominant follicle enters the wave approximately 6 hours earlier than other follicles in the cohort, which translates into a size advantage at the end of the common growth phase (Jaiswal *et al.*, 2004). Since all follicles of the common growth phase have potential for future dominance, selection is likely to involve suppression of other (subordinate) follicles (Ginther *et al.*, 2003). Selection may also involve the ability of a follicle to respond to the small rise in FSH that initiates the follicular wave with an increase in pregnancy-associated protein-A (PAPP-A), an IGF binding protein (BP)-4 protease. Cleavage of the IGFBP will allow IGF to bind to its receptor, which can act synergistically with FSH to increase follicular oestradiol production (Fortune *et al.*, 2004). During follicle selection increasing granulosa cell mRNA for the FSHR proceeds a significant increase in the mRNA for the LHR (Berisha *et al.*, 2000a).

Prior to the selection of the dominant follicle, levels of mRNA for cholesterol side-chain cleavage cytochrome P450 (CYP11A1), 17αhydroxylase, 17,20 lyase cytochrome P450 (CYP17) and cytochrome P450 aromatase (CYP19) are similar within a cohort of follicles (Xu *et al.*, 1995). Dominant follicle selection, therefore, is not associated with the expression of these steroidogenic enzymes. Growth of the dominant follicle has, however, been shown to be associated with lower follicular fluid IGFBP-4 (Mihm *et al.*, 2000) and IGFBP-2 (Stewart *et al.*, 1996) and decreased mRNA expression for FSHR, oestrogen receptor-β (oestrogen receptor 2) and inhibin A (Mihm *et al.*, 2006).

Deviation

Deviation in the growth rate between dominant and subordinate follicles manifests as a size difference that is initiated when the FSH concentration is low and follicles have reached a particular developmental stage (Fig. 5) (Ginther *et al.*, 1997; Berisha *et al.*, 2000a; Ginther *et al.*, 2003). The principle hormone controlling follicular growth is FSH, which, in turn, is controlled by oestradiol and inhibin A secreted from the large dominant follicle (Campbell *et al.*, 2003). mRNA for the LHR is detected only in healthy follicles >9 mm in diameter (Xu *et al.*, 1995), and exposure to FSH in the absence of LH can support the growth of follicles >10 mm in diameter (Crowe *et al.*, 2001; Hampton *et al.*, 2004b). LHR expression in granulosa cells occurs after selection and ensures the continued growth of the dominant follicle as the circulating concentration of FSH falls and subordinate follicles regress. LH stimulation of androgen secretion by thecal cells, progesterone production by granulosa cells and CYP19 activity combine to increase oestradiol production from the dominant follicle (Fortune *et al.*, 2001). Although Hampton *et al.* (2004b) found follicle size and number to be independent of LH concentration, changes in the frequency of LH pulses results in large increases in the concentrations of androstenedione and oestradiol in follicular fluid, this was not associated with changes in expression of mRNA for CYP11A1 or CYP19. In

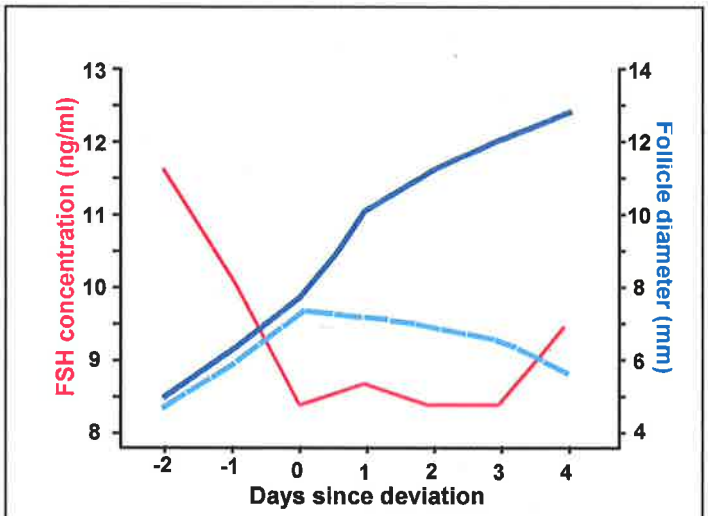


Figure 5. FSH concentration (▬) and diameter of dominant (〰) and subordinate (▬ ▬) follicles relative to the day of deviation (day 0). Ginther *et al.* 1997.

addition, LH concentrations positively correlate with the level of mRNA for CYP17 (Hampton *et al.*, 2004b).

Prolonged follicle growth and the increasing plasma oestradiol that is associated with follicular dominance are promoted by slight increases in LH pulse frequency. Upon luteal regression, reduced circulating progesterone concentration results in small increases in basal LH and increased LH pulse frequency (Fortune, 1994). When the dominant follicle reaches its maximum size and circulating oestradiol and progesterone concentrations are at the maximum and minimum, respectively, ovulation can occur (Fig. 6) (Sirois and Fortune, 1988; Noseir, 2003). Increased LH pulse frequency is also associated with sub-luteal progesterone (1.5 - 2.3 ng/ml) and a regressing dominant follicle, suggesting that regression of the non-ovulatory dominant follicle occurs as a result of the feedback effects of luteal progesterone (Stock and Fortune, 1993). Our own studies show that once the dominant follicle reaches a plateau in growth there is histological evidence of atresia in other follicles (Irving-Rodgers *et al.*, 2002a).

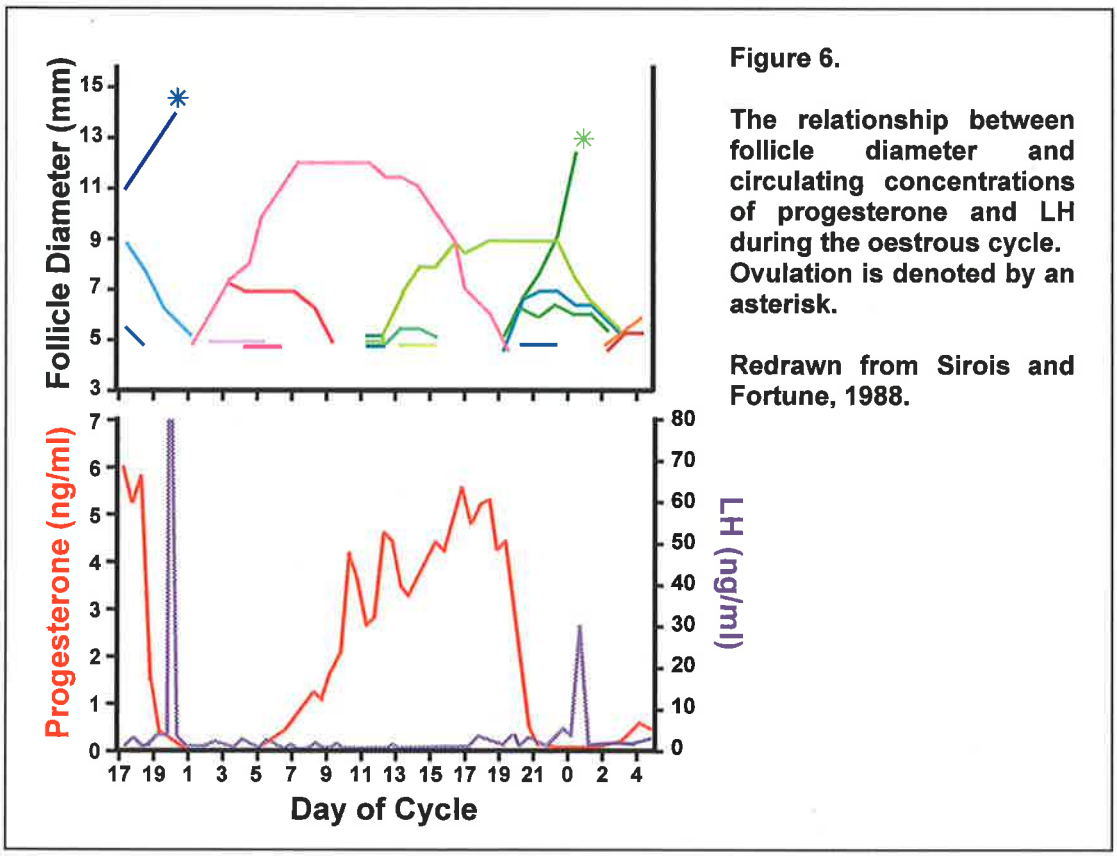


Figure 6.

The relationship between follicle diameter and circulating concentrations of progesterone and LH during the oestrous cycle. Ovulation is denoted by an asterisk.

Redrawn from Sirois and Fortune, 1988.

Peri-ovulatory Follicle Development

After luteolysis is initiated, and prior to the LH surge, granulosa and thecal cell levels of mRNA for CYP11A1 and 3β-hydroxysteroid dehydrogenase, Δ4Δ5 isomerase (HSD3B) are increased in the dominant follicle, as is CYP17 in thecal cells (Tian *et al.*, 1995). Since mRNA for CYP19 is elevated before luteolysis and does not change significantly, increased oestradiol synthesis prior to the LH surge is likely to be the result of increased thecal cell substrate (Tian *et al.*, 1995). In addition, LHR expression is increased in the thecal cells of the dominant follicle in comparison to subordinate follicles, as are IGF-I receptors (Stewart *et al.*, 1996). In granulosa cells, transient expression of the progesterone receptor occurs following the LH surge and is associated with cumulus cell expression of cyclooxygenase 2 (COX-2) (via diffusion of cAMP from granulosa cells through gap junctions) which is important in the process of ovulation (Hillier, 2001). In addition, granulosa cells are resistant to Fas ligand (FasL)-induced cell death following the LH surge (Porter *et al.*, 2000).

In vivo treatment with synthetic GnRH results in the ovulation of large healthy follicles and induces a new follicular wave and, following induction of luteal regression by treatment with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), there is emergence of a large growing follicle (Pursley *et al.*, 1995). Twelve hours after a second injection of GnRH, cows treated in this manner show a significant reduction in follicular fluid oestradiol concentration (Sirois, 1994; Komar *et al.*, 2001; Jo and Fortune, 2003). The LH surge occurred 2 hours after GnRH administration (Komar *et al.*, 2001), with ovulation occurring 24 – 31.5 hours following the LH surge (Sirois, 1994; Komar *et al.*, 2001). The preovulatory LH surge following GnRH induction attains a higher peak and is of shorter duration than that of naturally cycling cows, and the future dominant follicle emerges earlier following GnRH treatment (Haughian *et al.*, 2004). However the growth rate of the dominant follicle between 4.5 and 8.5 mm is not altered by GnRH treatment, neither is the number of follicles greater than 5 mm in diameter (Haughian *et al.*, 2004).

In granulosa cells the expression of progesterone receptors is transiently upregulated within 6 hours of the LH surge (Cassar *et al.*, 2002), and progesterone enhances its own secretion while suppressing oestrogen secretion and impeding the action of mitogens (Peluso, 2006). At this time the percentage of granulosa in the resting G_0/G_1 phase increases while the percentage of cells in the S phase decreases. By 14 hours post LH, the

percentage of replicating cells is reduced in the membrana granulosa, with no change in the theca interna (Quirk *et al.*, 2004). In addition, granulosa cells become resistant to FasL-induced cell death 14 hours after treatment with GnRH (Porter *et al.*, 2001).

Growth Factors

IGF System

This system is composed of:

- Two ligands (IGF-I and IGF-II).
- Two receptors.
    - The somatomedin-like actions of both IGFs are mediated by the type I receptor (IGF-IR). Ligand affinity; IGF-I>IGF-II>>insulin.
    - The type II receptor (IGF-IIR) binds IGF-II, with low affinity for IGF-I, no affinity for insulin; also binds TGFβ1.
- Six binding proteins (IGFBPs). These are present in serum and other fluids and modulate the actions of IGFs.

The actions of IGFs during folliculogenesis are mediated by changes in IGF bioavailability, rather than changes in IGF concentration. IGF-I is an important regulatory molecule of follicular development. IGF-I found in follicular fluid is of hepatic origin and derived from the serum. Bovine ovaries lack significant expression of IGF-I. Expression of IGF-IR increases in granulosa cells from primary to large follicle (Wandji *et al.*, 1992). IGF-II is expressed in the theca with higher expression in small follicles in comparison to large follicles. The IGF-IIR is expressed in the theca of healthy follicles and the granulosa cells of atretic follicles (Mazerbourg *et al.*, 2003).

Intrafollicular concentrations of IGFBP-2, -4 and -5 are inversely proportional to follicular size between 1-2 mm and preovulatory size, but increase independent of size in atretic follicles. These changes are due to both changes in mRNA expression and proteolytic cleavage of IGFBP's. For instance, changes in IGFBP-2 expression in follicular growth and atresia can be due to decreased and increased expression, respectively. The reduction in IGFBP-2 expression is FSH-dependent. IGFBP-4 expression is low in both granulosa and thecal cells and changes only slightly during folliculogenesis (Mazerbourg *et al.*, 2003), but is significantly reduced in the future dominant follicle in comparison to subordinate follicles (Mihm *et al.*, 2000) as is IGFBP-2 (Stewart *et al.*, 1996). IGFBP-5

expression increases significantly in the granulosa cells of atretic follicles. Follicular expression of IGFBP-3 is low and does not appear to have a role in follicle growth or atresia. Furthermore, IGFBP-2 and -4 undergo conformational changes upon binding to IGF, making them more susceptible to cleavage, and IGF enhances the degradation of IGFBP-2 (Mazerbourg *et al.*, 1999; Mazerbourg *et al.*, 2003). IGFBP-1 and -6 may also be present in the follicular fluid of atretic follicles. In addition, a large number of isoforms of IGFBPs exist, and post-translational modification may affect the bioactivity of these molecules (Nicholas *et al.*, 2002).

In vitro, IGF-I significantly increases IGFBP-2 mRNA levels and decreases IGFBP-5 from granulosa cells of large but not small follicles, while IGF-II has no effect. In addition, IGF-I increases production of IGFBP-2, -4 and -5 from thecal cells of large follicles, and IGF-II increases IGFBP-2 and -5 in these cells (Voge *et al.*, 2004b). IFGBP expression is also regulated hormonally. FSH and LH decrease IGFBP-3 mRNA in granulosa cells from small follicles and in the presence of insulin increase IGFBP-2. Oestradiol decreases IGFBP-2, -3 and -4 mRNA in thecal cells, enabling IGF-stimulated androstenedione production and enhancing oestrogen synthesis. Oestradiol also decreases IGFBP-5 mRNA in granulosa cells from large follicles, enhancing the paracrine regulation of oestradiol production (Voge *et al.*, 2004a).

In preovulotory follicles, the proteolytic degradation of IGFBP-4 and -5 is maximal. IGFBP-2, -4, and -5 are degraded by PAPP-A. Granulosa cell PPAP-A expression correlates with both LHR and CYP19 expression and is maximal in preovulatory follicles. Degradation of IGFBP-2 and -4 is inhibited by the heparin-binding domains of IGFBP-3 and -5, and connective tissue growth factor (CTGF) (Mazerbourg *et al.*, 1999). In addition, increased expression of IGFBP-5 in atretic follicles may enhance the inhibition of IGFBP-4 degradation via direct interaction between the heparin-binding domains of IGFBP-5 with IGFBP-4 proteinases (Mazerbourg *et al.*, 2003). Furthermore, the ability of IGF-I, basic fibroblast growth factor (FGF-2) and epidermal growth factor (EGF) to decrease FasL induced apoptosis of cultured granulosa cells has been linked to the ability of these factors to stimulate cell proliferation (Quirk *et al.*, 2004).

Inhibins and Activins

Activins and inhibins are TGFβ family members produced by granulosa cells that, together with the activin-binding protein follistatin, have been implicated as having a role in early follicular growth (McNatty *et al.*, 2000). Inhibin has a negative feedback effect on FSH secretion, while activin enhances FSH secretion in local regulatory roles. Activin promotes granulosa cell FSHR expression and FSH-induced mitosis and steroidogenic differentiation and predominates in immature follicles (Knight and Glister, 2003). However, in the follicular fluid of bovine follicles <6 mm in diameter, activin will be neutralised by binding to follistatin (Glister *et al.*, 2006). Inhibin and activin stimulate and inhibit thecal cell androgen production, respectively (Knight, 1996). As follicles mature, they produce increasing amounts of inhibin and follistatin, thereby increasing the amount of substrate available for oestrogen synthesis. Inhibin also enhances the androgen-stimulatory action of insulin, IGF-I and IGF-II (Hillier, 2001). Secretion of inhibin, activin and follistatin are enhanced by BMPs (Glister *et al.*, 2004). In addition, there is evidence of a positive effect of activin on bovine oocyte *in vitro* maturation (Silva and Knight, 1998).

Steroid Hormones

Synthesis of androgens by thecal cells is controlled by LH, and granulosa cells of antral follicles express androgen receptors throughout development (Hampton *et al.*, 2004a). Androgens may potentiate the stimulatory effect of FSH on granulosa cells by suppression of cAMP catabolism in an event that is receptor-mediated (Hillier and de Zwart, 1982). FSH-induced expression of CYP19 and the conversion of androgen to oestrogen is a feature of mature granulosa cells of preovulatory follicles. Oestrogen and progesterone production in granulosa cells is enhanced by androgens. In addition androgens potentiate levels of both IGF-I and IGF-IR receptor mRNA (Drummond, 2006). Late preovulatory follicle development is associated with declining expression of the androgen receptors, which diminishes granulosa cell responsiveness to gonadotrophins presumably delaying final granulosa cell maturation until the LH surge (Hillier, 2001).

Oestrogen receptors are present in the cytoplasm and nucleus, and have a zinc finger DNA-binding domain. Of the two oestrogen receptors cloned, oestrogen receptor-β is more abundant in ovarian tissues than oestrogen receptor-α, with highest expression in the

granulosa cells of growing follicles. Both FSHR and LHR expression are increased by oestrogen (Rosenfeld *et al.*, 2001), and oestrogen inhibits regulates androgen receptor expression (Drummond, 2006). Oestrogen also regulates the expression of the gap-junction protein connexin 43. Gap junctions allow transfer of molecules between granulosa cells and between granulosa cells and the oocyte and are also important channels of communication in the avascular environment within the follicle (Rosenfeld *et al.*, 2001). In addition, the LH surge down-regulates the oestrogen receptor (Hillier, 2001).

Anti-Mullerian Hormone

Anti-mullerian hormone (AMH), known also as Mullerian inhibiting substance (MIS) is a member of the large TGFβ family of growth and differentiation factors. AMH is first expressed in granulosa cells at the primary follicle stage and continues to be expressed until cyclic recruitment of follicles occurs in response to FSH stimulation. By affecting follicle sensitivity to FSH, AMH may have a role in the selection of the cohort of follicles to enter a wave of follicle growth, or to undergo atresia (Visser and Themmen, 2005).

BMP-15 and GDF-9

BMP15 is also a member of the TGFβ super family and is an oocyte-secreted factor, expressed in the oocyte from the primary follicle stage onwards (Galloway *et al.*, 2000). There is evidence that BMP's are involved in the transition of a primordial follicle to a primary follicle (Shimasaki *et al.*, 2004) and it has been suggested that BMP-15 and growth differentiation factor 9 (GDF-9) are determinants of the species-specific number of oocytes that will ovulate at each oestrous cycle (Moore *et al.*, 2004).

BMP-15 stimulates granulosa cell mitosis and proliferation and also promotes kit ligand expression by granulosa cells. Kit ligand binds to oocyte c-kit promoting oocyte growth (Packer *et al.*, 1994) and suppressing BMP-15 expression (Shimasaki *et al.*, 2004). In addition, rodent studies have shown FSH-induced expression of steroidogenic acute regulatory protein (StAR), CYP11A1, HSD3B, LHR and inhibin and activin subunits are inhibited by BMP-15 (Shimasaki *et al.*, 2004). The effect of BMP-15 is therefore to inhibit luteinisation. GDF-9 is another oocyte-derived growth factor with homology to BMP-15 that is expressed in all follicles (Bodensteiner *et al.*, 2000). The function of GDF-9 may vary between species (Shimasaki *et al.*, 2004) and differential *in vitro* effects observed may be due to the species origin of granulosa cells and growth factors used (McNatty *et*

al., 2005). In addition, there is evidence that other BMP family members produced by thecal cells have effects on thecal and granulosa cells (Knight and Glister, 2003). BMP-4 and -7 are expressed by thecal cells and granulosa cells and oocytes express BMP-6 while all these cell types express BMP receptors (Glister *et al.*, 2004).

Atresia

Atresia is an important process whereby cells of anovulatory follicles die, the follicular fluid is resorbed and remnants of the follicle are incorporated into the ovarian stroma. Oestradiol and inhibin, secreted by the dominant follicle, exert a negative feedback effect on FSH production, which ultimately results in atresia of subordinate follicles. Therefore selection of the dominant follicle and atresia of subordinate follicles are processes that co-ordinately regulate the number of follicles that will ovulate.

The cell death in atresia is likely to involve a balance between multiple molecules that are either survival-promoting factors [gonadotrophins, IGF-I, interleukin-1β, epidermal growth factors (EGFs), fibroblast growth factors (FGFs), TGFα] or atretogenic factors [TGFβ, interleukin-6, androgens, reactive oxygen species, FasL and tumour necrosis factors (TNF)] (Hussein, 2005). Atresia of primordial and primary follicles is initiated by oocyte death. In contrast, in growing follicles, granulosa and thecal cells undergo apoptosis and oocyte death is a later event (Reynaud and Driancourt, 2000). In addition, there is an association between a high rate of granulosa cell mitosis and follicular atresia (Lussier *et al.*, 1987; Quirk *et al.*, 2004).

Apoptosis

Apoptosis of granulosa cells has long been recognised as a feature of follicular atresia. The morphological features of apoptosis include (Hussein, 2005):

- nuclear condensation
- convolution of the nuclear and plasma membranes
- cellular fragmentation and budding into the membrane-bound 'apoptotic bodies' (Fig. 7A)
- phagocytosis of apoptotic bodies (Fig. 7B)

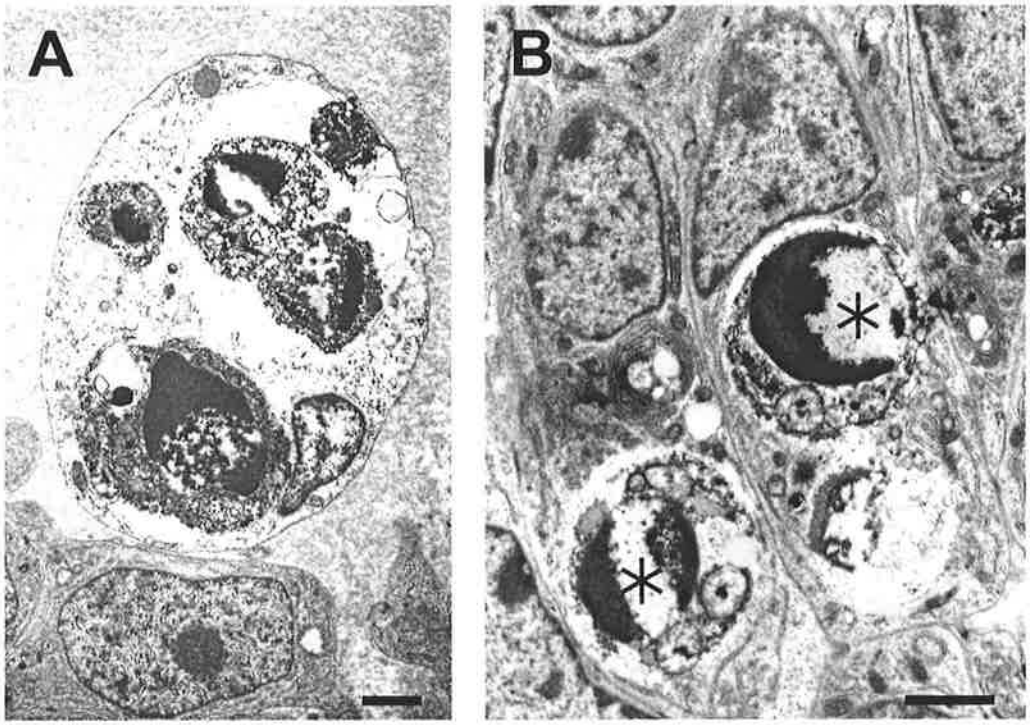


Figure 7. Apoptosis of granulosa cells.
A. Atretic body in the follicular antrum. B. Phagocytosis of apoptotic bodies (asterisks) by granulosa cells. Scale bar = 2μm. B from van Wezel *et al.* 1999.

Apoptosis is also a feature of cell death of thecal cells (Fig. 8) (Clark *et al.*, 2004). In addition, features of cell death associated with terminal differentiation have also been identified in granulosa cells (van Wezel *et al.*, 1999).

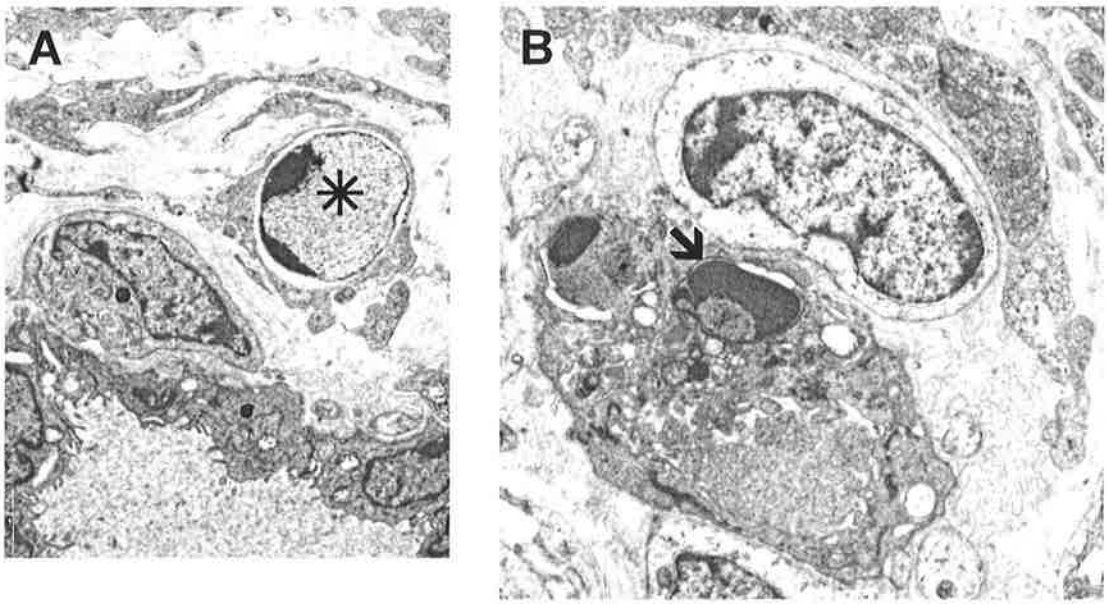


Figure 8. Cell death in the theca interna of atretic follicles involves apoptosis of stromal cells (asterisk in A) and endothelial cells (arrow in B). Scale bar = 2 μm.

Molecular Mediators of Apoptosis

Caspases are the main effector molecules in ovarian apoptosis and are a family of cysteine proteases that are activated by cell surface receptors (such as Fas) and Bcl-2 family proteins. The mitochondria are the intracellular site of the integration of apoptotic or cell survival signals. Bcl-2 is a nuclear and mitochondrial membrane-associated protein with pro-survival properties including modulation of mitochondrial release of cytochrome c, binding to Bax and blocking of c-Myc induced apoptosis (Hussein, 2005). Bax is a pro-apoptotic Bcl-2 antagonist. Heterodimers of Bax and Bcl-2 inhibit Bax homodimer formation and apoptosis (Oltvai *et al.*, 1993). Involvement of Bcl-2 in ovarian apoptosis is supported by reduced follicle numbers in mice deficient in Bcl-2, as well as reduced follicular atresia in mice over expressing Bcl-2, higher Bax in atretic follicles, and follicles with excessive numbers of granulosa cells in Bax-deficient mice (Hussein, 2005).

Following activation of receptors mediating apoptosis, complexes of protease-apoptosis activating factor-1 (Apaf-1), cytochrome c and caspase-9 (called the apoptosome) aggregate in the cytoplasm and caspase-9 then initiates cell death by activating downstream caspases, including caspase-3 (Fig. 9). Granulosa cells of early antral follicles express abundant Apaf-1, and *in vivo* gonadotrophin treatment suppresses Apaf-1 expression and granulosa cell apoptosis. Caspase-3 expression is also regulated by gonadotrophins and is expressed by granulosa cells of atretic follicles. Caspase-mediated cleavage of important cellular substrates eventually results in cell death. X-linked inhibitor of apoptosis (XIAP) prevents caspase activation, and treatment with gonadotrophins increases granulosa XIAP (Quirk *et al.*, 2004; Hussein, 2005).

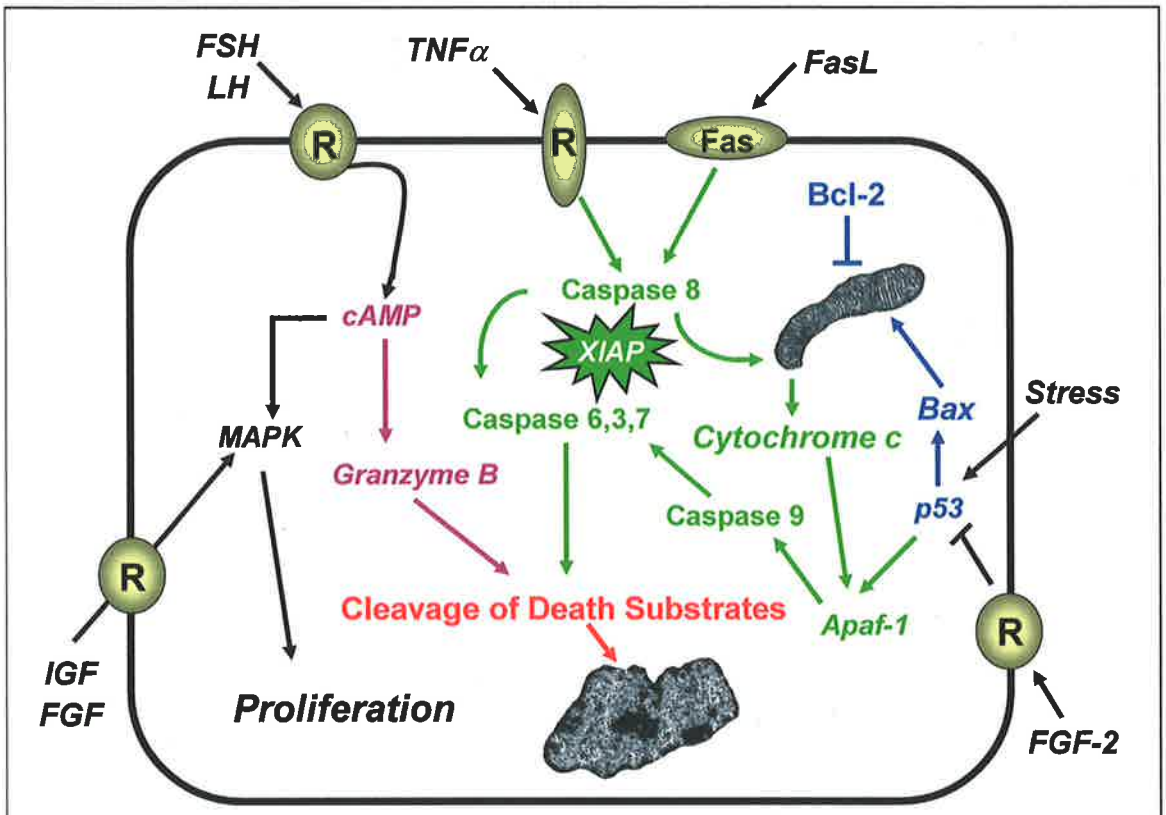


Figure 9. Pathways regulating the apoptosis of granulosa cells. Stimulation by TNFα or Fas ligand can cause release of mitochondrial cytochrome c, as can activation of p53. FGF-2 can protect against p53-induced apoptosis, and XIAP prevents caspase activation. Alternatively, cAMP activation of gramzyme B and the direct cleavage of death substrates. In addition, gonadotrophins, IGF and FGF can negate apoptotic signals. Redrawn from Amsterdam *et al.* 2003.

Fas and Fas Ligand

The majority of cell-surface death receptors mediating apoptosis of mammalian cells are members of the TNF receptor family, such as Fas. TNF family members are mostly type II transmembrane proteins. Granulosa cells express both Fas and FasL. Evidence for the involvement of TNF receptor family members in follicular atresia includes higher mRNA for FasL in granulosa and thecal cells of atretic follicles and Fas-induced granulosa cell apoptosis. Binding of FasL to Fas stimulates cytoplasmic protein-protein interactions and activation of caspase-8 and downstream caspases. In the first follicular wave, subordinate follicles express more Fas mRNA and are more susceptible to Fas-induced apoptosis [in the presence of interferon (IFN)] than healthy dominant follicles. IFN has been shown to induce Fas expression in bovine granulosa and thecal cells (Vickers *et al.*, 2000). Furthermore, thecal cells of dominant follicles also express less Fas mRNA than subordinate follicles, albeit at a level which is 10% of that of granulosa cells (Porter *et al.*, 2000). The LH surge is not associated with a change in granulosa cell expression of Fas

mRNA and protection of granulosa cells from Fas-mediated cell death is therefore likely to be due to the expression of an inhibitor of apoptosis proteins (Porter *et al.*, 2001).

Serum has been shown to modulate granulosa cell expression of both FasL/Fas and p53. p53 is a stress response gene encoding the p53 protein that accumulates in the cytoplasm during the G_1 phase of the cell cycle and migrates to the nucleus at the start of the S phase. p53 is expressed by apoptotic granulosa cells of atretic follicles and its inhibition is associated with fewer atretic follicles whereas over expression induces apoptosis of cAMP-stimulated cells (Hussein, 2005). Granulosa cells at the transition between the G_1 to S phase have been shown to be susceptible to apoptosis, whereas cells at other phases of the cell cycle are resistant. Activation of receptors for growth factors such as EGF, FGF and IGF induce the expression of anti-apoptotic genes protecting cells from apoptosis (Quirk *et al.*, 2004). In addition to decreasing apoptosis induced by FasL *in vitro*, IGF-I, FGF-2 and EGF stimulate cell proliferation, suggesting a link between the cell survival and proliferative effects of growth factors (Quirk *et al.*, 2004). Moreover, the effect of IGF-I to protect granulosa cells from FasL-induced apoptosis is mediated through the PI3K/Akt pathway only when progression through the G_0/G_1-to-S phase transition occurs (Hu *et al.*, 2004).

Atresia of the Dominant Follicle of the First Follicular Wave

Since atresia is the destiny of the dominant follicle of the first follicular wave, it provides a good model system for the study of factors important in the initiation of the atresia process. Valdez *et al.* (2005) found no evidence to support the hypothesis that proteins of the Bcl-2 family are involved in the activation of apoptosis during atresia of dominant bovine follicles. Caspase activity does not increase in dominant follicles when growth plateaus (days 4 – 8 following wave initiation), nor does oxidative stress appear to initiate granulosa cell apoptosis (Valdez *et al.*, 2005). These results suggest that other mechanisms are responsible for the observed decreased production of oestradiol and reduced numbers of viable granulosa cells early in atresia of the dominant follicle.

Other Mechanisms of Atresia

Granzyme B

Mitochondrial release of cytochrome c may not, however, be involved in the initial stages of the apoptosis of granulosa cells. An alternate pathway of cell death, which would preserve mitochondria, and hence steroidogenesis, until late in the atresia process, may involve the activity of granzyme B (Amsterdam *et al.*, 2003; Sasson *et al.*, 2003). An association between increased progesterone production and reduced oestrogen production by the granulosa cells of preovulatory follicles has been observed to precede atresia (Uilenbroek *et al.*, 1980). The reduced oestrogen synthesis is believed to be the result of reduced thecal androgen substrate (Gross *et al.*, 2001). Furthermore, an efflux of potassium ions has also been associated with the activation of apoptosis in a variety of cells (Bortner *et al.*, 1997; Orlov *et al.*, 2000) including granulosa cells (Perez *et al.*, 2000). Gross *et al.* (2001) demonstrated that decreased intracellular potassium facilitated FSH-stimulated progesterone biosynthesis and increased cAMP levels in granulosa cells in the absence of increased StAR or CYP11A1 expression. Our own studies of small antral follicles have shown that an elevation in the follicular fluid progesterone concentration of a sub group of atretic follicles (basal atretic follicles) is associated with precocious granulosa cell expression of HSD3B and CYP11A1 (Irving-Rodgers *et al.*, 2003a).

Hyaluronic acid

Hyaluronic acid has also been shown to inhibit the apoptosis of cumulus and mural granulosa cells via a mechanism that involves the cell surface hyaluronic acid receptor, CD44 (Kaneko *et al.*, 2000). Although the stability of gap junctions is also important for integrity of granulosa cells, a causal effect linking granulosa cell contacts and resistance to apoptotic signals has not been established (Amsterdam *et al.*, 2003). However it has been demonstrated that blocking N-cadherin junctions enhances granulosa cell apoptosis, and this is associated with cleavage (possibly by matrix MMPs) of the extracellular domain of the N-cadherin molecule. The protective effect of N-cadherin may be mediated by interaction with the FGF receptor (Peluso, 1997; Makrigiannakis *et al.*, 2000).

CART

The neuropeptide cocaine- and amphetamine-regulated transcript (CART) has recently been localised to the membrana granulosa of some antral follicles, and is absent from

dominant ovulatory follicles (Kobayashi *et al.*, 2004). *In vitro* CART is able to inhibit the oestrogen production of granulosa cells from oestrogenic follicles with no effect on progesterone production, indicating a specific effect in follicles. CART may therefore be a negative regulator of oestrogen, or promote the atresia process (Kobayashi *et al.*, 2004).

Androgen Receptor

Testosterone has been shown to enhance apoptosis granulosa cells (Billig *et al.*, 1993). Expression of the androgen receptor, which is present in granulosa cells of murine small follicles but not large healthy and preovulatory follicles, is maintained in atretic follicles (Cheng *et al.*, 2002). In the bovine follicle, the androgen receptor is expressed by granulosa cells of growing antral follicles as well as thecal cells of larger antral follicles (Hampton *et al.*, 2004a). Since increasing follicular fluid oestrogen concentration is associated with reduced granulosa cell expression of the androgen receptor, Cheng *et al.* (2002) hypothesised that activation of the oestrogen receptor β represses androgen receptor expression. Therefore follicles in which androgen expression remains elevated will not progress to oestrogen dominance and become atretic (Cheng *et al.*, 2002).

Ovulation

At ovulation the follicle ruptures and the oocyte is released. Ovulation is initiated by the LH surge and involves increased blood flow in the theca interna, extravasation of leukocytes and localised oedema. Final maturation of the oocyte occurs after the LH surge (Hyttel *et al.*, 1997). The steroidogenic cells of the theca interna hypertrophy in the periovulatory period (Priedkalns and Weber, 1968; McClellan *et al.*, 1975). Increased blood flow at the base of the follicle in conjunction with reduced blood flow at the apex may facilitate follicle rupture and extrusion of the oocyte (Brannstrom *et al.*, 1998). Follicle rupture involves degradation of the extracellular matrix and connective tissue, and involves a large number of proteinases, including MMPs, plasminogen activator, plasmin, and members of the disintegrin and metalloproteinase domain with thrombospondin motifs (ADAMTS) family (Fig.10) (Ohnishi *et al.*, 2005; Curry and Smith, 2006). In addition, recent evidence from rodent studies suggest that cells within the theca interna containing smooth muscle α actin contract in response to endothelin-2 (ET2) produced by granulosa cells of the periovulatory follicle and this contraction may contribute to follicular rupture (Ko *et al.*, 2006). The precursor to ET2, preproendthelin-2, is also expressed in the bovine

ovary (Uchide *et al.*, 2003). Recently it has been shown that it is the LH surge (and not follicle rupture *per se*) that is essential for the development of the bovine corpus luteum (Hayashi *et al.*, 2006).

In addition, granulosa cells express nuclear progesterone receptors after the LH surge, and progesterone enhances its own secretion, suppresses oestrogen secretion from granulosa cells and inhibiting proliferation. The anti-apoptotic effect of progesterone in granulosa and luteal cells may be mediated by binding to the plasma membrane protein complex of progesterone receptor membrane component 1 (PGRMC1) and sterol regulatory element binding protein 1 (SERBP1). Expression of PGRMC1 is upregulated at ovulation and SERBP1 increases in granulosa cells during folliculogenesis (Peluso, 2006).

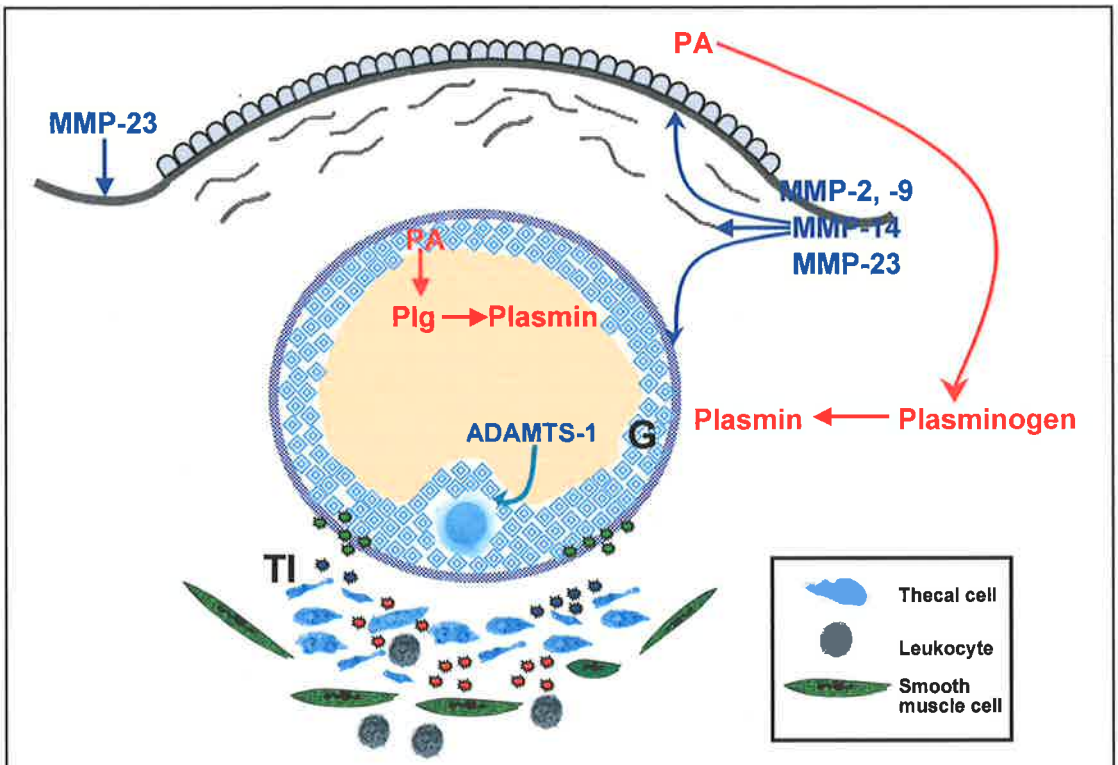


Figure 10. Proteinases involved in the process of ovulation.
MMP-2, -9 and -14 from thecal cells may degrade the follicle wall. MMP-23 from the theca externa and surface epithelium may degrade the basal lamina underlying the surface epithelium. Plasminogen (Plg) is converted to plasmin by plasminogen activator (PA) from the surface epithelium or granulosa cells and can active pro-MMPs. Proteinases released by leukocytes (red astrerisks), thecal cells (blue asterisks) or granulosa cells (green asterisks) weaken the follicular wall. Endothelin-2 produced by granulosa cells is able to diffuse through the partially degraded follicular basal lamina, and bind to smooth muscle cells causing constriction of the follicle and eventual extrusion of the oocyte and follicular fluid when the apex of follicle ruptures. From Ko *et al.* 2006, and Ohnishi *et al.* 2005.

Corpus Luteum

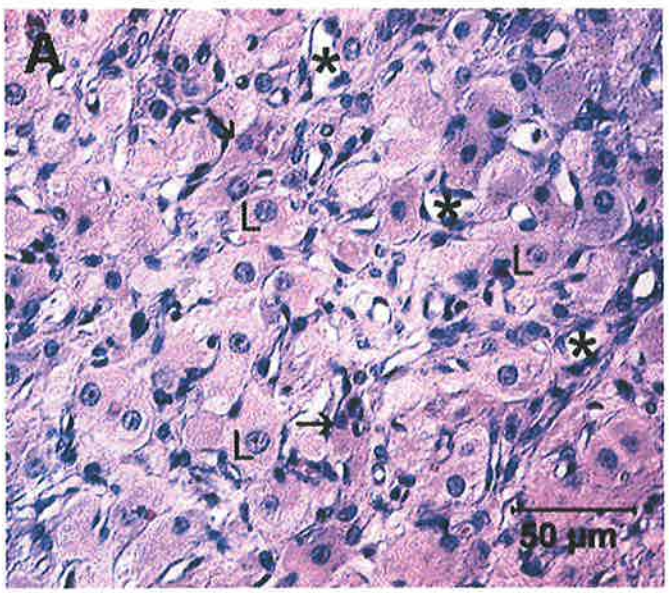
Formation

Ovulation signifies the first day of the corpus luteum (CL) lifespan. Following rupture of the follicle, the remaining wall collapses inwards, forming the structural basis for the connective issue septa of the developing CL. This is most apparent in the human corpus luteum where distinct thecal and granulosa-derived tissue layers are evident. Degradation of the follicular basal lamina permits migrating cells and sprouting capillaries from the theca interna to intermingle with granulosa cells (Amselgruber *et al.*, 1999). In the first 12 hours after ovulation, granulosa cells develop the organelle structures present in mature large luteal cells (LLC) and cease dividing (McClellan *et al.*, 1975). TNFα, produced by macrophages and endothelial cells, is a likely stimulator of CL development via PG-stimulated progesterone production. In addition, both TNFα and PGE_2 may regulate luteal angiogenesis (Okuda and Sakumoto, 2003). Vascular volume density and CL volume increase 2- to 3-fold between 3 and 4 days after ovulation (Acosta *et al.*, 2003) and the majority of cells undergoing mitosis immediately following ovulation are endothelial cells and fibroblasts (McClellan *et al.*, 1975). Endothelial cells comprise approximately 53% of cells in the mature bovine CL and the capillary network comprises about 14% of the volume, while fibroblasts comprise 10% of cells and 6% of volume (O'Shea *et al.*, 1989).

Monocytes constitute the most numerous leukocyte cell population present in the bovine CL, and these cells as well as macrophages and T lymphocytes increase significantly during the latter half of the luteal phase. This increase in immune cells is coincident with an increase in endothelial cell expression of monocyte chemoattractant protein-1 (MCP-1) (Townson *et al.*, 2002). In addition to its function as a chemoattractant for monocytes, MCP-1 is also a differentiation factor for macrophages (Tabata *et al.*, 2003). MCP-1 levels increase markedly between the follicular and late ovulatory phase. This late follicular phase increase in MCP-1 does not appear to be steroid dependent and secretion is stimulated by interleukin-1α and -1β, but not TNFα (Dahm-Kahler *et al.*, 2006). In addition, FGF-2 stimulates bovine aortic endothelial MCP-1 mRNA *in vitro* (Wempe *et al.*, 1997). Luteal endothelial cells increase MCP-1 synthesis in response to TNFα and IFN stimulation, but not $PGF_{2\alpha}$ or progesterone, suggesting that the role of MCP-1 in luteolysis is not mediated by $PGF_{2\alpha}$ (Cavicchio *et al.*, 2002).

Luteinisation

The differentiation of steroidogenic thecal and granulosa cells into small and large luteal cells, respectively (Fig. 11), after the LH surge is called luteinisation. Luteal cells comprise approximately 70% of the volume of the CL (Fields and Fields, 1996).



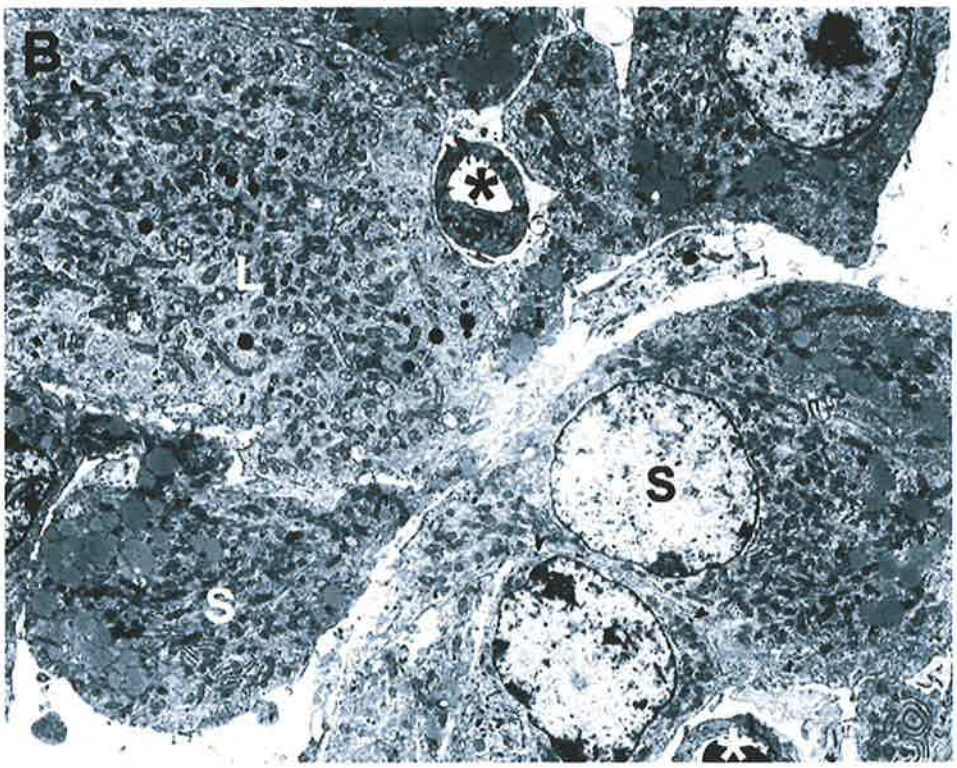


Figure 11.

Light (A) and electron (B) micrographs of luteal parenchyma showing large luteal cells (L), small luteal cells (arrows in A and S in B) and capillaries (asterisks).

Luteinisation is also associated with a change in steroid hormone production from that dominated by oestrogen synthesis from granulosa cells of the preovulatory follicle requiring CYP17 (thecal cells) and CYP19 (granulosa cells) expression, to progesterone

production catalysed by CYP11A1 and HSD3B. In addition, oestrogen synthesis is maintained in the primate CL. Growth of the CL involves hypertrophy of LLC as well as hyperplasia of small luteal cells (SLC), fibroblasts and endothelial cells (Niswender *et al.*, 2000). On day 3 of the bovine oestrous cycle, the CL has been estimated to weigh 0.6 $\pm$ 0.3 g, dramatically increasing to 3.6 $\pm$ 0.3 g by day 7, which is maintained until day 17 of the cycle (4.0 $\pm$ 0.3) before decreasing to 3.2 $\pm$ 0.4 g on day 19 (Fields and Fields 1996). SLC comprise 26% of luteal cells and 28% of the volume of the CL while LLC comprise only 3% of cells but 40% of luteal volume (O'Shea *et al.*, 1989). Luteinisation also involves loss of sensitivity to EGF, while sensitivity to FGF is maintained (Gospodarowicz *et al.*, 1977).

Progesterone is the principle product of the CL, and is required for the establishment and initial maintenance of pregnancy. Cholesterol is the substrate for steroid hormone production. Lipoproteins synthesised in the liver are transported to the CL and taken up by luteal cells via two different mechanisms. Most exogenous cholesterol is derived from low-density lipoprotein (LDL) particles via binding to LDL receptors localised to clathrin-coated pits on the plasma membrane. Following entry into a series of endocytic compartments, cholesterol and cholesterol esters are transferred to the lysosome. After hydrolysis in the lysosomes, cholesterol is transferred to the endoplasmic reticulum and/or Golgi apparatus. Rapid uptake of high-density lipoprotein (HDL) is mediated by non-endocytic means via a cell surface receptor localised to plasma membrane microdomains called caveolae. Cholesteryl esters then traffic to intracellular sites such as lipid droplets, the endoplasmic reticulum and Golgi complex by a non-lysosomal vesicular mechanism (Schroeder *et al.*, 2001).

Transport of cholesterol from the outer to the inner mitochondrial membrane requires StAR and is the rate-limiting step in steroidogenesis (Stocco, 2000). Within the mitochondrial matrix, cholesterol is converted to pregnenolone via the combined actions of the CYP11A1 and its electron transport chain members, adrenodoxin and adrenodoxin reductase. Pregnenolone is then converted in the endoplasmic reticulum to progesterone by HSD3B. Circulating progesterone concentration depends upon the number and size of SLC and LLC and their synthetic capacity as well as the blood flow through the CL (Niswender *et al.*, 2000). The pattern of progesterone secretion consists of a rising phase, a plateau phase and a regressing phase. In addition, it has been suggested that initial progesterone secretion is autonomous, and that progesterone may stimulate its own

secretion (Rothchild, 1981). In addition, both progesterone and oxytocin may regulate CL function via $PGF_{2\alpha}$. In the developing CL, luteal $PGF_{2\alpha}$, together with oxytocin and progesterone, function in an autocrine or paracrine manner to cause pulsatile progesterone release, but at the end of the luteal phase PGF2α initiates luteolysis (Bah *et al.*, 2006).

The observation that steroidogenic cells and fibroblasts of the CL express progesterone receptors throughout its lifespan supports the suggestion of a paracrine role for progesterone in the CL (Rothchild, 1981; Maybin and Duncan, 2004). Small and large luteal cells differ in their basal secretion of progesterone, and LLC produce up to 40 times more progesterone than SLC. Progesterone production by SLC is mediated by enhanced cholesterol transport that is stimulated by LH via phosphorylation of StAR and by PGI_2 via activation of a protein kinase. This response does not occur in LLC, despite the presence of LHR, possibly due to be the constitutive activation of the protein kinase C and the high basal secretion of progesterone by these cells. In LLC stimulation of progesterone production in response to prostaglandins, growth hormone and IGF-I is limited and not mediated by protein kinase A. Therefore primary regulation of progesterone in these cells appears to be negative (Niswender *et al.*, 2000).

IGF-I and growth hormone receptors are found on LLC and these ligands may be important in the maintenance of the high basal levels of progesterone. IGF-I is believed to activate intrinsic tyrosine kinase, increasing the activity of phosphoinositide 3-kinase which appears to affect the cytoskeleton and may be anti-apoptotic. The early luteal stage is associated with the production of high concentrations of PGE_2 and PGI_2 which have been proposed to have a role in luteal development. Both SLC and LLC have binding sites for PGI_2 and the binding of PGE_2 to its receptor on LLC results in increased progesterone synthesis (Niswender *et al.*, 2000).

Morphologically, SLC are distinguished by adherens junctions, whorls of smooth endoplasmic reticulum, abundant cytoplasmic lipid, prominent Golgi apparatus and elongated mitochondria with tubular cristae and crystalline inclusions (Fields and Fields, 1996). The nuclei of SLC are small and have a dense chromatin pattern and little folding of the plasma membrane. In contrast, the LLC has a relatively large nucleus with a light chromatin pattern and extensively folded plasma membrane (Priedkalns and Weber, 1968). These cells lack adherens junctions and have a paucity of lipid, at least early in the oestrous cycle.

LLC have an abundance of smooth endoplasmic reticulum, abundant spherical mitochondria with lamellar and tubular cristae and electron-dense inclusions during regression (Fields and Fields, 1996). The mitochondria of LLC are increased in size in comparison to those of granulosa cells (Priedkalns and Weber, 1968), and the cytoplasm contains an abundance of secretory granules forming a large single cluster at the periphery of the cell. Granules are released from the cells by exocytosis, induced by $PGF_{2\alpha}$, and occupy a region of the cytoplasm that is devoid of other organelles but contains an extensive microfilament network. These granules contain oxytocin and neurophysin and their formation is stimulated by the preovulatory LH surge. The proportion of LLC with granules increases from 3% on day 3 of the oestrous cycle to a maximum of 84% on day 7 and declining to 16% on day 17 and 8% on day 19. Luteal cells have oxytocin receptors, and highest oxytocin binding is seen at the mid-luteal stage (Okuda *et al.*, 1992). In addition, secretory granules containing tissue inhibitor metalloproteinases (TIMPs) have been identified in LLC (Fields and Fields, 1996; McIntush *et al.*, 1996). Biosynthesis of PGE_2 and $PGF_{2\alpha}$ is greatest in LLC in comparison of other luteal cell types with PGE_2 synthesis predominating during growth of the CL, and $PGF_{2\alpha}$ dominating during regression (Arosh *et al.*, 2004). Furthermore, the presence of a viable embryo and PGE_2 secretion from the endometrium can prolong the lifespan of the CL (Niswender *et al.*, 2000).

Vascularisation

Angiogenesis is an important process occurring after ovulation, when the avascular membrana granulosa and the vascularised theca interna are remodelled into the highly vascularised corpus luteum. Sprouting of new vessels occurs from post-capillary venules and capillaries as pericytes and then endothelial cells migrate into the membrana granulosa and form cylindrical cords. Luteinised granulosa cells of the developing CL are closely associated with vascular cells of the sprouts (Amselgruber *et al.*, 1999). Changes in the vascular associated extracellular matrix also occur at ovulation. A fine meshwork of fibronectin fibrils forms along sprouting capillaries and newly formed sprouts are associated with a discontinuous layer of collagen type IV and uneven deposition of lamina (Amselgruber *et al.*, 1999). Recently a role has been shown for monocytes/macrophages in tissue neovascularisation by the local degradation of extracellular matrix and arrangement of the tissue space, and in aiding the penetration of progenitor cells (Anghelina *et al.*, 2006).

Sugino *et al.* (2005) showed in women that the proportion of blood vessels doubles between days 1-3 and days 4-11 of the menstrual cycle, before declining at days 12-15. The number of pericytes showed a similar change, increasing significantly between days 1-3, 4-5 and 6-11 and declining at days 12-15. These changes are coincident with an increase in luteal cell expression of angiopoietin-2 seen at days 4-5 of the cycle and an increase in angiopoietin-1 staining seen at days 6-11. Angiopoietin-1 stabilises blood vessels by interacting with endothelial cells and pericytes, while angiopoietin-2 antagonises the effects of angiopoietin-1 and destabilises vessels. The late luteal phase (days 12-15) is associated with weaker angiopoietin-1 staining and strong angiopoietin-2 staining. These observations may reflect stabilisation of blood vessels in mid luteal phase (days 6-11 of the cycle) and destabilisation of blood vessels in the late luteal phase. In addition, declining angiopoietin-1 expression may induce endothelial cell apoptosis. It has been proposed that in the presence of vascular endothelial growth factor (VEGF), angiopoietin-2 promotes vessel sprouting (by blocking angiopoietin-1), while in the absence of VEGF, inhibition of angiopoietin-1 promotes blood vessel disintegration (Sugino *et al.*, 2005).

The most important angiogenic factors are VEGF A, FGF-1 and -2, IGF-I and -II, and the angiopoietins (Schams and Berisha, 2004). FGF-2 is localised to endothelial cells of vessels within the theca interna and externa (Berisha *et al.*, 2000a) and CL (Gabler *et al.*, 2004). VEGF is localised to the membrana granulosa and theca interna of bovine preovulatory follicles (Berisha *et al.*, 2000a) and LLC of the corpus luteum where it may act as a chemoattractant for sprouting endothelial cells (Berisha *et al.*, 2000b). In addition, DNA microarray analyses show treatment with FSH increases the expression of VEGF (Sasson *et al.*, 2003). FGF-2 has been shown to stimulate luteal endothelial VEGF receptor mRNA expression *in vitro,* while VEGF stimulates FGF receptor expression. Cross-talk between the FGF and VEGF systems may also effect angiogenesis indirectly via effects on extracellular matrix components (Gabler *et al.*, 2004).

Prokineticin-1 (PK-1) or endocrine gland vascular endothelial growth factor, is a heparin-binding growth factor that is selectively mitogenic for endothelial cells of endocrine tissues (LeCouter *et al.*, 2001; Kisliouk *et al.*, 2003) and anti-apoptotic for luteal endothelial cells (Kisliouk *et al.*, 2005). In addition there is conflicting evidence for an effect of hypoxia on the induction of PK-1, which may reflect differences in cell types examined in *in vitro* studies (LeCouter *et al.*, 2001; Kisliouk *et al.*, 2003). In the ovary, PK-1 is localised to the

granulosa and thecal compartments of follicles (LeCouter *et al.*, 2001) and PK-1 expression is stimulated by forskolin (Kisliouk *et al.*, 2003). Granulosa cell expression of PK-1 is highest in primordial and primary follicles, decreasing in larger follicles (Ferrara *et al.*, 2003). Receptors for PK-1 (PK-R1 and PK-R2) are expressed by luteal endothelial cells and there is also significant expression of PK-R1 in thecal cells and steroidogenic luteal cells (Kisliouk *et al.*, 2003). Expression of PK-1 is higher in LLC in comparison to SLC, and increases significantly between the early and late luteal phase (Fraser *et al.*, 2005). Taken together, these results suggest that PK-1 expression is regulated and that PK-1 has a role in the vascularisation of the CL.

Blood flow to the ovary containing the corpus luteum is specifically increased approximately 5-fold during the luteal phase (Niswender *et al.*, 1975; Niswender *et al.*, 1976). Ovulation and peak luteal function are associated with the highest intraovarian and ovarian artery blood flow during the menstrual cycle (Kupesic *et al.*, 1997). In the bovine, total ovarian blood flow declines 4 days prior to oestrus, primarily as the result of the redirection of flow through the ovarian artery toward the uterus. A decrease in the proportion of the corpus luteum occupied by capillaries is associated with a decrease in blood flow during the late luteal phase (Niswender *et al.*, 1976). In addition, total ovarian blood flow is correlated with systemic progesterone concentration and negatively correlated with oestradiol concentration (Ford and Chenault, 1981).

Corpus Luteum Regression

Regression of the CL is necessary in the non-pregnant animal to initiate the final maturation and then ovulation of a dominant follicle. Regression involves cessation of progesterone synthesis (functional) luteolysis, which is followed by tissue destruction (structural luteolysis) although morphological changes in LLC are seen on day 14 of the oestrous cycle before the initiation of luteolysis (Priedkalns and Weber, 1968; Fields and Fields, 1996). Inhibition of progesterone production is initiated by PGF2α released from the uterus, initially through the down regulation of StAR (Niswender *et al.*, 2000; Davis and Rueda, 2002). Inhibition of progesterone production is followed by apoptosis and/or necrosis of endothelial and luteal cells and a reduction in luteal blood flow (Berisha and Schams, 2005). The $PGF_{2\alpha}$ receptor is present on thecal cells and bovine SLC and LLC, whereas luteal endothelial cells do not possess significant $PGF_{2\alpha}$ receptors. Leukocytes

also have important roles in functional and structural leutolysis (Benyo and Pate, 1992; Nishimura *et al.*, 2004). Immune cells (monocytes, macrophages and T lymphocytes) increase significantly in number in the late luteal phase. Recruitment of leukocytes is mediated by endothelial expression of MCP-1 (Townson *et al.*, 2002) and is not regulated by PGF2α (Cavicchio *et al.*, 2002).

Activation of the $PGF_{2\alpha}$ receptor results in an elevation of intracellular calcium concentration following release of free calcium from the smooth endoplasmic reticulum, with a detrimental effect on luteal steroidogenesis. Another consequence of $PGF_{2\alpha}$ receptor activation is induction of nerve growth factor inducible protein-B (NGFI-B or Nur77), a transcription factor required of the induction of 20-alpha-hydroxysteroid dehydrogenase (AKR1C1) that converts progesterone to an inactive metabolite. $PGF_{2\alpha}$ also induces translocation of cytosolic protein kinase C isoforms to the plasma membrane enhancing their catalytic activity and causing a reduction in progesterone synthesis and mRNA for HSD3B and StAR. $PGF_{2\alpha}$ also activates extracellular signal-regulated mitogen-activated protein (Erk MAP) kinase signalling that represses the ability of gonadotrophins to elevate the expression of StAR and progesterone secretion. Down regulation of cholesterol transport mediated by StAR appears to be the principle mechanism by which $PGF_{2\alpha}$ blocks progesterone synthesis (Niswender *et al.*, 2000; Davis and Rueda, 2002).

Since luteal cells themselves synthesise $PGF_{2\alpha}$ and since $PGF_{2\alpha}$ and protein kinase C activators induce the expression of the prostaglandin rate limiting enzyme COX-2, $PGF_{2\alpha}$ may function in an autocrine manner to inhibit luteal steroidogenesis (Niswender *et al.*, 2000; Davis and Rueda, 2002). This mechanism is especially significant in primates since luteolysis is not mediated by uterine $PGF_{2\alpha}$ in these species (Niswender *et al.*, 2000). In addition, interleukin-1β, which is produced by macrophages, fibroblasts and endothelial cells, has been shown to stimulate luteal $PGF_{2\alpha}$ synthesis *in vitro* (Nothnick and Pate, 1990). Oestrogens may also be involved in luteolysis by increasing intraluteal prostaglandins, while progesterone has effects that tend to delay synthesis and release of prostaglandins from the CL (Rothchild, 1981).

$PGF_{2\alpha}$ also stimulates secretion of oxytocin in a process involving a protein kinase C-induced phosphorylation event and actin filament depolymerisation. These changes facilitate exocytosis (Niswender *et al.*, 2000; Davis and Rueda, 2002) and are dependent upon cell-to-cell contact (Shibaya *et al.*, 2005). Oxytocin decreases progesterone secretion. Changes to the cytoskeleton and exocytosis of oxytocin may also involve activation of the rho family of small G-coupled proteins. Rho activation also leads to cell rounding and may function in CL development and regression. Disruption of the microtubular system blocks cholesterol transport to the mitochondria and may be an early event in $PGF_{2\alpha}$ inhibition of progesterone synthesis (Niswender *et al.*, 2000; Davis and Rueda, 2002).

Morphological changes in luteal cells appear approximately 24–36 hours after exposure to $PGF_{2\alpha}$ (Sawyer *et al.*, 1990). A loss of LLC precedes the loss of SLC (Braden *et al.*, 1988; Niswender *et al.*, 2000). Morphological changes are first observed in LLC on day 14 of the oestrous cycle as an increase in large lipid droplets, electron dense inclusions in mitochondria, vesiculation of the endoplasmic reticulum and a reduction in secretory granules (Priedkalns and Weber, 1968; Fields and Fields, 1996). Structural changes occurring in regressing SLC include lipid accumulation and lysis of mitochondria and lipid droplets by lysosomes (Priedkalns and Weber, 1968). Following the decline in circulating progesterone, cytolysis is accompanied by increased secretion of TIMP and formation of collagen fibres (Fields and Fields, 1996). TIMP-2 and MMP-2 localise to SLC and connective tissue stroma of the human corpus luteum, while TIMP-1 localises to the LLC and MMP-1 to the connective tissue stroma (Duncan *et al.*, 1998).

Apoptosis of luteal cells is most likely mediated via the Fas/FasL system by leukocytes that increase in number in the CL at the time of regression. Luteal apoptosis is suppressed by progesterone via inhibition of Fas and caspase-3 (Okuda *et al.*, 2004). Progesterone protects endothelial cells from apoptosis induced by TNFα (Friedman *et al.*, 2000), illustrating the relationship between functional regression and structural regression. TNFα (but not IFNγ or FasL) can cause apoptosis of endothelial cells and elevation of reactive oxygen species (Pru *et al.*, 2003). T lymphocytes express Fas ligand and are also the primary source of IFN, while Fas expression in luteal cells is induced by TNFα and IFN (Taniguchi *et al.*, 2002; Okuda and Sakumoto, 2003; Rosiansky *et al.*, 2006). Production of bioactive TNFα by macrophages occurs after the loss of progesterone synthesis (Shaw

and Britt, 1995), TNFα stimulates luteal PGF2α synthesis, which together with PGF2α from the uterus, is responsible for luteolysis (Niswender *et al.*, 2000). In addition, the mid luteal phase is associated with high Bcl-2 and low Bax expression. A change in this ratio in the regressing CL is inversely related to the frequency of apoptosis, suggesting a role for this system in luteolysis (Sugino *et al.*, 2000) independent of progesterone (Okuda *et al.*, 2004).

Luteal regression may also be initiated by autoimmune type responses mediated by major histocompatibility gene complex (MHC) expression. Immune cells such as macrophages and B lymphocytes express MHC class II cell-surface glycoproteins important in antigen presentation to T lymphocytes. Class II MHC antigen expression by other cell types (such as pancreatic islet cells) in disease states enables antigen presentation to T lymphocytes. Expression of MHC class II by luteal cell is significantly enhanced by treatment with IFNγ *in vitro* (Fairchild and Pate, 1989). MHC class II is low in luteal cells from the early luteal phase, but the mid luteal phase is associated with a significant increase in LLC expression of MHC class II (Benyo *et al.*, 1991). Luteal cells from the regressing bovine CL, but not mid stage CL are able to stimulate T-cell proliferation *in vitro* (Petroff *et al.*, 1997). In addition, INFγ enhanced MHC Class II expression is attenuated by LH, indicating gonadotrophin regulation of expression during the luteal phase (Fairchild and Pate, 1989). Luteal cells may also participate in an MHC Class I dependent immune response (Cannon and Pate, 2003).

Vascular changes during luteolysis are stage- and PG-dependent, and include an initial acute increase in blood flow that is necessary for the release the vasoactive peptides endothelin-1 (ET1) and angiotensin II, followed by vasoconstriction and angiolysis (Acosta *et al.*, 2002). The initial vasodilation caused by $PGF_{2\alpha}$ may also be mediated by nitric oxide, and be required for the leukocytic infiltration accompanying luteolysis (Skarzynski *et al.*, 2003). In addition to the hypothesised role of ET2 in ovulation (Ko *et al.*, 2006), a role for ET1 is suggested in luteal regression (Davis and Rueda, 2002; Rosiansky *et al.*, 2006).

Conclusion

In contrast to the vast array of scientific investigation and knowledge regarding the genetic code and intracellular signalling, the importance of extracellular matrix on cellular fate and function is only really beginning to be fully appreciated and is relevant well beyond the ovary. For instance, it has been shown recently that the elasticity of the cellular microenvironment can determine mesenchymal stem cell differentiation and lineage specificity (Engler *et al.*, 2006). It has also been discovered that development of cirrhosis of the liver is associated with altered basement membrane composition, while the normal composition of the basement membrane maintains hepatocyte differentiation (Zeisberg *et al.*, 2006). The expression of laminin 10 ($\alpha5\beta1\gamma1$), collagen type IV and nidogen-2 but not perlecan, are lower in regions associated with neutrophil transmigration from venules (Wang *et al.*, 2006). In addition, laminin $\alpha4$ regions of endothelial basal laminas are permissive of T-cell transmigration in the central nervous system, whereas laminin $\alpha5$ is not (Sixt *et al.*, 2001). Laminin $\alpha4$ is found with $\beta1$ and $\gamma1$ in laminin 8 that is expressed by all endothelial cells and upregulated by cytokines and growth factors important in inflammatory events. Laminin $\alpha5$ is found in laminin 10 in conjuction with laminin $\beta1$ and $\gamma1$ and is up-regulated by proinfammatory signals (Hallmann *et al.*, 1995). These few examples exemplify how matrix is becoming increasingly recognised for its role in regulating cell fate and function.

In contrast to other systems, and with the exception of oocyte-associated matrices, the role of extracellular matrix in ovarian biology has been relatively under investigated. The studies constituting this present body of work contribute substantially to the literature in this area. It has been suggested that models of the biological regulation of cells and tissues should not be restricted to changes in molecular binding, but rather take into account the broader concepts of cellular architecture and micromechanics (Huang and Ingber, 1999). The studies described here contribute essential information which, with the development of analytical methodologies able to handle complex systems, will aid "the transition from molecular reductionism to cellular realism" (Huang and Ingber, 1999) in the understanding of ovarian function.

With this approach to the study of extracellular matrix, I hypothesised that changes in matrix composition would accompany changes in the behaviour of follicular cells. More

importantly, matrix, like growth factors and hormones, would contribute to the regulation of cellular behaviour. Thus alterations in the type of matrix and changes in its composition would regulate follicle development and/or atresia. Before these hypotheses could be tested it was necessary to characterise follicular matrix and identify changes accompanying development and atresia. Previous studies on ovarian matrix had identified classes of molecules only, such as the presence of laminin and collagen type IV, but largely failed to identify specific components of these molecules. Other studies with a biochemical approach in the absence of localisation studies, importantly failed to identify matrix associated with specific cell types.

Given the limitations of previous studies, I undertook a comprehensive series of studies investigating matrix changes associated with follicular development. In the course of these studies my immunohistochemical observations led to the discovery and identification of a novel type of basal lamina matrix. In addition, I discovered that healthy bovine ovarian follicles are of two phenotypes, as are atretic follicles. Although these phenotypes had been described in two early publications (Rajakoski, 1960; Marion *et al.*, 1968), they were inadequately or incorrectly described. I thus conducted several studies to fully describe these atresia types, including the matrix associated with them.

I observed different morphological phenotypes of bovine healthy antral follicles (Irving-Rodgers and Rodgers, 2000). The basal granulosa cells (those adjacent to the follicular basal lamina) were either all rounded or all columnar, with equal proportions of each present in the population of antral follicles $\leq$4 mm. Follicles with rounded basal cells were surrounded by a single layer of basal lamina and seen in antral follicles of all sizes. Follicles with columnar basal cells were rarely seen in follicles >5 mm in diameter. Such follicles have additional basal lamina material and were described as having a follicular basal lamina that was loopy in cross-sectional appearance (Irving-Rodgers and Rodgers, 2000). Follicles with this morphological phenotype were also observed in sections of human ovary (Irving Rodgers *et al.*, 2004). In addition, bovine oocytes harvested from follicles with an aligned basal lamina had significantly greater developmental capacity to become blastocysts following in vitro maturation and fertilization, than those from follicles with a loopy basal lamina (Irving-Rodgers *et al.*, 2004b). The cause of the loopy basal lamina is not known at this stage, but could be due to a lower rate of follicle antrum expansion (Rodgers *et al.*, 2001). Bovine follicles <5 mm in diameter in which the loopy phenotype occurs may expand at a slower rate than larger follicles, with matrix produced

in excess of that required for follicular expansion being shed and hence giving rise to loopy appearance. As a consequence of a slower rate of expansion such follicles may have older oocytes relative to follicles with a faster rate of follicular expansion. This could contribute to poorer quality oocytes in these follicles.

My studies also provided evidence that granulosa cells contribute to the follicular basal lamina. Culture of bovine granulosa cells from small antral follicles grown without anchorage promotes the growth of stem and/or progenitor cells (Lavranos *et al.*, 1999). Under such culture conditions colonies of granulosa cells form (Lavranos *et al.*, 1994), 20% of which produce an extracellular matrix resembling basal lamina (Rodgers *et al.*, 1995). These colonies produce collagen type IV, cellular fibronectin and proteoglycans. Treatment with FGF-2 doubled the number of cells/colony, increased colony diameter by 50%, and increased fibronectin production 6-fold (Rodgers *et al.*, 1996).

Using immunohistochemistry to identify and localise extracellular matrix components associated with follicles, laminin chains α1, β2 and γ1 were consistently localised to the follicular basal lamina (van Wezel *et al.*, 1998). Laminin α2, seldom present in the follicular basal lamina of healthy follicles, was more frequently observed in atretic follicles (van Wezel *et al.*, 1998). In addition, the follicular basal lamina of primordial and preantral follicles was positive for all collagen type IV chains (Rodgers *et al.*, 1998). While α3 - α6 chains were not present in the basal lamina of antral follicles, α1 and α2 continued to be expressed (Rodgers *et al.*, 1998). Perlecan and nidogen-1 were localised to the follicular basal lamina of follicles beyond the preantral stage, and versican was localised to the thecal layers (McArthur *et al.*, 2000). Therefore, there are changes in the composition of the basal lamina at critical stages of follicular development, such as activation and antrum expansion. This supports the hypothesis that basal lamina composition is important for changes in cell behaviour.

Components of the basal lamina were also localised to the membrana granulosa of large antral follicles. This material (termed focimatrix) appeared as aggregates between granulosa cells and was expressed prior to granulosa cell expression of CYP11A1 and HSD3B, suggesting a role for focimatrix in the regulation of granulosa cell behaviour (Irving-Rodgers *et al.*, 2004a). Focimatrix was observed to contain collagen type IV α1 and α2, laminin chains α1, β2, and γ1, and nidogen-1 and -2, and perlecan and therefore had the composition of a basal lamina (Irving-Rodgers *et al.*, 2004a). However unlike

other basal laminas, focimatrix did not appear to surround individual cells or separate groups or sheets of cells from surrounding stroma such as occurs for endothelium or epithelium. Therefore, focimatrix does not appear to be a physical barrier to the movement of solutes within the membrana granulosa, or to provide a structural support for granulosa cells. However, focimatrix could act as a reservoir for substances such as growth factors, thereby potentiating their action. In addition, the presence of focimatrix within the membrana granulosa is likely to alter the physio-mechanical forces to which the granulosa cells are subjected, possibly resulting in a change in cell behaviour (Huang and Ingber, 1999).

The degradation of extracellular matrix at the apex of the follicle that accompanies release of the oocyte at ovulation has been extensively studied. However, specific changes to the follicular basal lamina had not previously been investigated. In the peri-ovulatory period no loss of the follicular basal lamina content of laminin chains β2 and γ1, or nidogen-1 was observed (Irving-Rodgers *et al.*, 2006b). Immediately following ovulation, perlecan is absent from the follicular basal lamina and collagen type V α1 is discontinuously distributed (Irving-Rodgers *et al.*, 2006b). Focimatrix underwent similar composition changes at ovulation and is not present in either the bovine or human corpus luteum (Irving-Rodgers and Rodgers, 2000; Irving-Rodgers *et al.*, 2004a; Irving-Rodgers *et al.*, 2006c). Versican is lost from the theca interna following ovulation, however granulosa cells show strong cytoplasmic staining (Irving-Rodgers *et al.*, 2006b). Degradation of the follicular basal lamina and focimatrix at ovulation could result in release of matrikines; regulatory peptides generated by proteolysis of extracellular matrix molecules. Matrikines derived from basal laminas have been shown to have anti-angiogenic and anti-tumour activity, and to modulate MMP activity (Maquart *et al.*, 2005). The role(s) matrikines may play in folliculogenesis has yet to be investigated.

Also included in this thesis is work identifying a new type of atresia, based upon examination of follicular morphology and re-examination of the seminal papers of (Rajakoski, 1960; Marion *et al.*, 1968). Basal atresia is characterised by initial destruction of the basal layer of granulosa cells, and it occurs in approximately 50% of atretic antral follicles ≤5 mm (Irving-Rodgers *et al.*, 2001). Macrophages are observed to infiltrate the membrana granulosa of basal atretic follicles and phagocytose dead granulosa cells, while the remaining granulosa cells are hypertrophied and resemble luteinising granulosa cells (Irving-Rodgers *et al.*, 2001). Macrophage infiltration of the membrana granulosa does not

occur in antral atresia while the follicular basal lamina was aligned with granulosa cells, suggesting that the integrity of the basal lamina is maintained by the granulosa cells (Irving-Rodgers *et al.*, 2001). Detachment of granulosa cells from the basal lamina may also occur at ovulation as part of a mechanism permitting the migration of cells from the theca into the membrana granulosa.

Electron microscopy has revealed that the follicular basal lamina remains intact during atresia while the composition does not differ between basal and antral atretic follicles (Irving-Rodgers *et al.*, 2002a). Therefore, degradation of the follicular basal lamina, which is an important part of the process of ovulation, is not a feature of atresia. Granulosa cells of basal atretic follicles not undergoing apoptosis express CYP11A1 and HSD3B, and the follicular fluid of these follicles contains significantly elevated concentrations of progesterone, and reduced androstenedione and testosterone (Irving-Rodgers *et al.*, 2003a). Death of the basal layer of granulosa cells results in detachment of the remaining antrally-situated granulosa cells. Maturation of these surviving granulosa cells into progesterone-producing cells may involve mechanisms similar to those occurring at ovulation, or during the *in vitro* luteinisation of granulosa cells. Alternatively, progesterone could be up-regulated via a mechanism involving the synthesis of focimatrix, which is also produced by basal atretic follicles (Irving-Rodgers et al., unpublished observations).

Concentrations of insulin-like growth factor binding proteins (IGFBP)-2,-4 and -5 are greater in the follicular fluid of atretic follicles in comparison to healthy follicles, but do not differ between types of healthy or atretic follicles (Irving-Rodgers *et al.*, 2003b). This suggests that the type of atresia is independent of the bioavailability of IGF. Within the theca interna, death of endothelial- and CYP11A1-positive cells is greater in basal atretic follicles in comparison to antral atretic follicles (Clark *et al.*, 2004). This is the first study to examine the cell death in differing cell populations within the thecal interna. The death of steroidogenic cells in the theca interna of basal atretic follicles is the likely cause of the reduced follicular fluid steroid levels observed in these follicles (Irving-Rodgers *et al.*, 2003a). Pro-insulin-like growth factor 3, which had previously been shown to be expressed within the theca interna of bovine follicles, is colocalised to steroidogenic cells. Pro-Insl3 is reduced significantly in atresia and this is correlated with an up regulation of HSD3B in granulosa cells (Irving-Rodgers *et al.*, 2002b).

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Basal lamina and other extracellular matrix production by granulosa cells in anchorage-independent culture

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Kirsten Scott was a summer vacation student working in our laboratory after she completed second year of a BSc at the University of Adelaide. Under the supervision of Helen Irving-Rodgers and myself she performed some morphometric analyses that contributed to this manuscript and I hereby sign to this affect.

We have not seen or heard of her since she since that time. Believing that she wanted to undertake medicine we found reference on Google to a Kirsten Scott as a medical student at the University of Melbourne. She is no longer listed as a student either at University of Adelaide or Melbourne. We approached both the Alumni of University of Adelaide and University of Melbourne in order to contact her. This was unsuccessful. We phoned her old phone number (disconnected) and we searched the Yellow pages of Australia for all general practitioners in Australia but did not find any listings for her.

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Statement of Authorship

Distribution of the α1 to α6 chains of type IV collagen in bovine follicles

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BIOLOGY OF REPRODUCTION 59, 1334–1341 (1998)

Distribution of the α1 to α6 Chains of Type IV Collagen in Bovine Follicles[1]

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ABSTRACT

During follicular development the proliferative and differentiated state of the epithelioid granulosa cells changes, and the movement of fluid across the follicular basal lamina enables the formation of an antrum. Type IV collagen is an important component of many basal laminae. Each molecule is composed of three α chains; however, six different type IV collagen chains have been identified. It is not known which of these chains are present in the follicular basal lamina and whether the type IV collagen composition of the basal lamina changes during follicular development. Therefore, we immunolocalized each of the six chains in bovine ovaries using antibodies directed to the nonconserved non-collagenous (NC) domains. Additionally, dissected follicles were digested with collagenase to release the NC domains, and the NC1 domains were then detected by standard Western immunoblot methods. The follicular basal lamina of almost all primordial and preantral follicles was positive for all type IV collagen α chains. Colocalization of type IV collagen and factor VIII-related antigen allowed for discrimination between the follicular and endothelial basal laminae. Type IV collagen α1, α2, α3, α4, and α5 chains were present within the follicular basal lamina of only a proportion of antral follicles (17 of 22, 20 of 21, 15 of 18, 14 of 28, and 12 of 23, respectively), and staining was less intense than in the preantral follicles. Staining for the α1 and α2 chains was diffusely distributed throughout the theca in regions not associated with recognized basal laminae. The specificity of this immunostaining for α1 and α2 chains of type IV collagen was confirmed by Western immunoblots. As well as being detected in the basal lamina of approximately half of the antral follicles examined, type IV collagen α4 also colocalized with 3β-hydroxysteroid dehydrogenase-immunopositive cells in the theca interna. Type IV collagen α6 was detected in the basal lamina of only one of the 16 antral follicles examined. Thus, the follicular basal lamina changes in composition during follicular development, with immunostaining levels being reduced for all type IV collagen chains and immunoreactivity for type IV collagen α6 being lost as follicle size increases. Additionally, immunoreactivity for α1 and α2 appears in the extracellular matrix of the theca as it develops.

INTRODUCTION

Development of the ovarian follicle is characterized by dramatic changes in the granulosa compartment. The number of granulosa cells doubles 21 times from the primordial follicle to the 18-mm preovulatory follicle [1]. There is also differentiation of the membrana granulosa cells, with marked differences occurring between cells at different stages of follicular development and relative to their position within the membrana granulosa [2]. The follicular basal lamina surrounds the oocyte-(antrum)-granulosa complex throughout follicular development—excluding capillaries, white blood cells, and nerve processes from the granulosa compartment—until it is degraded at ovulation. It has been postulated that the follicular basal lamina regulates the fate of the granulosa cells [3], consistent with the role of basal laminae in influencing cell proliferation, differentiation, and migration and in maintaining polarity of other cell types. The follicular basal lamina might also play a role in filtering out the larger molecules of serum during the accumulation of follicular fluid [4].

The functions of basal laminae throughout the body are very tightly related to their composition. Basal laminae are often composed of a lattice-type network of type IV collagen intertwined with a network of laminin. This structure is stabilized by the binding of entactin to the collagen and laminin and by low-affinity interactions between type IV collagen and laminin [5, 6]. Fibronectin, heparan sulfate proteoglycans, and other molecules are associated with the type IV collagen-laminin backbone. Importantly, basal laminae in different regions of the body differ in the ratio of all these components. Furthermore, each "component" is a class of several isoforms. The type IV collagen molecule is composed of three separate α chains; but six different isoforms, encoded by separate genes, have been discovered to date. Potentially, any combination of the chains might be present, although some combinations such as ($[\alpha1]_2$, α2), (α3, α4, α5), and (α5 with α6) are more common than others [7–9]. It is considered that the unique composition of each basal lamina, determined by the ratios of the different components to each other and of the specific isoforms of each component present, contributes to its specific functional properties [10]. For example, changes in the composition of a basal lamina can affect its ability to selectively filter materials [11], as occurs in the renal glomerulus.

During follicular development, there is a 317 400-fold increase in the area covered by the basal lamina from the primordial follicle to the 18-mm follicle [1]. This implies that the follicular basal lamina is continually remodeled during follicle growth. We have previously hypothesized that the composition of the follicular basal lamina changes during follicular development and atresia [12]. Consistent with this, the presence of individual chains of laminin was

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shown to alter with follicular development and atresia. Immunolocalization studies of the follicular basal lamina have demonstrated the presence of type IV collagen [13–15] but have not identified which isoforms are present. In another study utilizing Northern blotting, the expression of the α2 chain was detected in granulosa cells, and the expression of the α3 chain was detected in both the theca and granulosa cells [16]. The chains were not specifically localized to the follicular basal lamina; this is important, as there are other basal laminae in follicles, such as those of the thecal vasculature, and our studies have identified an extracellular "thecal matrix" [12]. Furthermore, none of these studies have considered changes in the collagen component of the follicular basal lamina with follicular development and atresia.

As type IV collagen is one of the most important structural components of basal laminae, in the current work we investigated the specific type IV collagen composition of the follicular basal lamina and the ways in which it changes with follicular development. Type IV collagen was immunolocalized in bovine follicles, and Western blot analyses were conducted on isolated follicles using antibodies specific to the α1, α2, α3, α4, α5, and α6 chains of type IV collagen.

MATERIALS AND METHODS

Tissues

All bovine tissue was collected at a local abattoir, within 20 min of slaughter, from cows assessed visually as being nonpregnant. A slice of up to 5 mm was cut through the center of ovaries (n = 10) to be used for immunohistochemistry, and the slices were immediately immersed in Tissue-Tek OCT embedding compound (Miles Inc., Elkhart, IN) and snap-frozen; these blocks were stored at −70°C. Tissue sections (10 μm) were cut using a CM1800 Leica (Milton Keynes, Bucks, UK) cryostat, collected on glass slides freshly treated with 0.01% poly-L-lysine hydrobromide (cat. #P-1524; Sigma Chemical Co., St. Louis, MO) or 0.01% poly L-ornithine hydrobromide (cat. #P-4638; Sigma), and stored at −20°C until use. For Western blot analyses, tissues were placed into Hepes-buffered Earle's balanced salt solution without calcium or magnesium and placed on ice during transport to the laboratory. Antral follicles (3–10 mm) were dissected from the ovaries, and all adhering surface epithelium was removed. Follicles were pooled on the basis of diameter into four categories: < 3 mm (n = 10 follicles); 3–5 mm (n = 9 follicles); 5–10 mm (n = 7 follicles); > 10 mm (n = 4 follicles). The follicular fluid was gently removed by aspiration, and the remaining tissue of each follicle was weighed (pooled weights = 30 mg, 109 mg, 304 mg, 527 mg for categories 1–4, respectively) and stored at −20°C until required. Pieces of two kidneys were similarly weighed and stored.

Antibodies

The current study used both rat monoclonal antibodies and mouse monoclonal antibodies directed against individual α chains of type IV collagen. The rat monoclonal antibodies were to each of the type IV collagen α1 to α6 chains, and they were originally raised against synthetic peptides derived from amino acid sequences of the human non-collagenous (NC) 1 domain of each chain [17]. These antibodies have been screened by ELISA with a synthetic peptide and native NC1 fractions from human, rat, or bovine renal basal laminae and further screened by indirect immunohistochemistry of human kidney. They have also been shown previously to cross-react well with bovine tissue with the exception of the type IV collagen α6 antibody, which was less reactive (unpublished results). The mouse monoclonal antibodies were to the α1, α3, and α5 chains (Wieslab AB, Lund, Sweden). The type IV collagen α1 and α3 antibodies were raised against purified bovine NC1 domains; they were characterized by immunohistochemistry on human kidney sections and by Western blot analyses and ELISA against denatured and native NC1 hexamers from human and bovine kidney, human placenta, and bovine lens capsule [18]. The α5 antibody was raised against the collagenase-resistant residue of human glomerulus basement membrane. It was screened by ELISA and SDS-PAGE to recombinant human type IV collagen chains and further characterized by indirect immunofluorescence within human kidney sections [19, 20]. This detects the bovine α5 chain of type IV collagen (J. Wieslander, personal communication). All of these specific primary antibodies were diluted to 1:100 in antibody diluent (291 mM NaCl, 7.54 mM Na_2HPO_4, 2.50 mM $NaH_2PO_4 \cdot 2H_2O$, 0.01% NaN_3, pH 7.1). The rat monoclonal antibodies were used under denaturing and nondenaturing conditions, whereas the mouse monoclonal antibodies were used only under nondenaturing conditions.

Rabbit anti-human von Willebrand factor (factor VIII-related antigen) was obtained commercially (cat. #F-3520; Sigma, 1:50 in antibody diluent). Rabbit antiserum to 3β-hydroxysteroid dehydrogenase (3β-HSD) type II was kindly donated by J.I. Mason (Clinical Biochemistry, University of Edinburgh; 1:50 in antibody diluent); this antiserum was raised against human placental type I 3β-HSD [21, 22], but it cross-reacts with type II 3β-HSD [21–23], as type I 3β-HSD is 95% homologous to the type II 3β-HSD that is expressed in gonads and the adrenal. Normal sera used in this study for control sections were normal rabbit serum (cat. #R9133; Sigma), normal mouse serum (cat. #M5905; Sigma), normal rat serum (cat. #R9759; Sigma), and normal donkey serum (cat. #D9663; Sigma).

Fluorophore-conjugated secondary antibodies used in this study were from Jackson ImmunoResearch Laboratories (West Grove, PA) and included Cy3-conjugated AffiniPure donkey anti-rat IgG (cat. #712–165–153), Cy3-conjugated AffiniPure donkey anti-mouse IgG (cat. #715–165–150), and fluorescein (DTAF)-conjugated AffiniPure donkey anti-rabbit IgG (cat. #711–095–152), each at a dilution of 1:100 in antibody diluent. For further amplification, secondary antisera used were biotin-SP-conjugated AffiniPure F $(ab')_2$ fragment donkey anti-mouse IgG (H&L) (cat. #715–066–151) or biotin-SP-conjugated AffiniPure F $(ab')_2$ fragment donkey anti-rabbit IgG (cat. #711–066–152), in conjunction with Cy3-conjugated streptavidin (cat. #016–160–084) or fluorescein (FITC)-conjugated streptavidin (cat. #016–090–084).

Immunohistochemistry

Cryosections were dried at room temperature under vacuum (30 min) prior to fixation in acetone (5 min). After washing in hypertonic PBS solution (hPBS; 274 mM NaCl, 5.37 mM KCl, 8.10 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.2; three times, 5 min each), some sections were denatured with acidified urea solution (6 M urea, 0.1 M glycine, 0.1 M HCl, pH 3.5) [24] for 30 min and then washed again (three times, 5 min each in hPBS). The denaturation step

was omitted when the mouse monoclonal antibodies were used. Nonspecific staining was inhibited by the preincubation of sections with 10% normal donkey serum in hPBS (30 min). Sections were incubated overnight with either specific primary antiserum or nonimmune control serum. Sections were then washed (four times, 5 min each in hPBS) and incubated with the appropriate fluorophore-conjugated secondary antibodies for 2 h. To achieve further amplification of the immunostaining, in some instances sections were instead incubated with secondary antisera conjugated with biotin-SP (diluted with antibody diluent) for 2 h; they were then washed (four times, 10 min each in hPBS) and further incubated with Cy3- or FITC-conjugated streptavidin (1:100 diluted in antibody diluent) for 1 h. All incubations were carried out at room temperature in a humidified environment. After final washing (four times, 10 min each in hPBS), sections were mounted with buffered glycerol (0.167 M Na_2CO_3 in 67% glycerol, pH 8.6).

The protocol for dual labeling was essentially the same as for single labeling as described above, except that the sections were incubated concurrently with two primary antibodies of different species and were subsequently incubated concurrently with the two appropriate secondary antisera to enable discrimination between the primary antibodies. These were conjugated either with different fluorophores or with one conjugated to biotin-SP to allow further amplification by incubation with streptavidin conjugated to either Cy3 or FITC. Control sections for dual staining were as follows: the relevant polyclonal primary antibody and anti-mouse secondary antibody; the relevant mouse monoclonal primary antibody and anti-rabbit secondary antibody; the relevant rat monoclonal primary antibody and anti-rabbit secondary antibody; normal rabbit serum primary antibody and anti-rabbit secondary antibody; normal mouse serum primary antibody and anti-mouse secondary antibody; normal rat serum primary antibody and anti-rat secondary antibody; both normal rabbit serum and normal mouse serum primary antibodies and both anti-rabbit and anti-mouse secondary antibodies; both normal rabbit serum and normal rat serum primary antibodies and both anti-rabbit and anti-rat secondary antibodies.

Observations and Photography

Immunostaining was visualized using either an Olympus (Tokyo, Japan) AX70 fluorescent microscope, with the selective NG filter (575–615 emission) for detecting the Cy3 fluorophore and an NIB filter (515–545 emission) for DTAF fluorophore, or an Olympus Vanox AHBT3 fluorescence microscope using the IB filter (490 emission) to excite the DTAF fluorophore and the G filter (546 emission) to excite the Cy3 fluorophore. Images were captured using the AX70 microscope via a video linkage to an Apple Macintosh (Cupertino, CA) computer utilizing the program NIH Image 1.60b7 or were photographed using the Vanox microscope with Olympus C35AD-4 camera attachment. Photographs used in all the illustrations here were taken using Kodak (Eastman Kodak, Rochester, NY) T-Max 400 black-and-white film.

Western Blotting of Type IV Collagen NC1 Domains

To release the NC1 domain of type IV collagen, the tissues were homogenized and then digested at 37°C for 24 h with collagenase (50 mg/ml, CLS1, 238 U/mg; Worthington Biochemical Corporation, Freehold, NJ) in Hepes-buffered (50 mM; pH 7.5) calcium chloride solution (10 mM) containing protease inhibitors (4 mM *N*-ethylmalemide, 1 mM PMSF, 5 mM benzamidine hydrochloride, 25 mM ϵ-aminocaproic acid [25–27]); 2 ml of collagenase solution was used per gram of tissue. The digestion was terminated by the addition of EDTA (to a final concentration of 25 mM). Samples were then centrifuged (10000 × *g*; 10 min), and the supernatants were stored at −20°C until required.

Samples were denatured by the addition of an equal volume of 0.05 M Tris buffer, pH 6.8, containing 2% SDS, 10% glycerol, 2 mM EDTA, 0.005% bromophenol blue, 0.1 M 4,4′diaminodiphenyl sulfone, 12 M urea solution, at 100°C for 5 min. The samples were subsequently electrophoresed on 12.5% SDS-polyacrylamide gels, and the separated proteins were electroblotted onto nitrocellulose-coated nylon membrane (Hybond-C; Amersham, Castle Hill, NSW, Australia) at 175 mA overnight (transfer buffer: 20% methanol, 20 mM Tris, 150 mM glycine). A lane containing molecular weight markers (cat. #17–0446–01; Pharmacia Biotech, North Ryde, NSW, Australia) was removed and stained with Napthol blue black, and the remaining lanes were developed in a manner adapted from Towbin et al. [28] as reported previously [29]. Nonspecific binding sites were blocked by incubating the membrane (buffer A: 10 mM Tris pH 7.4, 150 mM NaCl, 5% BSA, 0.2% Nonidet P-40) for 1 h at 37°C in a shaking incubator. Each membrane was subsequently incubated with one of the antibodies directed against a type IV collagen α chain diluted 1:2500 in buffer A for 2.5 h at room temperature. Each blot was then washed (three times, 15 min each) in buffer A without BSA, but with the addition of 0.25% deoxycholic acid and 0.1% SDS, followed by a further rinse (once, 10 min) in a solution of 10 mM Tris and 0.15 M NaCl. The appropriate secondary antisera (goat anti-rat IgG; cat. #R5005; Sigma) and goat anti-mouse IgG (cat. #M8645; Sigma) were iodinated using the lactoperoxidase method as described previously [29, 30] and were diluted to 1×10^6 cpm/ml in buffer A. Blots were incubated with the secondary antisera for 45 min at room temperature and then washed as described above and air dried. Blots were subjected to autoradiography (Kodak XAR film) or analyzed using a Molecular Dynamics (Sunnyvale, CA) PhosphoImager and ImageQuant (Molecular Dynamics) software, version 4.1.

RESULTS

There are many structures in the ovary that contain basal laminae, but comments in this paper will be confined to ovarian follicles and their basal laminae. Specific staining for each of the individual type IV collagen chains was observed in basal laminae associated with ovarian follicles (summarized in Table 1; described in more detail below). All illustrations in this paper are of staining produced using the rat monoclonal antibodies unless otherwise specified. Only one antibody was available for type IV collagen α6, and this antibody cross-reacted with bovine α6 only weakly, as shown previously (Y. Sado, unpublished results). Dual labeling was also carried out to identify cell types such as endothelial cells (positive for factor VIII-related antigen) and steroidogenic thecal cells (positive for 3β-HSD). A comprehensive series of controls was carried out to ensure that no nonspecific staining or autofluorescence was misinterpreted as positive staining.

Table 1. Summary staining patterns produced by antibodies specific to the individual type IV collagen chains.*

Tissue	α1 (n = 6)	α2 (n = 6)	α3 (n = 5)	α4 (n = 9)	α5 (n = 6)	α6 (n = 6)
Follicular basal lamina						
Primordial and preantral	+++	+++	+++	+++	+++	+
Antral	+++	+++	+/−	+/−	+/−	−
Thecal matrix	+++	+++	−	−	−	−

* n, The number of ovaries examined for immunostaining. Intensity of staining: −, none; +, weak; ++, moderately intense; +++, very intense; comparison of intensity should only be within a column (i.e. between follicles of different classes) and not within a row (i.e. between collagen chains), as different antibodies have been used.

Follicular Basal Laminae of Primordial and Preantral Follicles

The follicular basal laminae of almost all primordial and small preantral follicles were positive for type IV collagen α1, α2, α3, α4, and α5 (Fig. 1) and stained weakly for α6 (not shown). Only a very few primordial follicles did not appear to contain detectable α3, α4, α5, or α6 in their follicular basal laminae. No staining with antibodies to factor VIII-related antigen was observed in the region of the ovary containing the primordial follicles, confirming that this region of the ovary is avascular [1].

Type IV Collagen α1 and α2 in Antral Follicles

Antibodies to the α1 and α2 chains of type IV collagen in antral follicles produced similar staining patterns (Fig. 2). In all of the follicles examined for α1(IV) staining and all except one of the antral follicles examined for α2(IV) staining, intense uneven staining was observed extracellularly throughout the theca interna (Fig. 2, a–c and e–g). Its close proximity to the follicular basal lamina made staining of the follicular basal lamina difficult to interpret. Of the 22 follicles examined for α1(IV) staining, 17 were considered to have basal laminae that stained positively; of these, 14 stained with intensity equal to that for the thecal matrix, 2 stained more intensely, and 1 stained less intensely. In 5 of the 22 follicles examined for α1(IV), we were not confident in classifying the basal lamina as staining positively; if there was staining, it was at the same intensity as the thecal staining. Of the 21 follicles examined for α2(IV) staining, 20 were considered to have basal laminae that stained positively, and 1 did not stain for this chain. Of the basal laminae that did stain, 18 stained with intensity equal to that in the theca, although staining was often uneven in intensity along the basal lamina. The basal lamina of two follicles stained more intensely than the theca.

Some of the extracellular staining observed in the theca interna was clearly associated with the endothelial basal laminae, but there were more areas staining positively for the α1 and α2 chains of collagen type IV than could be accounted for by the vasculature alone (compare Fig. 2, c with d and g with h).

Type IV Collagen α3, α4, α5, and α6 in Antral Follicles

Although the follicular basal laminae of some antral follicles stained for individual collagen chains (Fig. 3), this was not the case for all follicles; and the intensity of staining was much reduced compared to that of primordial and small preantral follicles. Staining was present in the follicular basal lamina of 15 of 18 follicles examined for α3 staining but was discontinuous along the basal lamina and/or punctate. The follicular basal laminae of only 14 of 28 follicles examined for α4(IV) staining were positive. However, staining for α4(IV) was faintly present intracellularly in cells in the theca interna (Fig. 3g) of all except 2 follicles. Colocalization studies with anti-3β-HSD (Fig. 3h) showed that the cells positive for α4(IV) also stained for 3β-HSD. The follicular basal lamina of 12 of 23 follicles examined stained positively for α5(IV); the remainder had no detectable staining. Of 16 follicles examined, 15 contained no detectable α6(IV) in their follicular basal laminae,

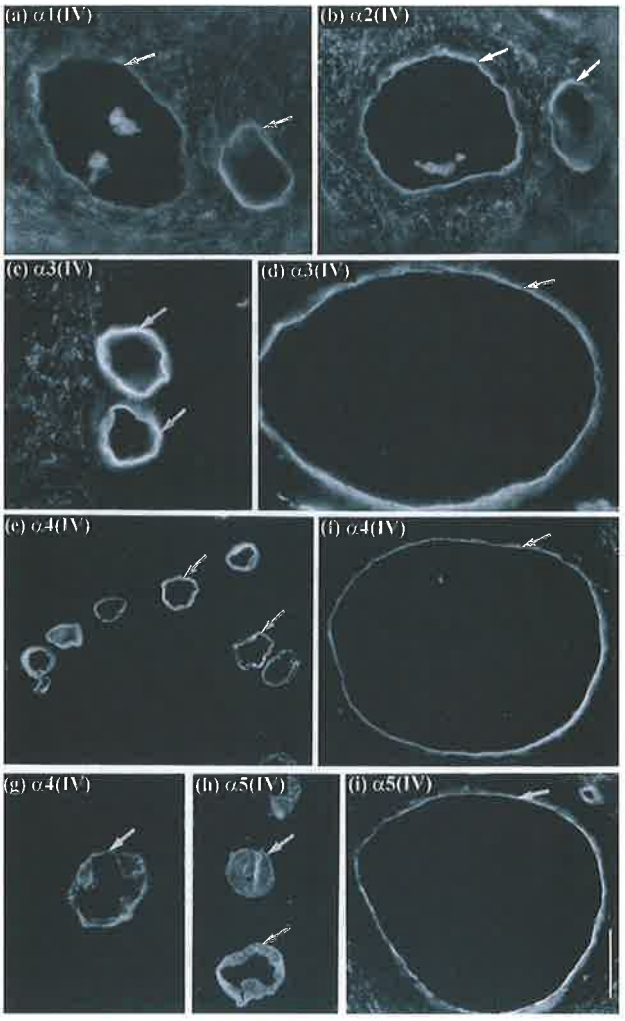

FIG. 1. Immunofluorescent localization of individual α chains of type IV collagen to preantral follicles (including primordial follicles). Primary antibodies used were directed against: **a**) the α1 chain, **b**) the α2 chain, **c, d**) the α3 chain (mouse monoclonal), **e–g**) the α4 chain, **h, i**) the α5 chain. Arrows indicate the position of the follicular basal laminae. Bar (lower right in **i**) = 50 μm in **a–e, g, h**; 100 μm in **f, i**.

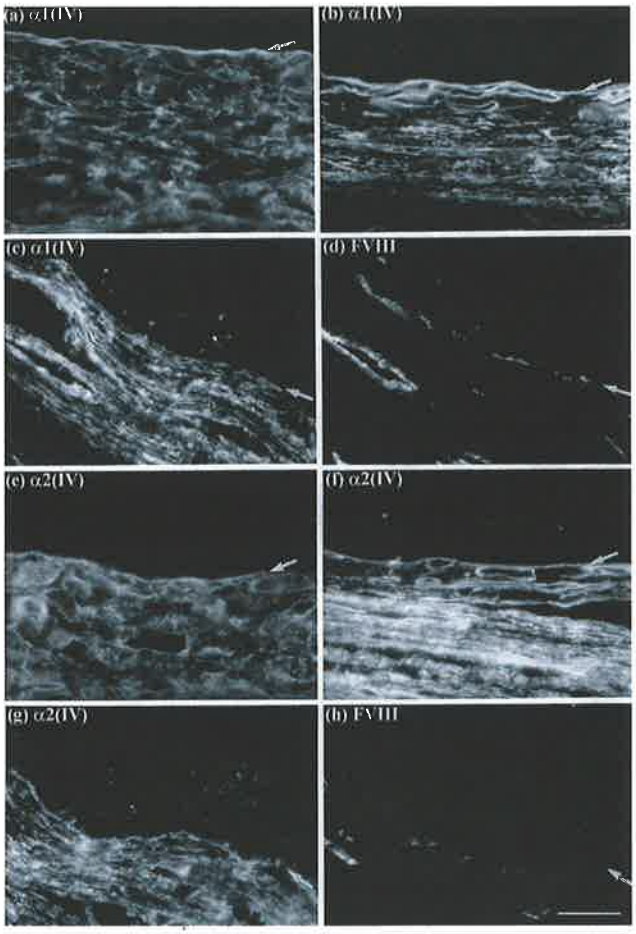

FIG. 2. Immunofluorescent localization of type IV collagen α1 and α2 chains to antral follicles, and colocalization of factor VIII-related antigen. In each photograph, the location of the basal lamina is indicated by arrows, the membrana granulosa lies above the basal lamina and the theca below it, and the follicular antrum is uppermost. **a–c**) Positive staining for type IV collagen α1 in the follicular basal lamina as well as widespread staining in the theca are shown. Counterstaining of the section shown in **c** for factor VIII-related antigen, shown in **d**, demonstrated that the type IV collagen α1 staining is more widespread than the thecal vasculature. Similarly, staining for type IV collagen α2 (**e–g**) is present in the follicular basal lamina and widespread in the theca. Counterstaining of the section shown in **g** for factor VIII-related antigen, shown in **h**, demonstrated that the type IV collagen α2 staining is more widespread than the thecal vasculature. Follicle sizes: **a**, 3 mm; **b, f** (same follicle), 10 mm; **c, d** (same follicle), 5 mm; **e**, 9 mm; **g, h** (same follicle), 8 mm. Arrowheads indicate the vasculature. Bar = 50 μm.

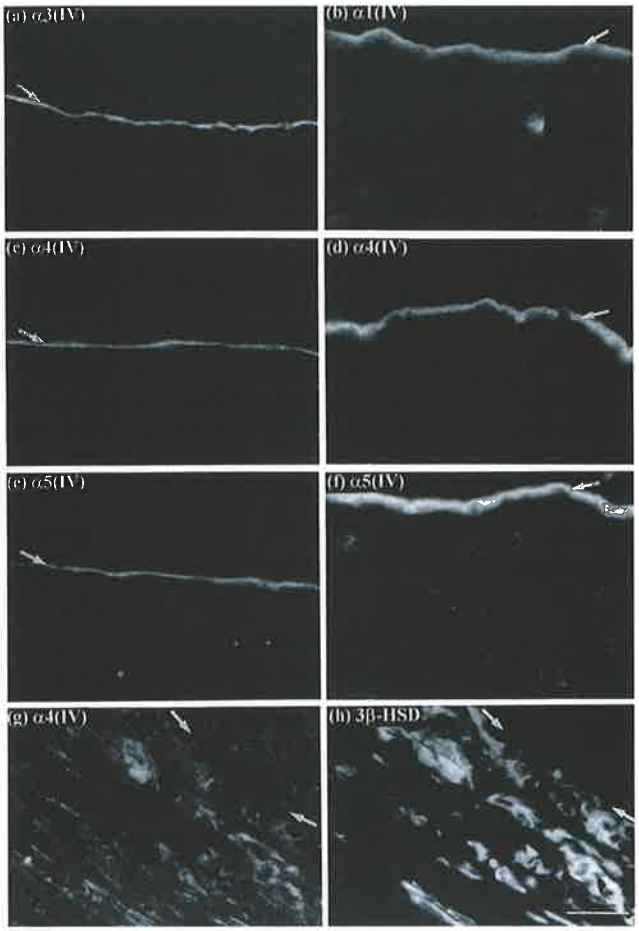

FIG. 3. Immunofluorescent localization of individual type IV collagen α chains to antral follicles, and colocalization of type IV collagen α4 with 3β-HSD. In each photograph, the location of the follicular basal lamina is indicated by arrows, the membrana granulosa lies above the basal lamina and the theca below it, and the follicular antrum is uppermost. **a–f**) Show positive staining for type IV collagen α chains in the follicular basal lamina. **a, c, e**) Healthy follicles; **b, d, f**) all are of the same atretic follicle. **g**) Staining of the theca, using the primary antibodies directed against type IV collagen α4; **h**) the same section counterstained for 3β-HSD. Arrowheads indicate the vasculature. Bar = 50 μm.

and staining was punctate in the follicular basal lamina of the other follicle.

Immunostaining of the Thecal Vasculature

The endothelial cell marker, factor VIII-related antigen, was colocalized with each of the type IV collagen α chains. In antral follicles, the arterioles and venules were located mainly at the extremities of the theca interna and in the externa, and capillaries were common in the theca interna. Intense fluorescence was observed in the subendothelial basal lamina of arterioles, venules, and capillaries with antibodies to α1 and α2, and the basal laminae of smooth muscle cells of arterioles also contained α1 and α2. In the thecal layers, no fluorescence was observed in the subendothelial cell basal laminae or the basal laminae of the vascular smooth muscle cells using antibodies to α3, α4, α5, or α6.

Western Blotting

In basal laminae, type IV collagen molecules assemble into a meshwork via disulfide bonds and by other nonreducible covalent cross-linking bonds [31]; therefore we digested the collagenous domains of type IV collagen with collagenase, releasing the NC1 domains. Each NC1 domain is a trimer of three chains, and each NC1 domain is bonded to the NC1 domain of another molecule of type IV collagen. Upon reduction, these hexameric structures yield monomers (approximately 25 kDa) and dimers (approximately 50–60 kDa) of single peptide chains from the NC1 domains [31]. The dimers are due to nonreducible, covalent cross-links [31]. Bovine kidney was chosen as a positive control as this tissue had previously been reported to contain four type IV collagen chains: α1, α2, α3, and α4 [27].

All of the rat monoclonal antibodies to type IV collagen α1 to α5 chains reacted well with bovine kidney (shown

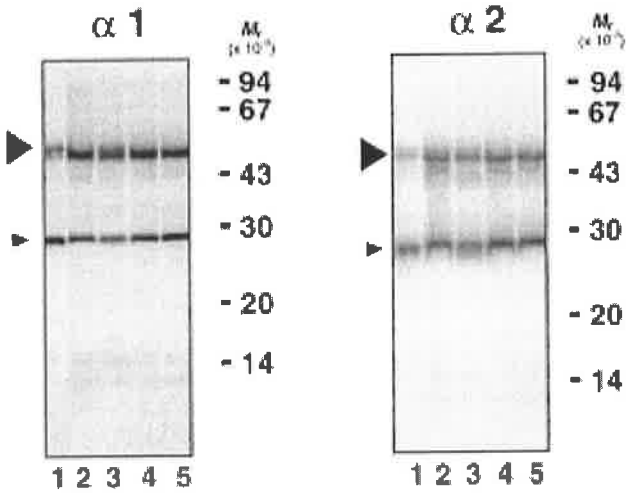

FIG. 4. Western blot analyses of type IV collagen α1 and α2. Samples were digested with collagenase, and monomers (small arrowhead) and dimers (large arrowhead) were detected. Lane 1, bovine kidney; lane 2, follicles < 3 mm; lane 3, follicles 3–5 mm; lane 4, follicles 5–10 mm; lane 5, follicles > 10 mm.

only for α1 and α2; Fig. 4). The mouse monoclonal antibodies were not tested. Strong specific bands of approximately equal intensity were observed at 54 and 28 kDa for both α1(IV) and α2(IV). Staining for α3(IV) (53 kDa and a stronger band at 28 kDa), α4(IV) (54, 28, and 25 kDa), and α5(IV) (54 kDa and a strong band at 28 kDa) was observed. The type IV collagen α6(IV) antibody barely detected any positive bands. The presence of two monomers detected using type IV collagen α4 antibody is likely to be due to interchain, nonreducible, covalent cross-links as has been observed previously [25]. The rat monoclonal antibodies to the α1 to α5 chains also reacted with nonreduced NC1 domains (not shown) but more strongly with denatured products.

Western blot analyses of dissected antral follicles showed that α1(IV) and α2(IV) were readily detectable at levels similar to that in kidney (Fig. 4). In comparison, the levels of the other type IV collagen chains α3, α4, or α5 were far lower in the follicles. This is consistent with the immunostaining patterns observed; by immunostaining, a high level of staining for α1 and α2 was present throughout the theca, whereas the α3 and α5 chains were observed only in the follicular basal laminae, and the thecal intracellular staining for α4 was very weak.

DISCUSSION

This is the first study to localize any of the six α chains of type IV collagen within the follicular basal lamina of any species. The follicular basal lamina of primordial and small preantral follicles contained all of the α chains of type IV collagen. In antral follicles, there was widespread extracellular staining throughout the theca interna for the α1 and α2 chains, often making it impossible to determine whether the follicular basal lamina itself contained these chains. The α3, α4, and α5 chains were not detected in some antral follicles. When it was present, the level of staining was less intense than at earlier stages of follicular development. The α6 chain was not detected in the follicular basal lamina of any antral follicles. In the light of the current study and a previous study of individual laminin chains in the ovarian follicle [12], it is clear that important changes take place during follicular development, both in the composition of the follicular basal lamina and in the development of a thecal matrix in the mesenchyme, adjacent to the follicular basal lamina.

There are currently 15 different classes of collagen; however, only type IV collagen is found predominantly in basal laminae. Each type IV collagen molecule contains three α chains wound into a triple helix. In all, six different α chains have been discovered, and a type IV collagen molecule may contain any combination of these chains although some combinations, such as $\alpha1(IV)_2$ α2(IV), are more common than others [8]. Here, we have demonstrated for the first time that the presence of individual type IV collagen α chains in the follicular basal lamina alters with follicular development. This is consistent with studies in other developing tissues. For example, during development of the kidney, collagen α1(IV) and α2(IV) are lost from the glomerular basement membrane, whereas collagen α3(IV), α4(IV), and α5(IV) are accumulated [11]. Additionally, studies of the type IV collagen α1 to α5 chains in murine seminiferous tubules have shown a temporal expression pattern of these chains during tubule development [32, 33]. Similar changes in individual chains of laminin within basal laminae of developing tissues are correlated with functional changes ([11]; see [12]). Therefore it is likely that the changing expression patterns reflect specific functional roles for individual type IV collagen chains. Such functional roles, though, have yet to be determined for any of these chains. Additionally, it remains to be seen which α chains combine to form type IV collagen molecules, how the type IV collagen molecules are combined in the follicular basal lamina (see [9]), and whether different arrangements of the molecules affect the function of the follicular basal lamina.

In the current study, follicular development was associated with a decrease in intensity or a complete loss of type IV collagen chains from the follicular basal lamina. Additionally, staining for α1(IV) and α2(IV) was frequently unevenly distributed along the follicular basal lamina of antral follicles, being present in some areas and absent in others. Conversely, in our previous study of laminin chains, the intensity of staining increased with increasing follicle size [12]. Thus there appears to be a transition from a type IV collagen-rich to a laminin-rich follicular basal lamina with increasing follicle size. Type IV collagen molecules can be covalently cross-linked to form a much more rigid network than the laminin network, and absence or discontinuity of the type IV collagen network might reflect the need for active basal lamina expansion during follicular development. It has been calculated that the surface area of the bovine follicle, the area covered by the follicular basal lamina, increases some 317 400-fold or doubles 19 times in developing from the primordial follicle stage to the 18-mm preovulatory-sized follicle [1]. Thus there must be continued synthesis and substantial remodeling of the follicular basal lamina. Incorporation of a new segment into the follicular basal lamina would require that this network be broken, and segments of the basal lamina lacking type IV collagen would easily be able to expand by incorporating new components. Follicles that did not contain any of the type IV collagen α chains might be those that are actively growing and require rapid basal lamina expansion, as a basal lamina containing no type IV collagen would be easily remodeled. Basal laminae lacking type IV collagen have been

found in a range of tissues during their development [34–36] and in a basal lamina produced in vitro [37].

The ovarian cell type that is responsible for producing the components of the follicular basal lamina remains controversial, although there is mounting evidence to suggest that the granulosa cells make a substantial, if not the sole, contribution to its synthesis. In other systems it is predominantly epithelial rather than stromal cells that synthesize basal lamina components, although in some tissues both cell types make a contribution [38]. In the ovarian follicle, it is the granulosa cells that are present throughout follicular development associated with the follicular basal lamina. The theca, on the other hand, only differentiates in bovine follicles at approximately the time of antrum formation, well after the basal lamina of the follicle has started increasing. Cultured granulosa cells have been shown to produce a basal lamina [39] that structurally resembles the follicular basal lamina [40]. By Northern blot analysis, granulosa cells were shown to express the α3 chain of type IV collagen and the γ1 (old nomenclature: B2) chain of laminin [16]. In a previous immunoelectron microscopy study [41], laminin was localized to Call-Exner bodies, which are ultrastructurally similar to basal lamina and have been observed within the membrana granulosa of antral follicles in vivo (cow: unpublished results; rabbit: [42]). Conversely, in some systems, mesenchymal production of basal lamina components have been shown [43], suggesting that cells of the theca might play a role in production of the follicular basal lamina. The thecal compartment of antral follicles has been shown by Northern analyses to express the α2 and α3 chains of type IV collagen and the laminin β1 and γ1 chains [16], and our observations of the α1 and α2 chains of type IV collagen (current study) and laminin γ1 [12] in the 'thecal matrix' suggest that cells in the thecal layer are capable of producing basal lamina components. However, the thecal expression of these molecules is not necessarily a contribution to the follicular basal lamina. This was particularly demonstrated for the laminin β1 chain, which was immunolocalized to the thecal vasculature but undetectable in the follicular basal lamina at most stages of follicular development [12]. Nevertheless, it remains possible that in vivo, the follicular basal lamina receives a contribution from both the granulosa cells and the stroma or thecal cells.

Type IV collagen has been reported to be found in basal laminae. However, in the current immunolocalization study, relatively large amounts of α1(IV) and α2(IV) were found within the extracellular regions of the theca interna of antral follicles. These large diffuse areas of staining were not associated with the follicular basal lamina or the subendothelial or smooth muscle basal laminae of the vasculature. The staining was not likely to be nonspecific, as Western immunoblot analyses of dissected follicles using the same antibodies produced bands only at the expected molecular weights for type IV collagen α chains, namely NC1 dimers (54 kDa) and monomers (28 kDa). Furthermore, staining patterns similar to those observed in the current study were reported by Bagavandoss et al. [13] using an antibody to the EHS sarcoma type IV collagen; α1(IV) and α2(IV) are found in the EHS sarcoma. Widespread staining in the theca interna has also been observed for laminin using antibodies directed against either the EHS laminin [12, 13, 41, 42], which contains the α1, β2, and γ1 laminin chains, or against the γ1 chain alone [12]. It is possible that the collagen α1(IV) and α2(IV) chains and the laminin γ1 chain are present as free molecules in the theca interna, as there are no recognized conventional basal laminae in these regions. However, it is also possible that this staining is associated with small fragments of basal lamina-like electron-dense material found extracellularly in the theca. This material has been observed at the electron microscope level in the theca of sheep [44], rats [41], and cows (see Fig. 1 in [29]). The origins and functions of this thecal matrix are not known.

From our studies, we conclude that the composition of the follicular basal lamina changes during the course of follicle growth and development. We believe, but have still to prove, that these changes reflect changes in the function of the follicular basal lamina, particularly in filtration properties and their ability to regulate functions of granulosa cells. We have also to determine for type IV collagen (and also laminin) how the individual chains are combined in each molecule and whether this also changes with follicle development. The large amounts of α1(IV) and α2(IV) present in the matrix of the thecal layer of the follicle are not associated with the follicular basal lamina or the basal laminae of the vasculature. The role of this matrix in the differentiation of thecal cells or other functions has still to be determined.

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Statement of Authorship

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Analysed samples and interpreted data.
Wrote manuscript.

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Assisted with experimental design and data interpretation.
Provided laboratory facilities and most reagents.
Assisted with writing the manuscript and acted as corresponding author.

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Differential localization of laminin chains in bovine follicles

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The composition of a basal lamina markedly affects its ability to filter material and affects the fate of adjacent epithelial cells. Therefore, basal laminae differ in composition with tissue development, and between different tissues in the body. Laminins are a component of basal laminae and consist of one α, one β and one γ chain, of which there are at least five, three and two isoforms, respectively. This is the first study to immunolocalize a range of these individual laminin chains (α1, α2, β1, β2, γ1) in ovarian follicles. Frozen sections of bovine ovaries ($n = 6$) were immunostained using specific antisera to laminin chains and factor VIII-related antigen (to identify endothelial cells). Secondary antisera were labelled with one of two different fluorochromes (DTAF and Cy3), and dual localization of laminin chains and factor VIII-related antigen was performed. The α1, β2 and γ1 chains were consistently localized to the follicular basal lamina in all healthy follicles. Staining was less intense in the atretic antral follicles. Conversely, α2 and β1 were rarely present in the follicular basal laminae of healthy antral follicles. Two of nine healthy antral follicles observed stained weakly for α2 in their basal lamina, and β1 was present at low concentrations in growing preantral follicles. In atretic antral follicles, the follicular basal lamina stained positively for α1, α2, and β2 but no β1 was detected and the γ1 staining was less intense than in healthy follicles. Antisera to Englebreth Holm-Swarm tumour laminin stained basal laminae of all follicles. In the theca of antral follicles, β1 and β2 chains were both present in the vasculature. Staining for the γ1 chain was present in the thecal vasculature and generally throughout the theca of healthy and atretic antral follicles. Therefore, the composition of the follicular basal lamina alters during development and atresia, and potentially plays a role in the changing identity of the granulosa cells and the accumulation of antral follicular fluid.

Introduction

Basal laminae are specialized sheets of extracellular matrix that separate epithelial cell layers from underlying mesenchyme in organs throughout the body, including the ovary. They influence epithelial cell migration, proliferation and differentiation, and can selectively retard the through-passage of material. Basal laminae are a lattice-type network of collagen IV intertwined with a network of laminin. This structure is stabilized by the binding of entactin to the collagen and laminin, and by low-affinity interactions between collagen IV and laminin (Yurchenco and Schittny, 1990; Paulsson, 1992). Fibronectin, heparan sulfate proteoglycans (HSPGs) and other molecules are associated with the collagen IV–laminin backbone. It is significant that basal laminae in different regions of the body differ in the ratio of all these components. Furthermore, each 'component' is in fact a class of several components. Thus, each collagen IV molecule is composed of three α chains, but six different types of α chain have been discovered to date, and any combination of these might be present (Hay, 1991; Zhou *et al.*, 1994). Similarly, each laminin molecule is composed of one α (A in the old nomenclature), one β (B1 in the old nomenclature) and one γ (B2 in the old nomenclature) chain (Burgeson *et al.*, 1994), yet five different α chains, three β chains and two γ chains have been discovered. Owing to alternative splicing of mRNA, at least 20 different isoforms of fibronectin exist. The unique composition of each basal lamina is considered to contribute to its specific functional properties (Engvall, 1993).

Numerous studies *in vitro* have shown that cell morphology is altered according to the type of extracellular matrix component on which the cells are cultured (Watt, 1986). Thus, alterations to the basal lamina composition will affect the fate of the associated cells. The composition of basal laminae also affects their ability to selectively filter materials. For example, in normal neonatal mice, laminin β1 is replaced by β2 in the kidney glomerular basement membrane as the kidney develops. However, mice with a null mutation in the laminin β2 gene continued with β1, but then failed to retard the through-passage of plasma proteins despite having a structurally intact glomerular basement membrane; these mice died of proteinuria within one month of birth (Noakes *et al.*, 1995). In the ovary, the membrana granulosa of each ovarian follicle is enveloped by a follicular basal lamina, which separates it from the surrounding stromal elements in primordial follicles (van Wezel

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and Rodgers, 1996) or theca in antral follicles (Gosden *et al.*, 1988; Luck, 1994). The follicular basal lamina is believed to play a role in influencing granulosa cell proliferation and differentiation (Amsterdam *et al.*, 1989; Richardson *et al.*, 1992; Luck, 1994). In addition, in healthy follicles, it excludes capillaries, white blood cells and nerve processes from the granulosa compartment until ovulation, at which time it is degraded. It probably also has a role retarding entry of larger molecular weight plasma proteins and molecules, for example low density lipoproteins (LDL), into the follicular antrum (Andersen *et al.*, 1976). Conversely, it may trap in the follicular fluid large molecules, for example some proteoglycans that are synthesized by granulosa cells and oocytes.

The bovine follicle is estimated to double in surface area nineteen times during development, implying that continual remodelling of the follicular basal lamina occurs (van Wezel and Rodgers, 1996). The composition of the follicular basal lamina is hypothesized to alter during follicular development, particularly at the time follicular fluid accumulates to form an antrum, and during follicular atresia. Immunolocalization studies have demonstrated the presence of collagen IV (Bagavandoss *et al.*, 1983; Kaneko *et al.*, 1984; Palotie *et al.*, 1984), laminin (Wordinger *et al.*, 1983; Bagavandoss *et al.*, 1983; Palotie *et al.*, 1984; Leu *et al.*, 1986; Christiane *et al.*, 1988; Yoshinaga-Hirabayashi *et al.*, 1990; Leardkamolkarn and Abrahamson, 1992; Fröjdman *et al.*, 1995), and fibronectin (Bagavandoss *et al.*, 1983; Yoshimura *et al.*, 1991; Figueiredo *et al.*, 1995) in the follicular basal laminae of antral follicles. However, none of these studies has sought to differentiate between the different isoforms of any of these components, except one study which compared the localization of α1 with that of β1-γ1 laminin in the ovary of the mouse fetus (Fröjdman *et al.*, 1995). Other studies using western and northern blotting identified the expression of a few of the subtypes of collagen and laminin (Zhao and Luck, 1995; Iivanainen *et al.*, 1995) but did not specifically localize these components to the follicular basal lamina; this is important since there are other basal laminae in follicles, such as those of the vasculature.

The laminin components in bovine follicles were immunolocalized using antibodies specific to the α1, α1, β1, β2 or γ1 chains of laminin to identify a range of individual laminin chains present in the follicular basal lamina.

Materials and Methods

Tissues

One ovary was obtained from each of six nonpregnant cows within 20 min of slaughter at a local abattoir. A slice of up to 5 mm was cut through the centre of the ovaries and they were immediately immersed in Tissue-Tek OCT Compound (Miles Inc, Elkhart, IN) and snap-frozen; these blocks were stored at − 70°C. Tissue sections (10 μm) were cut using a CM1800 Leica cryostat, collected on glass slides freshly treated with poly-L-lysine (Sigma Chemical Co., St. Louis, MO) and stored at − 20°C until use. Sections from each ovary were also fixed in acetone for 30 min and stained with haematoxylin and eosin by standard methods to facilitate assessment of follicular morphology. At least one section from each ovary was used for the immunolocalization of each laminin chain, and as a negative control.

Classification of follicle health

The term 'preantral' includes primordial follicles with one layer of flattened or flattened and cuboidal cells (van Wezel and Rodgers, 1996) and growing follicles with variable numbers of granulosa cell layers but without a visible antrum. On the basis of our previous observations using well preserved material (Faddy and Gosden, 1995), it would be expected that a negligible number of preantral follicles would be atretic. In the present study, even though the morphology of preantral follicles in frozen sections was only sufficient to identify changes taking place during moderate to severe atresia, most follicles appeared to be healthy. In antral follicles, a progression of marked changes to the membrana granulosa takes place during atresia (Rajakoski, 1960; Roy and Terranova, 1988). Therefore, the health of these antral follicles was classified according to the integrity of the membrana granulosa, and the presence or absence of cellular debris in the antrum. Antral follicles were classified as (1) 'healthy' if their membrana granulosa was intact and uniform in width, and if there was little or no cellular debris; or (2) 'atretic' if the membrana granulosa was not uniform in width, giving it a tattered appearance, or if there was a large amount of cellular debris. Follicles at more advanced stages of regression, such as those lacking granulosa cells and follicular scars, were not considered in this study.

Antibodies

Individual laminin chains were immunolocalized using rabbit polyclonal antisera raised against mouse recombinant laminin α1 chain (no. 317; Durbeej *et al.*, 1997; 1/50–1/100 dilution), or against human α2 chain (no. 321 in preliminary studies, no. M4 in comprehensive studies; donated by M. Paulsson; 1/50 dilution). Antiserum no. 321 has been described by Paulsson and Saladin (1989). Antiserum no.M4 was raised by immunizing a rabbit with the 300 kDa α2 band from a preparative gel, after purification of human placental laminin as described by Lindblom *et al.* (1994). This antiserum was found to be specific for α2 when tested by immunoblot analyses (M. Paulsson, personal communication), and in our preliminary studies no. 321 and no. M4 produced equivalent staining patterns. Mouse monoclonal antibodies were those raised against human laminin β1 chain (Cat. no. A004; Gibco BRL, Gaithersberg, MD; 1/500 dilution), bovine laminin β2 chain (no. C4, 1/100 dilution; Sanes and Chiu, 1983; Hunter *et al.*, 1989), or human laminin γ1 chain (Cat. no. A005; Gibco; 1/500 dilution). In addition, rabbit polyclonal antiserum (Cat. no. L9393; Sigma Chemical Co., St Louis, MO; 1/100 dilution) raised against laminin-1 isolated from Englebreth Holm-Swarm (EHS) tumours and, therefore, detecting α1, β1 and γ1 chains, was used.

Non-laminin antibodies used in this study were rabbit anti-human von Willebrand factor (factor VIII-related antigen) IgG (Cat. no. F-3520; Sigma Chemical Co.; 1/100 dilution), for

detection of endothelial cells (van Wezel and Rodgers, 1996), and mouse monoclonal anti-bovine synaptophysin (Cat. no. 902 314; Boehringer Mannheim GmbH, Mannheim; 1/25 dilution), which was used as an 'irrelevant antibody' negative control for the laminin monoclonal antibodies. As a range of antibodies to laminin chains was used, which produced different staining patterns in the ovary, they were also able to act as controls for each other. Normal rabbit serum (Cat. no. R9133; Sigma Chemical Co.) or normal mouse serum (Cat. no. M5905; Sigma Chemical Co.) also served as negative controls.

Immunohistochemistry

All sections were post-fixed by immersion in ethanol (if using polyclonal antibodies) or acetone (if using monoclonal antibodies) for 20–30 min, rinsed in hypertonic PBS (10 mmol sodium/potassium phosphate l^{-1} with 0.274 mol NaCl l^{-1}, 5 mmol KCl l^{-1}; pH 7.3), and then incubated in 10% normal donkey serum (Cat. no. D9663; Sigma Chemical Co.) in hypertonic PBS (30 min). The sections were then incubated overnight with one of the primary antibodies (diluted in hypertonic PBS), rinsed in hypertonic PBS (3 × 5 min), and then incubated as appropriate with either Fluorescein (DTAF)-conjugated AffiniPure donkey anti-rabbit IgG (Cat. no. 711-095-152; Jackson ImmunoResearch Laboratories, West Grove, PA; 1/150 dilution in hypertonic PBS; 45 min) or with Cy3-conjugated AffiniPure donkey anti-mouse IgG (Cat. no. 715-165-150; Jackson ImmunoResearch; 1/150 dilution in hypertonic PBS; 45 min). In some instances, sections that had been incubated with polyclonal antibodies were further incubated with biotin-SP-conjugated goat anti-rabbit IgG (Cat. no. 711-066-152; Jackson ImmunoResearch; 1/100 dilution in hypertonic PBS; 45 min) to achieve further amplification of the immunostaining. Sections were then rinsed in hypertonic PBS (3 × 5 min) and further incubated with Cy3 conjugated-streptavidin (Cat. no. 016-160-084; Jackson ImmunoResearch; 1/100 dilution in hypertonic PBS; 1 h). All incubations were at room temperature. All sections were finally rinsed in hypertonic PBS (3 × 5 min), and mounted with buffered glycerol (0.167 mol Na_2CO_3 l^{-1} in 67% glycerol, pH 8.6).

Dual labelling

As the α1 and β2 staining of the preantral follicular basal lamina was very faint, and it was conceivable that not all follicles of this stage were labelled, dual labelling of the α1 chain with the γ1 chain (which gave more intense staining of the basal laminae of preantral follicles; see results), and then dual labelling of the α1 and β2 laminin chains was conducted. In addition, sections were dual labelled for the β2 laminin chain and factor VIII-related antigen to confirm that the former was labelling the basal lamina of the vasculature. The protocol for dual labelling was essentially the same as described above, except that the sections were incubated concurrently with two primary antibodies, and that the sections were incubated concurrently with Fluorescein (DTAF)-conjugated AffiniPure donkey anti-rabbit IgG and Cy3-conjugated AffiniPure donkey anti-mouse IgG. Control sections were: the relevant polyclonal primary antibody and anti-rabbit secondary antibody; the relevant monoclonal primary antibody and anti-mouse secondary antibody; the polyclonal primary antibody and anti-mouse secondary antibody; the monoclonal primary antibody and anti-rabbit secondary antibody; the normal rabbit serum primary antibody and anti-rabbit secondary antibody; the normal mouse serum primary antibody and anti-mouse secondary antibody; both the normal rabbit serum and normal mouse serum primary antibodies; and both the anti-rabbit and anti-mouse secondary antibodies.

Photography

Sections were observed and photographed with an Olympus Vanox AHBT3 fluorescence microscope with Olympus C35AD-4 camera attachment, using the IB filter to excite the DTAF fluorochrome and the G filter to excite the Cy3 fluorochrome. Photographs were taken using Kodak T-Max 400 black and white film.

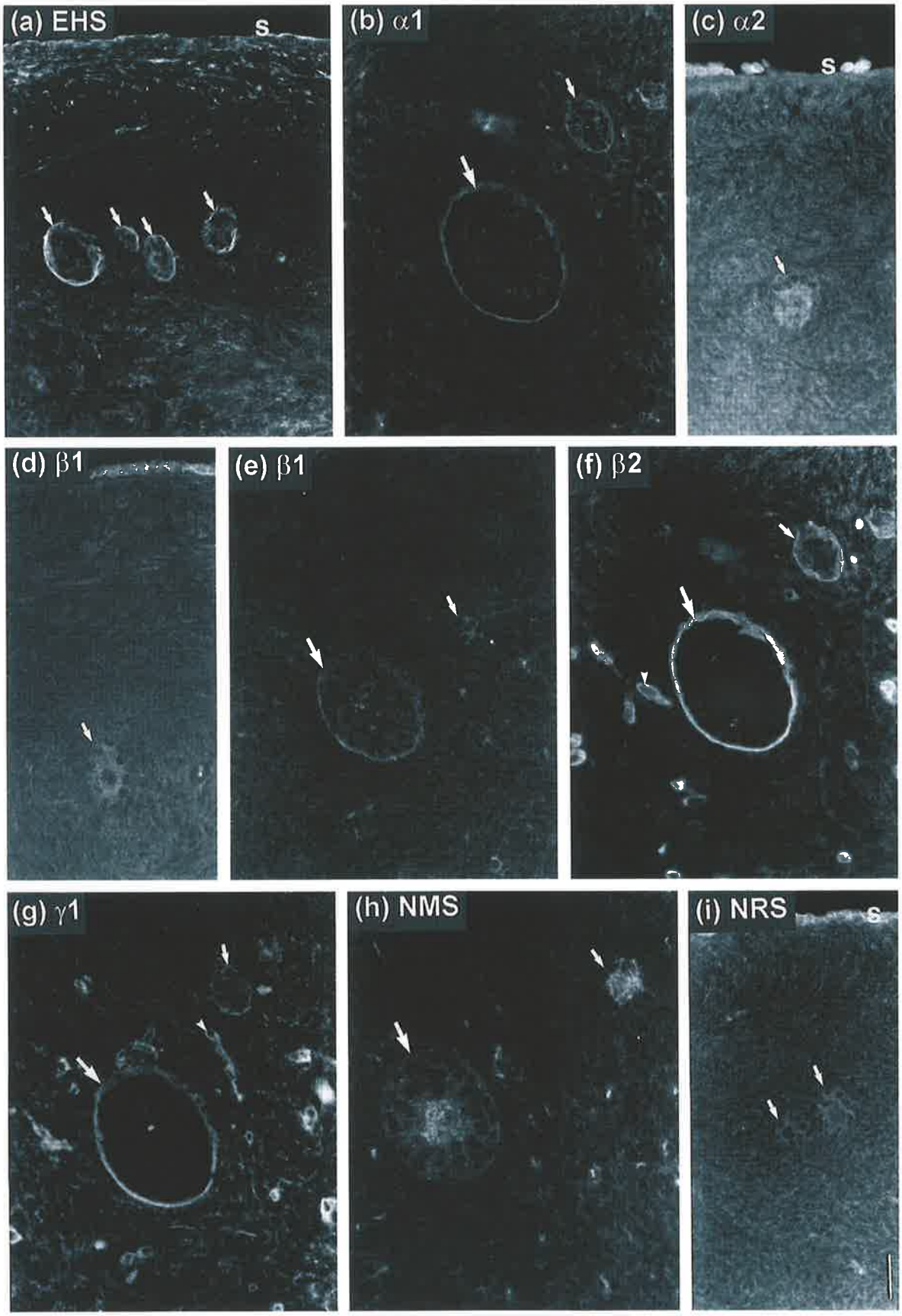
Electron microscopy

Three bovine ovaries were processed for electron microscopy as reported by van Wezel and Rodgers (1996). Briefly, the ovarian artery was cannulated, and the associated ovary was flushed with Earle's balanced salt solution (EBSS) and then 2.5% (v/v) glutaraldehyde in 0.1 mol 4-morpholine–propanesulfonic acid l^{-1}. Tissue from the ovarian cortex was then cut into pieces approximately 1 mm × 0.5 mm × 2 mm, and these were postfixed with osmium tetroxide, dehydrated and embedded in epoxy resin by standard methods as reported in detail by van Wezel and Rodgers (1996). Thick sections (2 μm) were stained with 1% (w/v) methylene blue, and thin sections were stained with uranyl acetate and lead nitrate and observed and photographed with a JOEL (Peabody, MA) CS1200 electron microscope.

Results

General observations

Five individual laminin chains were immunolocalized in bovine ovaries. In addition, an antibody raised against EHS laminin and, therefore, detecting the α1, β1 or γ1 chains, was used as a positive control. The results presented here are confined to follicles and the basal lamina structures therein, including the follicular basal lamina, basal laminae associated with endothelial cells and smooth muscle cells of the vasculature, and lamina-like material in the theca in areas where there are no recognized conventional basal laminae. Each of the primary antibodies used in this study resulted in staining of the follicular basal lamina which was abolished when that antibody was either omitted entirely from the immunostaining protocol or replaced by normal rabbit serum or normal mouse serum. The staining patterns produced by the different antibodies differed from each other when preantral follicles ($n > 150$; Fig. 1), antral follicles that were 'healthy' ($n = 9$; Fig. 2) or 'atretic' ($n = 10$; Fig. 3) were compared. Cells of the ovarian surface epithelium in normal rabbit serum controls were stained and,


(a) EHS
s
(b) α1
(c) α2
s
(d) β1
(e) β1
(f) β2
(g) γ1
(h) NMS
(i) NRS
s

therefore, staining of the surface epithelium produced by using the α1 or α2 antibodies could not be considered as specific. The follicular staining patterns produced by each antibody are summarized (Table 1).

α1β1γ1 Laminin

When the antibody directed against EHS laminin (laminin-1; Burgeson *et al.*, 1994) was used, staining was present in the follicular basal lamina of all preantral follicles (Fig. 1a) and healthy antral follicles (Fig. 2a). The follicular basal lamina of atretic antral follicles also stained, but with great variation in intensity between follicles (Fig. 3g,h). The theca also stained positively using the EHS antibody, with staining in areas where there are no recognized conventional basal laminae (Fig. 3g,h).

α1, α2 Laminin chains

The α1 (Fig. 1b) but not the α2 (Fig. 1c) laminin chain was immunolocalized to the basal lamina of preantral follicles. The α1 staining was usually not very intense, in contrast to strong staining for γ1 in the primordial follicular basal lamina. Owing to the variation in intensity of staining of α1, the possibility that the α1 chain might not be present in some preantral follicles was considered. Sections were dual-stained for both the α1 and γ1 chains and this confirmed that the α1 chain was localized to all the preantral follicles that were positive for the γ1 chain. The α1 chain was also present in the follicular basal lamina of healthy antral follicles (Fig. 2b) and stained with a much greater intensity than at the preantral follicle stage. Of the nine healthy antral follicles examined, the basal laminae of only two were positive for the α2 laminin chain and, in these cases, the staining was not evenly distributed from one region to another; the basal laminae of the other healthy antral follicles were unstained (Fig. 2c). Both the α1 (Fig. 3b) and the α2 (Fig. 3c) chains were localized in the basal laminae of all of the ten atretic antral follicles examined. Punctate fluorescence was observed in the membrana granulosa of some atretic follicles (Fig. 3b), but this was not specific staining as it was also seen in control sections (Fig. 3a). The α1 staining along the basal lamina was generally wider and less intense in atretic follicles than in healthy antral follicles.

β1, β2 Laminin chains

The β1 chain of laminin was not detected in the follicular basal laminae of any of the small preantral follicles (Fig. 1d,e), but large preantral follicles were stained weakly (Fig. 1e). In contrast, the β2 chain was detected in the basal laminae of all preantral follicles that were positive for the α1 laminin chain (and all α1-positive preantral follicles had been positive for γ1) and the staining intensity increased with increasing size of the follicle (Fig. 1f). In both healthy and atretic antral follicles, the β1 staining was present in the vasculature, which at times closely abutted the follicular basal lamina. However, staining of the follicular basal lamina itself was minimal or absent (Figs 2d,e and 3d). In contrast, the β2 chain was detected in both the follicular basal lamina and the vasculature (Figs 2f,h and 3e). Vascular staining for the β1 chain was limited to the endothelial cell basal laminae, while the β2 chain was also present in the smooth muscle layers of the arterioles (compare Fig. 3d with 3e). Blood vessels throughout the theca and ovarian stroma were positive for factor VIII-related antigen, with all β2 laminin localizing adjacent to cells positive for factor VIII-related antigen (Fig. 2h,i). Similar to the pattern of α1 staining, the region of β2 staining of the follicular basal lamina was wider and the staining was less intense in atretic follicles than in healthy follicles.

γ1 Laminin chain

The follicular basal laminae of all the preantral follicles (Fig. 1g) and healthy antral follicles (Fig. 2g) observed stained strongly for the γ1 laminin chain. In atretic antral follicles, staining of the follicular basal lamina was considerably less intense than in healthy antral follicles (Fig. 3f). Staining that was widely dispersed throughout the theca of healthy and atretic antral follicles, and not limited to the vasculature or other structures with distinct basal laminae, was also observed.

Electron microscopy

In general, the follicular basal lamina of primordial follicles was continuous and closely abutted the granulosa cells to envelop the follicle. The lamina appeared in some regions of most follicles to be composed of only a single layer of material while, in other regions, several layers of lamina material were observed. In regions where there were several layers of basal lamina material, these usually lay close together. Within growing follicles in most regions, the follicular basal lamina was generally multilayered. Often these layers were interconnected (Fig. 4). The inner layer of basal lamina closely abutted the granulosa cells, as for primordial follicles, but the other layers were not usually tightly packed and often this

Fig. 1. Immunofluorescent localization of laminin-1 (EHS laminin, composed of α1β1γ1; rabbit polyclonal antisera), and individual laminin chains (α1 and α2 are rabbit polyclonal antisera; β1, β2 and γ1 are mouse monoclonal antisera) to preantral bovine follicles. (a) Positive staining of the follicular basal laminae for laminin-1 (EHS laminin) (b) Positive staining for the α1 chain but (c) no detectable α2 chain. Staining of the ovarian surface epithelial cells in (c) is also seen in the normal rabbit serum (NRS) control (i) and, therefore, could not be considered as specific. (d, e) Nonspecific staining between the granulosa cells in small preantral follicles, which is also seen in the normal mouse serum control (h), but no staining of the basal lamina for the β1 chain. In contrast, the larger preantral follicle shown in (e) was positive for this chain, but the slight staining of the oocyte was also seen in the normal mouse serum (NMS) control (h), and considered nonspecific. (f, g) Positive staining of the follicular basal lamina for the β2 and γ1 chains, respectively, and staining of the stromal vasculature. The follicles in (c, d, i) were exposed to show the outline of the follicles and do not reflect high background concentrations. Arrows indicate the position of the follicular basal laminae, small arrows to small preantral follicles, large arrows to large preantral follicles; arrowheads indicate the stromal vasculature; S = the ovarian surface epithelium. Scale bar represents 50 μm.

region of basal lamina layers spanned 1–2 μm in cross-section. The arrangement of the basal lamina layers differed among follicles: some follicles apparently having sheets of basal lamina that ran parallel to the basal surface of the membrana granulosa, while the basal lamina layers of other follicles were more convoluted (Fig. 4).

Discussion

This is the first study to immunolocalize a range of individual laminin chains to the follicular basal lamina, and to investigate the changing composition of this basal lamina during follicular development and atresia. The α1 chain was present in the follicular basal lamina at all stages of follicle development, while the α2 chain was present only in atretic antral follicles and a few healthy antral follicles and absent from primordial and growing preantral follicles. The β2 chain was present in the follicular basal lamina of follicles of all stages, but the β1 chain was detected only in the basal lamina of large preantral follicles. Staining for the α1, β2 or γ1 chains appeared to increase in intensity from the preantral to healthy antral stages, and the basal lamina of atretic antral follicles appeared thicker but stained less intensely than that of healthy follicles. The basal laminae of the thecal vasculature stained positively for the β1 and β2 chains, while the γ1 chain localized more generally throughout the theca in areas that do not have a recognized conventional basal lamina.

Fröjdman *et al.* (1995) showed that mouse primordial, primary and antral follicles contain the α1 chain and, in the present study, we have shown a similar result in the bovine ovary. Antral follicles contain several layers of granulosa cells, and those cells adjacent to the follicular basal lamina differ from those adjacent to the follicular antrum in shape (columnar versus rounded or flattened; M. Krupa, I. L. van Wezel and R. J. Rodgers, unpublished) and the expression of a range of genes (Amsterdam *et al.*, 1975; Bortolussi *et al.*, 1977; Zoller and Weisz, 1978, 1979; Zlotkin *et al.*, 1986). Studies using kidney cells suggest that the α1 laminin chain may have a role in maintaining this polarity of the granulosa layer. Kidney cells in culture became polarized when laminin α1 was expressed, and antibodies to laminin α1 prevented polarization (Klein *et al.*, 1988, 1990; Ekblom, 1989).

Laminin α2 (merosin in the old nomenclature; Burgeson *et al.*, 1994) was found in the follicular basal lamina of atretic follicles and in a few healthy antral follicles. These follicles also had the laminin α1 chain; at this point it is unclear whether both chains were being produced in these follicles or whether, before the onset of atresia, there was a switch from the production of α1 to α2 chains, and no ongoing production of the α1 chain. It is not clear whether laminin α2 plays a major role in the induction of follicular atresia, or is expressed as a consequence of atresia. An alternative explanation is that the laminin α2 chain is produced as a normal event in late follicular development, and its expression coincides with atresia only because atresia of these follicles is commonplace. Evidence from other tissues suggests that this could be the case, as the α2 chain plays a role when cells are differentiating or maturing (Ehrig *et al.*, 1990). Thus, there is no expression of α2 by cultured cell lines, limited expression in malignant tumours, and expression in mouse basal laminae occurs postnatally not prenatally (Leivo and Engvall, 1988; Leivo *et al.*, 1989a,b). Similarly, in the epithelium of the human small intestine, whereas expression of the α1 chain is evident at 7 weeks of gestation, the α2 chain is not present until 16 weeks of gestation (Perreault *et al.*, 1995). However, to ascertain the role of the laminin α2 chain in the follicular basal lamina, it will be necessary to study follicles of known developmental stage.

The observation that both β1 and β2 laminin chains were present in the follicular basal lamina before antrum formation, but only the β2 chain was present after antrum formation, parallels previous studies in the developing kidney: that is, the laminin β1 chain was expressed at three early stages (the comma and s-shaped structures, and the capillary loop) and β2 was expressed later as the glomerulus developed (see Noakes *et al.*, 1995). Furthermore, Noakes *et al.* (1995) showed that changes in β1 and β2 expression are important for proper filtering of plasma proteins by the glomerular basement membrane. Others have shown that cultured mouse embryonic lung cells can be induced to polarize and form a lumen by treatment with a fragment of laminin β1 and heparan sulfate proteoglycan (Schuger *et al.*, 1996). On the basis of these studies, it is conceivable that changes in laminin β1 or β2 concentration during follicular development are important in controlling the filtration properties of the follicular basal lamina and initiating the formation of the follicular antrum.

The present study localizing individual laminin chains did not identify which laminin type was present in the follicular basal lamina. Laminin-3 (s-laminin; α1β2γ1; Burgeson *et al.*, 1994) may be present in the follicular basal lamina, as suggested by the presence of α1, β2 and γ1 chains throughout follicular development. However, the presence of the α2 and β1 chains in the follicular basal lamina at specific stages of development indicated that other laminin isoforms were present, at least during select stages. The α3, α4, α5, β3 and γ2 laminin chains have not yet been examined in the follicular

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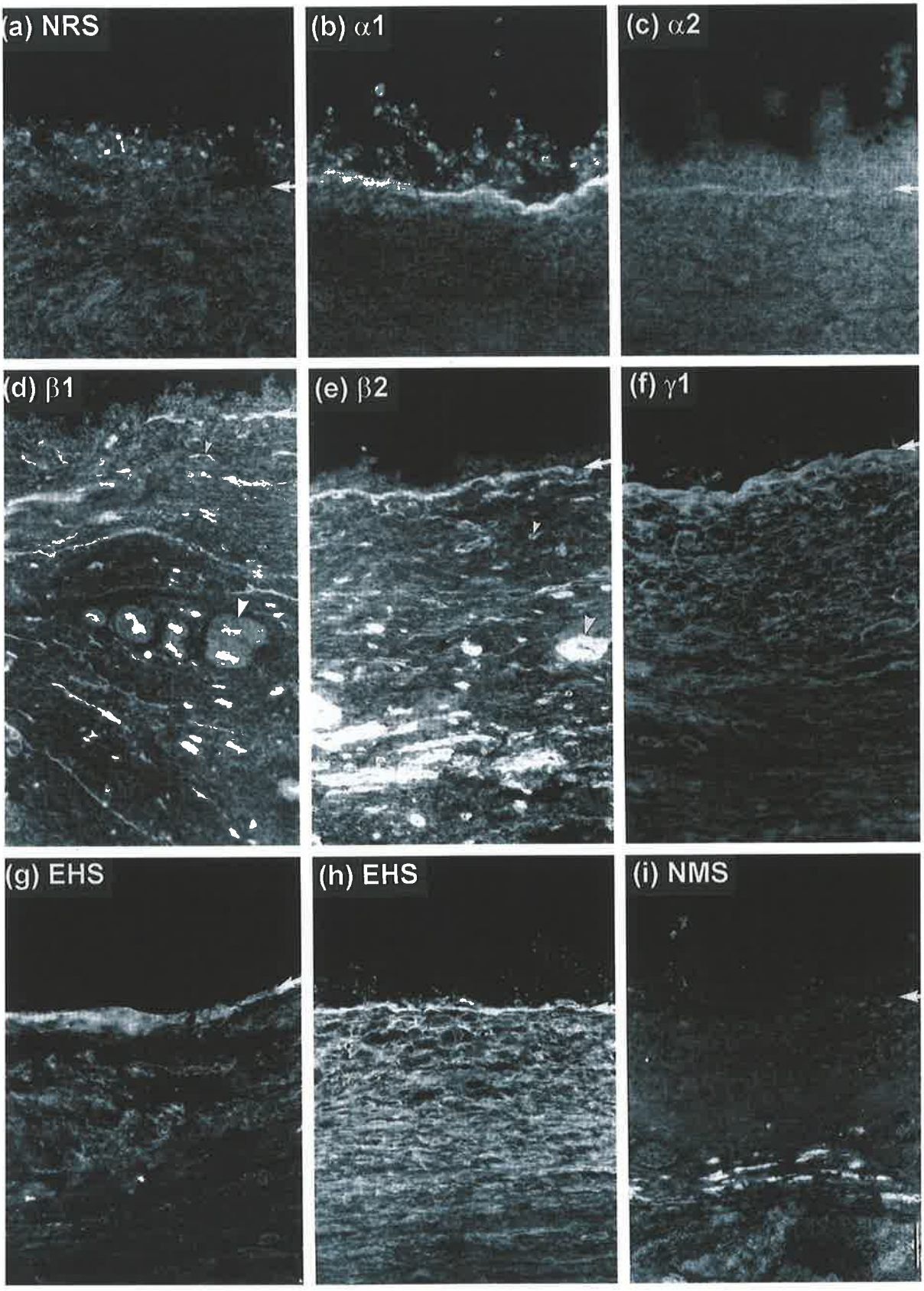
(a) NRS
(b) α1
(c) α2
(d) β1
(e) β2
(f) γ1
(g) EHS
(h) EHS
(i) NMS

region of basal lamina layers spanned 1–2 μm in cross-section. The arrangement of the basal lamina layers differed among follicles: some follicles apparently having sheets of basal lamina that ran parallel to the basal surface of the membrana granulosa, while the basal lamina layers of other follicles were more convoluted (Fig. 4).

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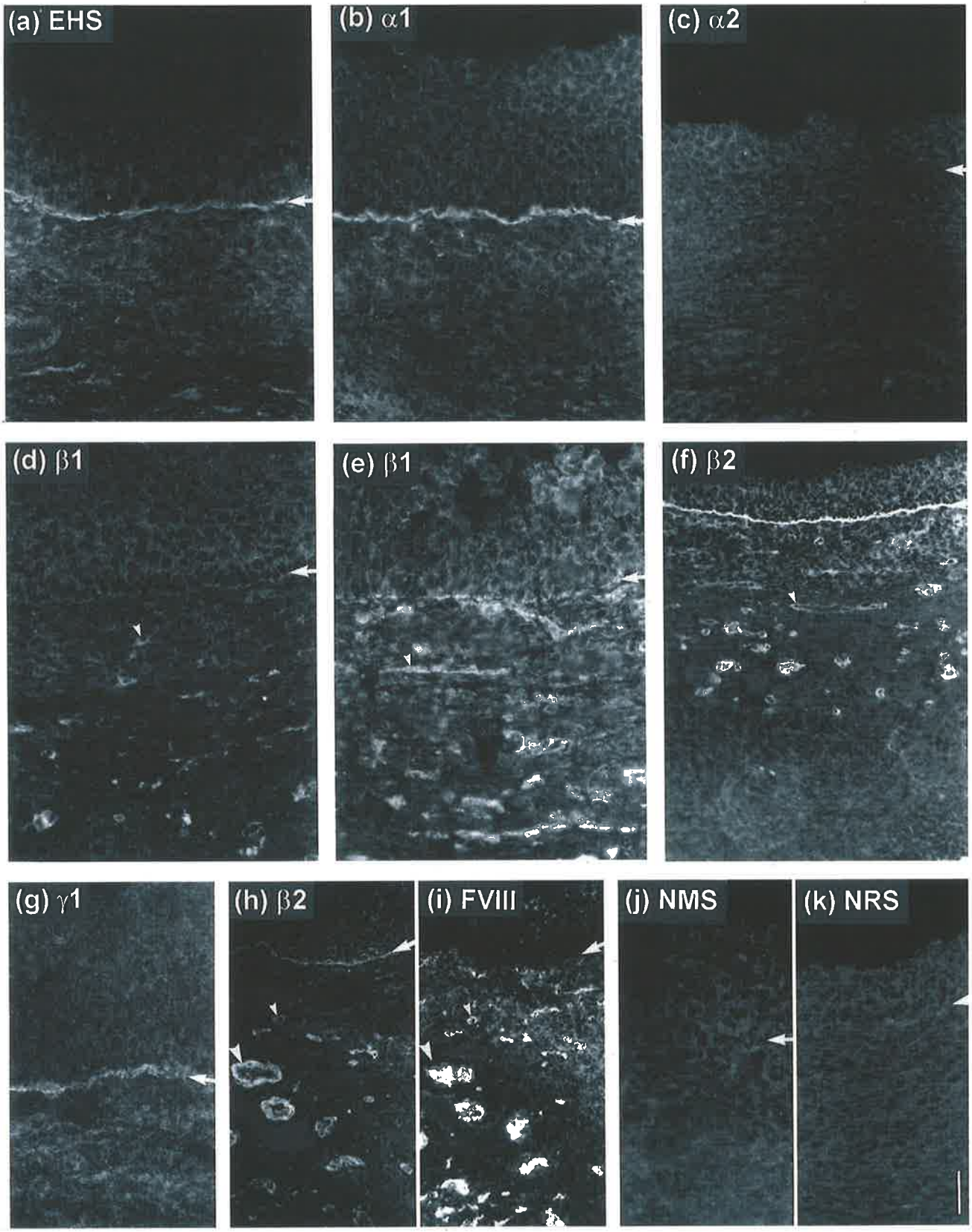
(a) EHS
(b) α1
(c) α2
(d) β1
(e) β1
(f) β2
(g) γ1
(h) β2
(i) FVIII
(j) NMS
(k) NRS

Table 1. Staining patterns produced by antisera specific to the individual α1, α2, β1, β2 and γ1 laminin chains and to EHS (α1β1γ1) laminin in bovine ovarian follicles

	α1	α2	β1	β2	γ1	EHS
Follicular basal lamina[a]						
Primordial/primary	+	−	−	+	+	+
Large preantral	++	−	+	++	++	++
Healthy antral	+++	−/+	−	+++	++	+++
Atretic antral	++	+	−	++	+	++
Other follicular						
Thecal matrix	−	−	−	−	++	++
Vascular subendothelial	−	−	+++	+++		
Vascular smooth muscle	−	−	−	+++		

Intensity of staining: −, none; +, weak; ++, moderately intense; +++, very intense. Comparison of intensity should only be made within a column (that is, between follicles of different classes) and not within a row (that is, between laminin chains) as different antisera have been used.

[a]The same nine healthy and ten atretic antral follicles were examined for each antibody. > 150 preantral follicles were examined for each antibody (these were not necessarily the same follicles).

basal lamina of any species, but may also contribute to laminin molecules in the follicular basal lamina. Gene expression for the laminin α4 chain has been detected in the ovary (Iivanainen *et al.*, 1995). However, whether this is the follicular basal lamina or one of the many other types of basal lamina or non-lamina structures in the ovary is not known.

Evidence presented here and previously (van Wezel and Rodgers, 1996) suggests that the basal lamina is continually remodelled during follicular development. Electron microscopic examination of the bovine follicular basal lamina has found that it is closely associated with the granulosa cells in primordial follicles (van Wezel and Rodgers, 1996). The follicular basal lamina of larger follicles was composed of many layers of basal lamina material joined together to form a branching network (this study and see Rodgers *et al.*, 1995), which is consistent with the notion that the basal lamina is shed and replaced by a newly synthesized basal lamina closer to the granulosa cells. Under the light microscope, multilayers of basal lamina would only have been detected as either an increased thickness or increased intensity of staining, as observed for the laminin α1, β2 and γ1 chains during follicular development. However, the more obvious explanation that this staining pattern was due to increased amounts of the specific laminin chains per unit surface area of basal lamina requires further investigation. Increasing thickness of the basal lamina staining during follicular atresia was also observed. This finding, together with the decreased intensity of staining for the α1, β2 and γ1 in atretic follicles, suggests that there may have been degradation of some basal lamina components – perhaps the collagen IV backbone – causing the basal lamina to lose its compact structure. Alternatively, simple convolution of the basal lamina may have occurred when the follicular fluid was resorbed and the atretic follicle decreased in size, producing the apparent increase in thickness of the basal lamina.

The cellular origin of the follicular basal lamina is a contentious issue (van Wezel and Rodgers, 1996). Within the laminin component of the follicular basal lamina, northern blot analysis detected the expression of the γ1 (old nomenclature: B2) chain but not β1 (old nomenclature: B1) by granulosa cells (Zhao and Luck, 1995), which is consistent with the present study localizing the γ1 but not the β1 laminin chain to the follicular basal lamina of antral follicles. Furthermore, a previous immunoelectron study in the rat ovary (Leardkamolkarn and Abrahamson, 1992) localized laminin to Call–Exner bodies, which are ultrastructurally similar to basal lamina and have been observed within the membrana granulosa of antral follicles *in vivo* in cows (I. L. van Wezel and R. J. Rodgers, unpublished) and rabbits (Gosden *et al.*, 1988). Laminin was also localized intracellularly in both granulosa and thecal cells, but this latter observation may represent degradation rather than synthesis. The contribution of the theca, or indeed the stroma surrounding preantral follicles, to the production of the follicular basal lamina is not known. In other systems, it is predominantly epithelial rather than stromal cells that

Fig. 3. Immunofluorescent localization of individual laminin chains and laminin-1 (EHS laminin) to atretic bovine antral follicles. In each photograph, the basal lamina is indicated by the arrow on the right side; the membrana granulosa lies above the basal lamina and the theca below it, and the follicular antrum is uppermost. (a) Normal rabbit serum (NRS) control showing punctate staining in the membrana granulosa. Similar staining for any laminin chain was, therefore, not considered as specific. Positive staining of the basal lamina for the α1 (b) and α2 chain (c). (d) No continuous staining of the basal lamina for the β1 laminin chain but localization of this chain to the vasculature, some of which closely abuts the follicular basal lamina. The smooth muscle of arterioles is not stained. (e) β2 chain localized to the follicular basal lamina and the vasculature, including arteriole smooth muscle. (f) Weak staining pattern produced by antibodies directed against γ1 laminin. (g, h) Localization patterns produced by antibodies directed against laminin-1, with staining of the basal lamina being either very weak (g) or very strong (h) relative to the thecal staining. (i) Normal mouse serum (NMS) control. Small arrowheads indicate the vasculature, large arrowheads indicate the vasculature with smooth muscle. Scale bar represents 50 μm (a–c, f–h) and 100 μm (d, e, i).

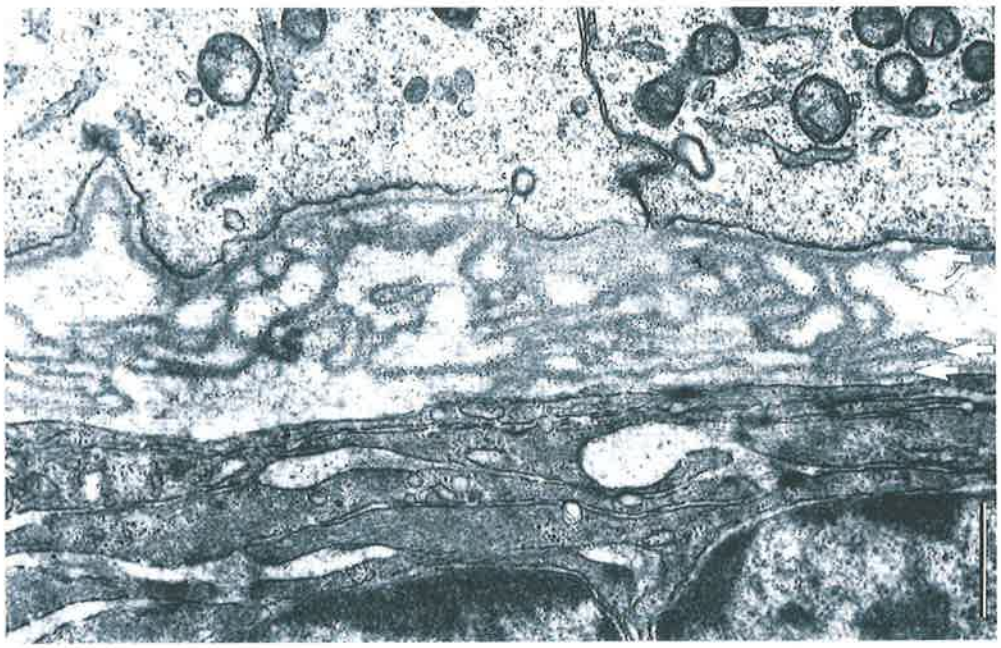

Fig. 4. Electron micrograph of the follicular basal lamina of a growing bovine preantral follicle. The many layers of basal lamina are indicated by arrows. Granulosa cells are uppermost, and thecal–stromal cells are beneath the follicular basal lamina. Scale bar represents 0.5 μm.

synthesize basal lamina components, although in some tissues both cell types make a contribution (Timpl and Dziadek, 1986). The thecal compartment of antral follicles has been shown by northern analyses to express laminin β1 and γ1 chains (Zhao and Luck, 1995). The present study showed laminin β1 in the thecal vasculature but not in the follicular basal lamina of antral follicles, suggesting that the thecal expression of β1 is not necessarily a contribution to the follicular basal lamina. Similarly, laminin γ1 was found widely distributed in the theca and, although it is present in the follicular basal lamina, expression in the theca cannot be considered as proof that the theca contributes laminin γ1 to the follicular basal lamina. However, it is possible that the follicular basal lamina *in vivo* requires a contribution from both the granulosa cells and the stroma or thecal cells.

Laminin chains were also detected in the theca interna and externa of antral follicles. Both β1 and β2 were localized to subendothelial basal laminae of the blood vessels (arterioles, capillaries and venules). The laminin β2 chain was also localized to the smooth muscle layers surrounding arterioles, which were located principally in the theca externa, consistent with studies in other tissues, in which the β1 chain was limited to subendothelial basal laminae, while the β2 chain was present between smooth muscle cells (Walker-Caprioglio *et al.*, 1995). Staining for the laminin γ1 chain or EHS laminin was present throughout the theca interna and externa in areas where there are no recognized conventional basal laminae. This pattern of staining has been observed using antibodies to some types of collagen IV (H. F. Rodgers and R. J. Rodgers, unpublished) and to EHS (α1β1γ1) laminin in rats (Bagavandoss *et al.*, 1983; Leardkamolkarn and Abrahamson, 1992) and mice (Fröjdman *et al.*, 1995), and in the interstitial tissue of developing gonads in these species (Fröjdman *et al.*, 1989, 1992a,b, 1993, 1995; Smith and MacKay, 1991). It is possible that this staining in the theca is due to free γ1 chains or free laminin not associated with a basal lamina. However, it is also possible that it is associated with small fragments of basal lamina-like, electron-dense material found extracellularly in the theca. This material has been observed by electron microscopy in the theca of sheep (O'Shea *et al.*, 1978), rats (Leardkamolkarn and Abrahamson, 1992) and cows (see Fig. 1 in Rodgers *et al.*, 1986). The origins and functions of this 'thecal matrix' are not known.

In conclusion, this is the first detailed immunolocalization study of individual members of any of the broad classes of basal lamina components, in the ovarian follicle. The observations made here confirm and extend previous studies, showing that the α1, α2, β1, β2 and γ1 laminin chains are differentially expressed in the ovarian follicle, and that the composition of the follicular basal lamina, at least in terms of laminin, changes during follicular development and atresia. On the basis of studies using other cell types it seems likely that the different laminin chains have specific roles in the regulation of antrum formation and the development of the follicular epithelium.

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Statement of Authorship

Ultrastructure of the basal lamina of bovine ovarian follicles and its relationship to the membrana granulosa

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Statement of Authorship

Identification and immunolocalization of decorin, versican, perlecan, nidogen, and chondroitin sulfate proteoglycans in bovine small-antral ovarian follicles

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Identification and Immunolocalization of Decorin, Versican, Perlecan, Nidogen, and Chondroitin Sulfate Proteoglycans in Bovine Small-Antral Ovarian Follicles[1]

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ABSTRACT

Proteoglycans (PGs) consist of a core protein and attached glycosaminoglycans (GAGs) and have diverse roles in cell and tissue biology. In follicles PGs have been detected only in follicular fluid and in cultured granulosa cells, and the composition of their GAGs has been determined. To identify PGs in whole ovarian follicles, not just in follicular fluid and granulosa cells, small (1–3-mm) bovine follicles were harvested. A proportion of these was incubated with $^{35}SO_4$ for 24 h to incorporate radiolabel into the GAGs. The freshly harvested and cultured follicles were sequentially extracted with 6 M urea buffer, the same buffer with 0.1% Triton X-100 and then with 0.1 M NaOH. Proteoglycans were subjected to ion-exchange and size-exclusion chromatography. The GAGs were analyzed by chemical and enzymic digestion, and on the basis of their composition, we chose a list of known PGs to measure by ELISA analyses. Versican, perlecan, decorin, but not aggrecan or biglycan, were identified. These, excluding decorin for technical reasons, as well as a basal lamina glycoprotein, nidogen/entactin, were immunolocalized. Versican was localized to the thecal layers, including externa and the interna particularly in an area adjacent to the follicular basal lamina. Perlecan and nidogen were localized to the follicular basal lamina of antral follicles, both healthy and atretic, but not to that of preantral follicles. Both were localized to subendothelial basal laminas, but the former was not readily detected in arteriole smooth muscle layers. This study has confirmed the presence of versican and perlecan, but not the latter as a component of follicular fluid, and identified decorin and nidogen in ovarian antral follicles.

follicle, follicular development

INTRODUCTION

Development of mammalian ovarian follicles and oocytes is a complex process involving tissue growth and remodeling, fluid accumulation, and cell replication, specialization, and differentiation. Proteoglycans (PGs) are ubiquitous molecules of extracellular matrices that have been implicated in these processes in a variety of other tissues [1]. Proteoglycans consist of glycosaminoglycans (GAGs) covalently attached to a protein core. Glycosaminoglycans consist of chains of repeating disaccharides that vary in composition thus forming different GAG chains. Proteoglycans are therefore a diverse range of molecules with diverse functions, documented to be involved in cell growth and differentiation, water homeostasis, and the regulation of growth factors.

Glycosaminoglycans have been identified in the ovarian follicular fluid of pigs [2–4], cows, [5–9], humans [10–12], and rats [13, 14]. The predominant GAGs in bovine and porcine follicular fluid are dermatan sulfate (DS) and chondroitin sulfate (CS) [2, 6]. The CS/DS-containing GAGs were shown to be attached to a protein core in bovine follicles by Grimek and Ax [9], while the GAG heparan sulfate (HS) was observed not to be bound to a protein core. The concentration of GAGs in bovine follicular fluid varied with size and health of the developing follicles [8, 9]. Chondroitin sulfate concentrations were higher in the follicular fluid of small-antral follicles as compared with large antral follicles [9]. The concentration of CS was also reported to vary with the health of the ovarian follicle but only when follicular atresia was assessed biochemically, not histologically [8].

The size of follicular fluid PGs was estimated to range from 7.5×10^5 to 2×10^6 [2, 9]. Molecules of this size are too large to traverse the basal lamina readily, thus they must be produced within the follicle by the granulosa cells [2]. Bovine granulosa cells in anchorage-independent culture produced an extracellular matrix rich in PGs, as determined by ruthenium red staining [15]. Cultured rat granulosa cells also produced PGs; determined by the incorporation of radiolabeled sulfate. These PGs included three hydrodynamic sizes of DS-containing PGs and two different hydrodynamic sizes of HS-containing PGs [16–19]. These DS- and HS-containing PGs were also found to be either intracellular, cell surface associated, or bound within the cell membrane. Production of CS/DS PGs by granulosa cells has been shown to increase in response to FSH in vitro [4, 7, 20]. Recently some of the PGs in human follicular fluid were identified as a versican-like PG and perlecan [12].

In addition to these PGs, other PGs could be present in follicular fluid, and no studies of PGs in the other follicular compartments have been conducted. We therefore analyzed, identified, and immunolocalized a number of PGs in bovine whole antral follicles. Small follicles were used as they are mostly healthy and at an early stage of antrum expansion.

MATERIALS AND METHODS

Tissues

All bovine ovaries were collected at a local abattoir, within 20 min of slaughter, from cyclic cows visually assessed not to be pregnant. The ovaries were immediately placed into Hepes-buffered Earle's balanced-salt solution without calcium and magnesium, containing 10 mM *N*-ethylmalemide (NEM), 5 mM benzamidine, 0.5 mM PMSF, 0.1 M δ-amino-caproic acid, and 0.01 M EDTA, on ice for return to the laboratory. Antral follicles of 1–3 mm diam-

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eter were dissected from the whole ovary. Most of these were weighed, snap frozen, and stored at −20°C for analyses of PGs. For analyses of PGs, 18 separate batches of 1- to 3-mm follicles from 18 ovaries were isolated. These were subsequently pooled and weighed 4.633 g. Three antral follicles from each batch were randomly chosen and fixed for histological assessment of follicular health. Other follicles were taken for culture as below and, following culture, were pooled with the unicubated batches prior to isolation of PGs.

Histological Assessment of Follicular Health

Dissected follicles were fixed by immersion in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 24 h at 4°C. Subsequently, specimens were washed in 5% sucrose in 0.1 M phosphate buffer, postfixed in aqueous 1% osmium tetroxide for 60 min, dehydrated using increasing concentrations of acetone, then embedded in epoxy resin (49% dodecenyl succinic anhydride, 39.2% Araldite, 9.8% Procure 812, 2% 2,4,8-tridimethylaminoethyl phenol), and cured at 60°C. Sections of 1 μm were cut, stained with 1% methylene blue in 1% sodium tetraborate, and examined by light microscopy. Follicles were assessed as atretic if pyknotic nuclei were present in the membrana granulosa.

Radiolabeling of Follicular PGs

In order to increase the sensitivity of detection the GAGs they were radiolabeled during culture of follicles. To do this, ovaries were collected as described above and immediately placed into Hepes-buffered Dulbecco's-modified Eagle's medium (DMEM):Ham's F12 medium (50:50) containing 100 μg/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone on ice. Antral follicles of 1–3 mm diameter were dissected from the ovaries, then washed in the same medium but without Hepes. Three batches of follicles were collected from three ovaries, weighing 0.78 g collectively. Dissected follicles were cultured in 35-mm dishes in DMEM/Ham's F12 medium containing 1% fetal bovine serum, antibiotics, and 20 μCi/ml $^{35}SO_4$ (562.5 Ci/mM) for 24 h in a humidified atmosphere of 5% CO_2 in air at 39°C. Follicles were then harvested, snap-frozen on dry ice, and stored at −20°C for further analyses.

PG Extraction

The batches of ^{35}S-labeled follicles were combined with the batches of unlabeled follicles. These pooled follicles (5.43 g) were minced with a razor blade and then extracted with five volumes of 6 M urea solution containing 0.05 M sodium acetate, 0.1 M δ-amino-caproic acid, 0.1 M disodium salt EDTA, 5 mM benzamidine, 0.5 mM PMSF, and 10 mM NEM, pH 5.0, by continuously rotating the solution at 4°C for 48 h. Following centrifugation (6000 × *g* for 15 min), the supernatant was retained for analyses and stored at −20°C. This will be referred to as the urea extract.

The pellet was then placed into five volumes of the urea extraction buffer described above but also containing 0.1% Triton X-100 and homogenized using a Dounce teflon/glass homogenizer for 10 min and rotated at 4°C for 48 h. Following centrifugation (6000 × *g* for 15 min), the supernatant was retained for analyses and stored at −20°C. This will be referred to as the urea/Triton X-100 extract.

The pellet was placed into 10 ml of 0.1 M sodium hydroxide to solubilize the remaining tissue components by rotation at 4°C for 48 h. The extract was then centrifuged (6000 × *g* for 15 min), and both the pellet and the supernatant were retained for analyses at −20°C. The supernatant will be referred to as the sodium hydroxide extract.

Glycosaminoglycan and Protein Measurements

After each step of chromatographic analyses the GAG content of samples was measured using either the carbazole method published by Blumenkrantz and Asboe-Hansen [21], or the dye-binding assay published by Farndale et al. [22]. Protein analyses were carried out using the bicinchoninic acid method of Smith et al. [23]. Radioactivity of samples was measured by scintillation counting.

Ion-Exchange Chromatography

The three sequential follicle extracts were each chromatographed using ion-exchange chromatography. The pH of the sodium hydroxide extract was first adjusted to pH 5.0 with hydrochloric acid. Each extract was rotated for 1 h with 2 ml of DEAE Sephacel beads (Pharmacia, Uppsala, Sweden) at 4°C. The DEAE Sephacel columns were then washed with 6 M urea, 0.05 M sodium acetate, 0.1 M δ-amino-caproic acid, 0.1 M disodium salt EDTA, pH 5.0, to remove tissue components unbound to the column beads. The PGs were then sequentially eluted from the columns in urea buffer above containing 0.15 M NaCl, followed by urea buffer containing 2.0 M NaCl. All eluted fractions were assayed for GAGs using the carbazole method. Glycosaminoglycan containing-fractions from the urea and urea/Triton X-100 extracts were pooled together due to the low amount of GAG present in the extracts. This will now be referred to as the urea-urea/Triton X-100 extract. The GAG-containing fractions from the sodium hydroxide extract were also pooled. All pooled fractions were retained for analyses and stored at −20°C.

Size-Exclusion Chromatography

Pooled GAG-containing fractions from above were dialyzed against 0.1 M Tris acetate buffer and then against deionized water before lyophilization and resuspension in 500 μl of column buffer containing 2 M guanidine, 0.1 M sodium acetate, 0.05 M Tris, pH 7.5. The resuspended urea-urea/Triton X-100 extract was chromatographed on a 90-cm sepharose CL2B (Pharmacia) column at a flow rate of 4 ml/h. Forty milliliters of column buffer was eluted (80 fractions). Fractions were assayed for GAG using the dye-binding method, and aliquots were also taken for liquid scintillation counting. Aliquots (200 μl) of each fraction were dialyzed against 0.1 M Tris acetate buffer, pH 7.0, followed by deionized water, and then lyophilized. The PG composition of the column fractions from the urea-urea/Triton X-100 extracts was determined by ELISA using a panel of antibodies specific to various PGs as described below.

The resuspended sodium hydroxide extract was similarly chromatographed but on a 90-cm sepharose CL4B column. The fractions containing GAGs were pooled, dialyzed, lyophilized, and resuspended in 150 μl H_2O, and the GAG composition determined by nitrous acid cleavage and chondroitinase digestions as described below.

Glycosaminoglycan Characterization of Sodium Hydroxide Extract

Nitrous acid cleavage. An aliquot (20 μl) was mixed with nitrous acid to cleave HS side chains as described by Hopwood [24]. The sample was incubated for 1 h at room

temperature and then neutralized with sodium carbonate. Samples (20 μl mixed with 480 μl column buffer as above) were rechromatographed on a sepharose CL4B column as described above.

Chondroitinase digestion. An aliquot (20 μl) was treated with chondroitinase ACII lyase (EC 4.2.2.5; ICN Immunobiologicals, Costa Mesa, CA) to digest CS side chains. The digestion was carried out in 250 μl of 0.08 M Tris acetate buffer, pH 6.0, containing 0.02 units of enzyme for 4 h at 37°C [25]. An aliquot was also digested with chondroitinase ABC lyase (EC 4.2.2.4; ICN Immunobiologicals) to digest both CS and DS side chains. The digestion was carried out in 240 μl of 0.1 M Tris acetate buffer, pH 8.0, using 0.02 units of enzyme for 4 h at 37°C [25]. Samples were rechromatographed on a sepharose CL4B column as described above.

Primary Antibodies and Purified PGs

Primary antibodies directed against epitopes present on 4-sulfated CS/DS (murine monoclonal antibody 2B6, IgG purified from ascites fluid, recognizes a disaccharide containing a nonreducing 4,5 unsaturated hexuronate adjacent to a 4-sulfated *N*-acetylgalactosamine that is produced by chondroitinase digestion of native CS or DS chains), 6-sulfated CS/DS (murine monoclonal antibody 3B3, IgM purified from ascites fluid, recognizes a disaccharide containing a nonreducing unsaturated hexuronate adjacent to a 6-sulfated *N*-acetylgalactosamine which is produced by chondroitinase digestion, or on nondigestion it recognizes an epitope on native CS chains containing a nonreducing unsaturated or saturated hexuronate adjacent to a 6-sulfated *N*-acetylgalactosamine) [26, 27], and aggrecan (murine monoclonal antibody 1C6, raised against reduced and alkylated rat chondrosarcoma PGs) [27] were kindly donated by Bruce Caterson, Department of Surgery, University of North Carolina at Chapel Hill, NC. Antibodies recognizing the bovine decorin (rabbit serum, antibody LF94, raised against a synthetic peptide) and the bovine biglycan (rabbit serum, antibody LF96, raised against a synthetic peptide) were kindly donated by Larry Fisher, Bone Research Branch, National Institute of Dental Research, National Institutes of Health, Bethseda, MD [28–30]. The antibody recognizing the bovine versican, was affinity-purified IgG from a rabbit immunized with a histidine-tagged recombinantly expressed and purified fragment (amino acid numbers 1340–1613) of bovine versican called GAG-β [31]. It was kindly donated by Dieter Zimmermann and Maria Teresa Dours-Zimmermann, Department of Pathology, University of Zurich, Schmelzbergstrasse, Zurich, Switzerland. The antibody recognizing the HS-containing PG perlecan (murine monoclonal antibody A76, raised against bovine corneal endothelial cell extracellular matrix) was kindly donated by Anne Underwood, CSIRO Molecular Science, North Ryde, NSW, Australia [32]. The antibodies recognizing nidogen/entactin, called 913 and 914, were rabbit sera raised against purified nidogen isolated from mouse EHS tumor [33] and were kindly donated by Dr. Marie Dziadek, Department of Anatomy, University of Melbourne. Purified decorin was isolated from bovine articular cartilage in house.

Enzyme-Linked Immunosorbent Assay Analyses of Urea-Urea/Triton X-100 Extract

Samples (10 μl) to be tested were diluted in 140 μl of PBS (10 mM) and incubated (1 h at 37°C) in 96-well plates to allow attachment to wells. The wells were then washed in PBS and incubated with 150 μl of 0.01 M PBS containing 1% BSA (1 h at 37°C). Primary antibody at the appropriate dilution (1/5000 for 2B2 and 3B3, and 1/1000 for 1C3, LF94, LF96, GAG-β, and A76) in 150 μl of the same buffer was added to each well and incubated (1 h at 37°C). The secondary antibodies were horseradish peroxidase-conjugated sheep anti-mouse IgG (Silinus Laboratories, Vic, Australia) for monoclonal primary antibodies, and horseradish-peroxidase-conjugated sheep-anti-rabbit IgG (Silinus Laboratories, Vic, Australia) for polyclonal antibodies were applied at a dilution of 1/500 in same buffer and incubated (1 h at 37°C). The peroxidase activity was measured as a color reaction using 100 μl of 2,2′-azino-di-(3-ethylbenzthiazoline-6-sulfonic acid) as substrate with 10% hydrogen peroxide, prepared as described in the BioRad horseradish peroxidase substrate kit (BioRad, Hercules, CA). The optical density was read at 415 nm.

Samples to be tested for the presence of aggrecan were, prior to attachment to the wells, first reduced by treatment with 10 mM DL-dithiothreitol (100°C for 10 min) and then alkylated in 20 mM iodoacetic acid (in dark for 2 h) in order to expose the antigen. Samples to be incubated with the 2B6 or 3B3 antibodies required prior digestion with chondroitinase ABC lyase to expose the antigen. Chondroitinase ABC lyase was prepared at 0.5 units per 10 ml, 0.1 M Tris acetate buffer, pH 8.0, containing 1% BSA. The digestion occurred in the 96-well plates in which the samples had previously been incubated to allow attachment, by adding 150 μl to each well and incubating at (37°C for 1 h).

Immunohistochemistry

The antibodies used for immunohistochemistry were LF94 recognizing decorin, A76 recognizing perlecan, 2B6 recognizing chondroitin/dermatan-4-sulfate, GAG-β recognizing versican, and 913 recognizing nidogen/entactin are described above. Formalin-fixed and paraffin-embedded human rib sections were used as the positive control tissue for 2B6 antibody. Paraffin-embedded sections were dewaxed and rehydrated by sequential incubations in xylene, absolute ethanol, 90% ethanol, 70% ethanol, and water. Frozen kidney sections (10 μm) were used as positive control tissue for LF94 and A76. Sections of frozen tissues used for decorin, chondroitin/dermatan-4-sulfate, versican, and nidogen localization were fixed in 4% paraformaldehyde solution for 20 min (n = 6 ovaries). Frozen sections to be used for perlecan localization were fixed in periodate-lysine-paraformaldehyde fixative for 20 min (n = 4 ovaries).

After fixing or rehydrating, sections were washed in hypertonic PBS containing 0.274 M sodium chloride, 5.37 mM potassium chloride, 10 mM sodium phosphate, pH 7.2. Sections to be incubated with 2B6 or LF94 required predigestion with chondroitinase ABC lyase to expose the antigen. Fifty microliters of chondroitinase ABC lyase at a concentration of 0.05 units/ml in 0.1 M Tris acetate buffer, pH 8.0, was applied to each slide for 1 h at 37°C. The slides were washed in hypertonic PBS. All slides were blocked in hypertonic PBS containing 10% normal donkey serum (NDS) for 30 min at room temperature and then incubated with primary antibody diluted in hypertonic PBS containing 10% NDS (antibody dilutions for A76 was 10 μg IgG/ml or 1/200, LF94 1/200, 2B6 1/1000, GAG-β 1/1000 and for 913 1/200, negative controls normal mouse serum and normal rabbit serum 1/100) for 1 h. Sections were then rinsed

and incubated in a 1/25 dilution of biotin conjugated anti-mouse or anti-rabbit IgG (biotin-SP-conjugated AffiniPure F (ab')$_2$ fragment donkey anti-mouse or rabbit IgG (H&L); Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 min. Sections were then rinsed and incubated in avidin-biotin complex (VectorStain ABC Kit PK 4001 from Vector Laboratories Inc., Burlingame, CA) diluted 1/100 in hypertonic PBS for 15 min. Sections were then rinsed and then peroxidase detected using substrate diaminobenzidine from the DAKO Liquid DAB+ Substrate-Chromagen System (DAKO Corporation, Carpinteria, CA; catalog no. K3467). The sections were then rinsed and mounted in buffered glycerol and examined by light microscopy.

RESULTS

Ovarian Follicles

A total of 21 batches of 1–3-mm follicles from 21 ovaries were isolated, weighing a total of 5.43 g. Histological assessment was carried out on a random sample size of about 10% of the isolated follicles and revealed that 85% of follicles were healthy, while 15% were in early atresia, as determined by pyknosis of the granulosa cell layer. There were no regressed follicles present. The freshly isolated follicles and the ^{35}S-labeled follicles were pooled for further analyses.

Extraction of PGs

Three sequential extractions were carried out on the pooled follicles to separate the PGs into groups based on their cellular location. The initial urea extraction was designed to remove all soluble PGs, the urea plus Triton X-100 extraction to extract membrane-associated PGs, and the sodium hydroxide extraction to solubilize the remaining tissue components. Thus, all constituents of the follicles were examined. Protein yields were 172 mg in the urea extract, 81 mg in the urea/Triton X-100 extract, and 85.3 mg in the sodium hydroxide extract. Glycosaminoglycan analyses of these samples were not possible due to high protein levels interfering with the GAG assay.

Ion-Exchange Chromatography

The urea, urea/Triton X-100, and sodium hydroxide extracts were chromatographed on a DEAE Sephacel column to separate the PGs from the majority of the proteins present. The majority of GAGs were recovered from the column with 2.0 M NaCl buffer (Fig. 1). The urea extract elution profile (Fig. 1A) revealed that 94.6% of protein and 98.7% of radiolabeled components were present in the flow through and urea buffer washes, while 85% (110.29 μg) of the GAG was eluted with 2.0 M NaCl. Thus, the majority of protein was separated from the PGs present. This pattern was also observed in the urea/Triton X-100 extract (Fig. 1B) in which 95.9% of protein and 88.7% of radiolabeled components were present in the flow through and the urea wash, separated from 80% (34.59 μg) of GAG that eluted with 2.0 M NaCl. The sodium hydroxide extract (Fig. 1C) showed 93.8% of protein and 42% of radiolabeled components in the flow through and urea wash. Eighty-eight percent (29.5 μg) of GAG eluted with 2.0 M NaCl as did 44% of radiolabeled components. Thus, in contrast to the previous two extracts, the GAGs in the sodium hydroxide extraction were radiolabeled.

The GAG-containing fractions for each extract that eluted with 2 M NaCl were pooled for further analyses. As the

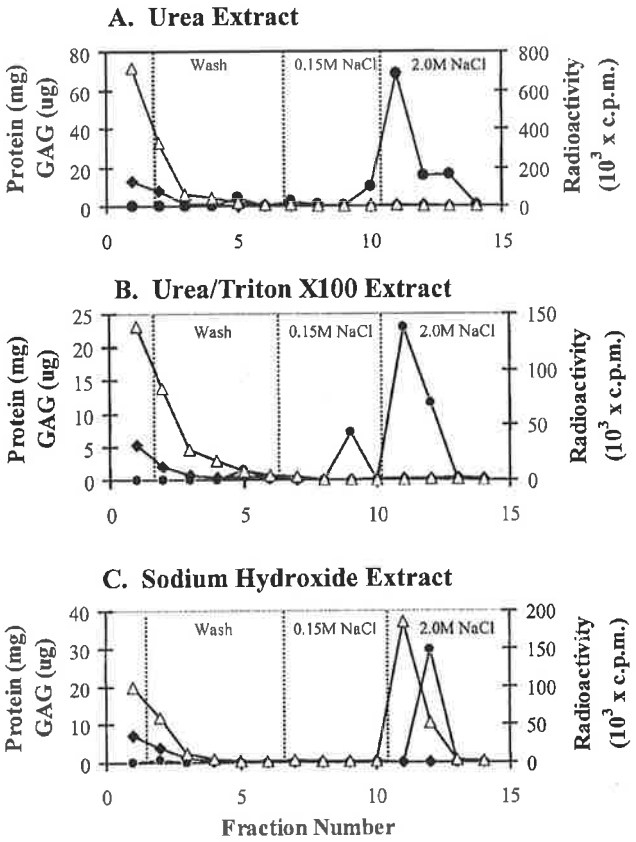


FIG. 1. Anion-exchange chromatography on a DEAE cellulose column (2 ml) of each of the sequential tissue extracts made from small-antral bovine follicles: **A**) urea extract, **B**) urea/Triton X-100 extract, and **C**) sodium hydroxide extract. The column was developed with the urea extraction buffer (6 M urea, 0.05 M sodium acetate, 0.1 M δ-amino-caproic acid, 0.1 M disodium salt EDTA, pH 5.0; urea wash), followed by 0.15 M NaCl, and then 2.0 M NaCl in the urea extraction buffer. The eluted fractions were analyzed for their concentrations of protein (♦), GAGs (●), and ^{35}S (△).

urea/Triton X-100 extract contained only 34.6 μg of uronic acid (fractions 11–12, Fig. 1B) these were pooled with the urea extract (fractions 11–12, Fig. 1A) to allow further analyses. This will now be referred to as the urea-urea/Triton X-100 extract. The sodium hydroxide extract contained only 29.5 μg of uronic acid, but as the PGs were affected by the sodium hydroxide treatment and because the GAGs in this extract were radiolabeled, these fractions (fractions 11–12, Fig. 1C) were maintained as a separate pool for further analyses.

Size-Exclusion Chromatography

The GAG-containing fractions of the urea-urea/Triton X-100 extracts (fractions 10–13 in Fig. 1A and fractions 11–12 in Fig. 1B) were combined and chromatographed on a sepharose CL2B column (Fig. 2A). A small peak of a large PG eluted with a K_{av} range 0.24 to 0.57 (Fig. 2A, bar 1). A large polydisperse peak of GAG eluted with a K_{av} range of 0.57 to 0.81 (Fig. 2A, bar 2) and had a shoulder ranging up to 1.0 (Fig. 2A, bar 3). All column fractions were retained for ELISA analyses.

The GAG-containing fractions of the sodium hydroxide extract (fractions 11–12, Fig. 1C) were combined and chromatographed on a sepharose CL4B column (Fig. 2B). A

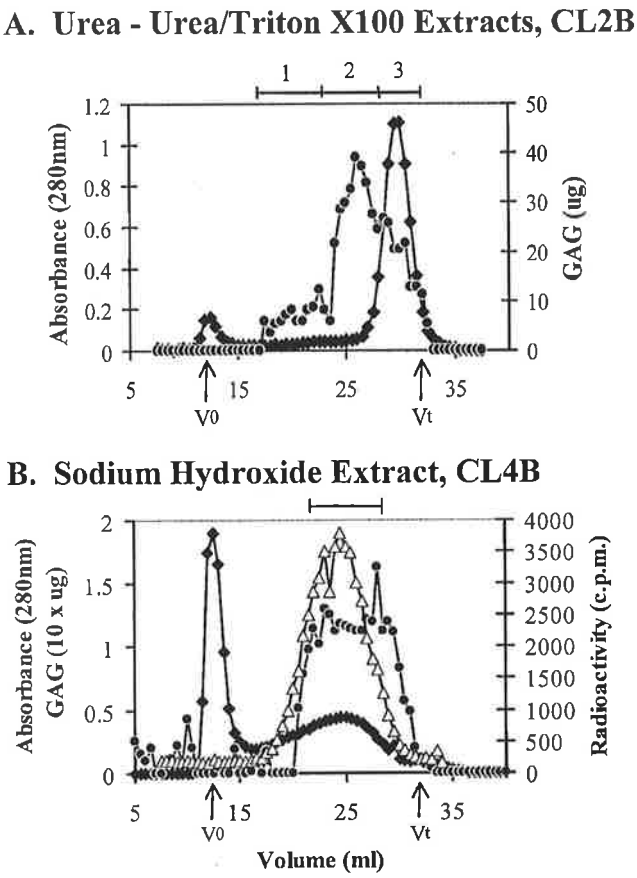


FIG. 2. Size-exclusion chromatography of PGs isolated by anion-exchange chromatography of three sequential extracts of small-antral bovine follicles. **A)** The urea and urea/Triton X-100 isolates were combined and then chromatographed on 90-cm size-exclusion sepharose CL2B column (elution buffer of 2 M guanidine, 0.1 M sodium acetate, 0.05 M Tris, pH 7.5); 1, 2, 3 represent three peaks of PGs. **B)** The sodium hydroxide isolates were chromatographed on 90-cm size-exclusion sepharose CL4B column; 1 represents a peak of PG. The eluted fractions were analyzed for their concentrations of protein (♦), GAGs (●), and ^{35}S (△). No ^{35}S was detected in **A**.

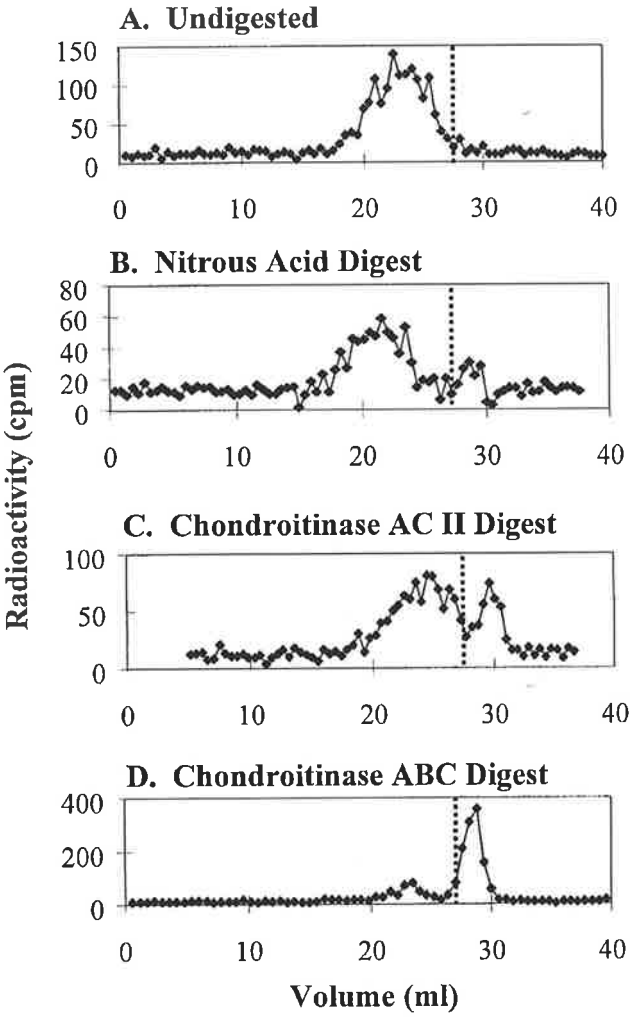


FIG. 3. Glycosaminoglycan characterization of PGs isolated from the sodium hydroxide extract of small-antral bovine follicles by ion-exchange chromatography. These PGs were either **A)** untreated, **B)** treated with nitrous acid to cleave HS, **C)** digested with chondroitinase ACII lyase to remove CS, or **D)** digested with chondroitinase ABC lyase to remove CS and DS. These digests were each separately chromatographed on a 90-cm size-exclusion sepharose CL4B column (elution buffer of 2 M guanidine, 0.1 M sodium acetate, 0.05 M Tris, pH 7.5), and the ^{35}S in each eluted fraction was measured.

single polydisperse peak of GAG, corresponding to a peak of radioactivity eluted with a K_{av} range of 0.5–0.9 (Fig. 2B, bar 1). Fractions from this peak were pooled for GAG identification by enzyme digestion.

Characterization of GAGs

Aliquots of the sodium hydroxide extract were digested with GAG-degrading enzymes and rechromatographed on the sepharose CL4B size-exclusion column. The degree of GAG digestion was assessed by calculating the percentage of radiolabeled PGs that had shifted from the undigested PG peak (elution volume 17–27 ml, Fig. 3A) after enzyme degradation or nitrous acid cleavage. Of the PGs present 20.3% were susceptible to nitrous acid cleavage, indicating the presence of HS (Fig. 3B), 38.7% were susceptible to chondroitinase ACII digestion (Fig. 3C), and 70% were susceptible to chondroitinase ABC lyase digestion (Fig. 3D). Thus, the PG(s) present in the sodium hydroxide extract contained a majority of CS, followed by DS, followed by a small amount of HS.

Identification of GAGs and PGs

The presence of different GAGs and PGs in individual column fractions from the CL2B chromatograph of the urea/Triton X-100 extract was determined by testing column fractions for reactivity to antibodies against defined GAG and PG epitopes. Positive controls of purified PG preparations and PGs containing the appropriate GAGs were included.

The antibody 2B6 that recognizes 4-sulfated CS/DS GAG identified three peaks of reactivity (Fig. 4A) that corresponded to the GAG profile observed earlier (Fig. 2A). This indicates the presence of more than one CS/DS-containing PG in the urea-urea/Triton X-100 extract. Reactivity to the antibody 3B3 was also tested; however, no reactivity was observed, indicating that there was no or little CS/DS-6-sulfated GAG in this extract.

Reactivity to the antibody GAG-β that recognizes the

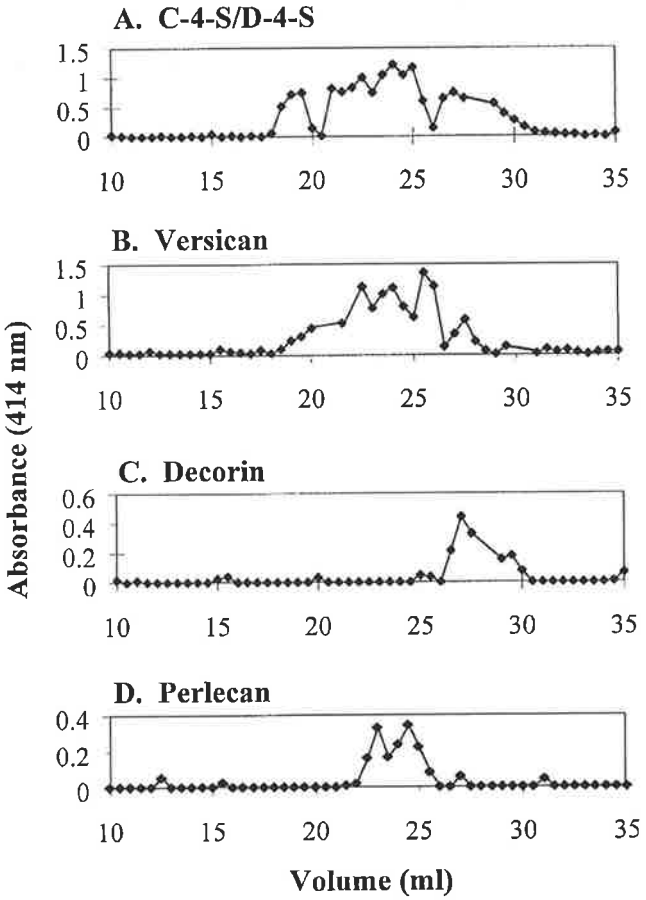


FIG. 4. Characterization of PGs isolated by ion-exchange chromatography of the urea-urea/Triton X-100 extract of small-antral bovine follicles. These PGs were chromatographed on a size-exclusion 90-cm sepharose CL2B column and the eluted fractions were analyzed by ELISA for A) chondroitin/dermatan-4-sulfate, B) versican, C) decorin, and D) perlecan.

CS PG versican was also tested (Fig. 4B). A polydisperse peak of reactivity was observed that overlapped the eluted second peak of 2B6 reactivity. Antibodies to components of the large aggregating cartilage PG aggrecan and to the PG biglycan were also tested; however, no reactivity was observed (data not shown). There was no reactivity observed in the sodium hydroxide extract to any of the antibodies tested. This may have been due to sodium hydroxide extract containing only a small amount of PG or to a reduction in the ability of any PGs to bind to the ELISA tubes following the sodium hydroxide treatment.

The antibody LF94 recognizes the core protein of decorin, which is a 4-sulfated CS/DS PG. One peak of reactivity to LF94 was observed (Fig. 4C) that corresponded to the third eluted peak of CS/DS-containing PGs observed using the 2B6 antibody (Fig. 4A). Thus, it appears that decorin is present in the follicle extract. To confirm that this peak corresponded to decorin, purified decorin isolated from bovine articular cartilage was also chromatographed on the CL2B column. This purified decorin preparation eluted in the same position as both the decorin and 4-sulfated CS/DS antibody reactive peaks observed in the extract (results not shown).

Reactivity to the antibody A76 that recognizes the HS PG perlecan was also tested (Fig. 4D). One peak of reactivity was observed indicating the presence of perlecan. This peak coincided with the later fractions that eluted in the second peak of 2B6 reactivity.

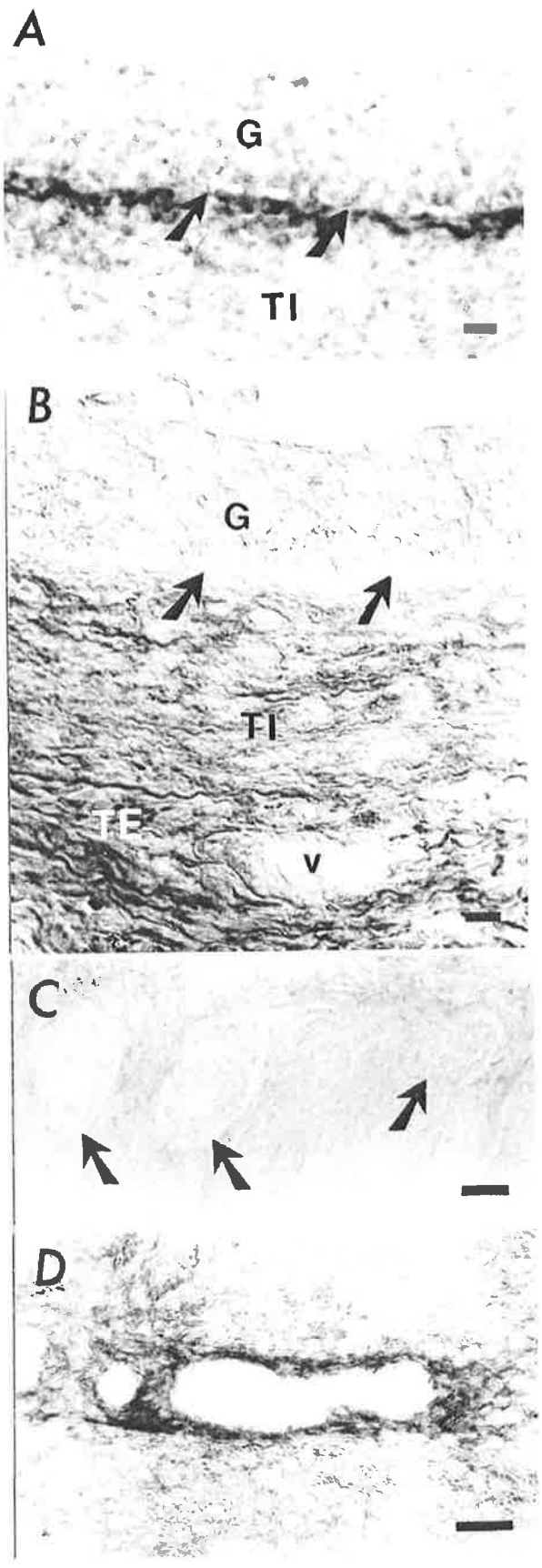
These results indicate the presence of a large amount of 4-sulfated CS/DS reactivity in the urea-urea/Triton X-100 extract. One portion of this CS/DS reactivity has been identified as the DS SLRP decorin in peak 3 (Fig. 4), and versican was also identified in peak 2. The modular HS PG perlecan was also observed in the extract. The remainder of CS/DS reactivity was unaccounted for, indicating the presence of larger unidentified PGs in peak 1.

Immunolocalization of PGs

In antral follicles CS/DS-4-sulfate immunoreactivity was observed extracellularly in the theca interna of healthy (Fig. 5A), atretic, and regressing antral follicles, with highest concentration adjacent to the follicular basal lamina (n = 6 ovaries examined). The follicular basal lamina may have been positively stained, but Call-Exner bodies (result not shown) were not stained, suggesting that the CS/DS-4-sulfate may not be a component of the follicular basal lamina. Weaker staining was observed in the membrana granulosa, and the staining was extracellular. Not all follicles (Fig. 5B) had the same staining pattern; in some the staining was weaker adjacent to the basal lamina. In addition CS/D-4-sulfate immunoreactivity was localized to filamentous material within the theca externa layers (Fig. 5B) and in the connective tissue septa surrounding large blood vessels (Fig. 5D). No staining was associated with primordial or preantral follicles (Fig. 5C). Negative control sections were those in which normal mouse serum was substituted for primary antisera (Fig. 6, A and B) or where primary antisera was employed but without prior chondroitinase digestion required to expose the antigen (not shown). No staining was observed in these controls.

Primordial and preantral follicles showed little or undetectable levels of immunoreactivity for versican (n = 6 ovaries examined) (Fig. 7A). Immunoreactivity was observed extracellularly in the theca interna of healthy antral follicles (Fig. 7B), in high concentration at or near the follicular basal lamina. Additional staining was observed extracellularly in the more antrally situated granulosa cells. No staining was observed in Call-Exner bodies (result not shown). Strong immunoreactivity was seen in atretic (Fig. 7C) and regressed (Fig. 7D) follicles in the area occupied by the theca interna. Normal rabbit serum was used in place of primary antisera in negative control sections (Fig. 6, C and D).

In all healthy antral follicles examined perlecan was immunolocalized (n = 4 ovaries) to the follicular basal lamina separating the membrana granulosa from the surrounding thecal layers (Fig. 8, A and B). Call-Exner bodies (Fig. 8A) present in some early or small-antral follicles were positively stained. Perlecan was also detected in the follicular basal lamina of atretic antral and regressed follicles, indicating that perlecan is not lost during follicular regression. Staining was weak or undetectable in preantral follicles (results not shown). No staining was observed in the membrana granulosa or in the thecal cell layers apart from that associated with blood vessels in the theca. Strong perlecan immunoreactivity was seen in the subendothelial basal laminas of capillaries, arterioles, and larger blood venules in both the theca and ovarian stroma. Less intense staining was localized to the basal laminas associated with ovarian arteriolar smooth muscle cells. The negative control used was normal mouse serum. There was no staining observed



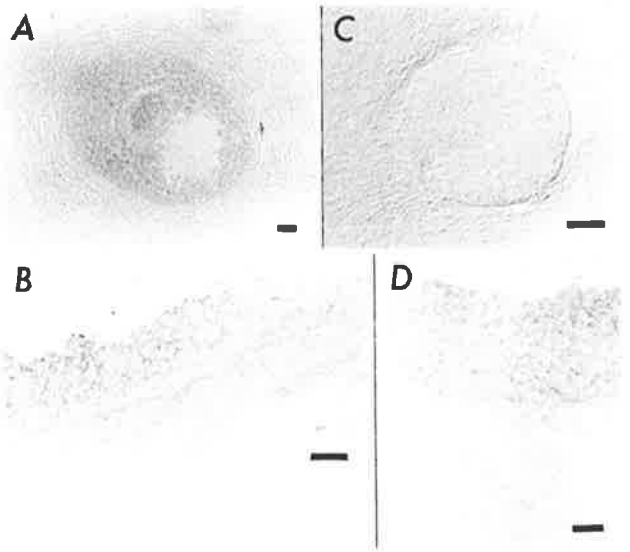


FIG. 6. **A, B**) Control sections of bovine antral follicles in which the primary antisera have been replaced by normal mouse serum show no positive staining. **C, D**) Control sections of bovine follicles in which the primary antisera have been replaced by normal rabbit serum show no positive staining. Sections **B** and **D** were pretreated with chondroitinase ABC lyase. **A** = 170 × 240-μm follicle; **B** = 1-mm follicle; **C** = 90-μm preantral follicle; **D** = 275 × 375-μm antral follicle. Scale bars = 20 μm in **A, C,** and **D,** and 50 μm in **B.**

within the ovary (Fig. 6B) when normal mouse serum was substituted for the primary antibody.

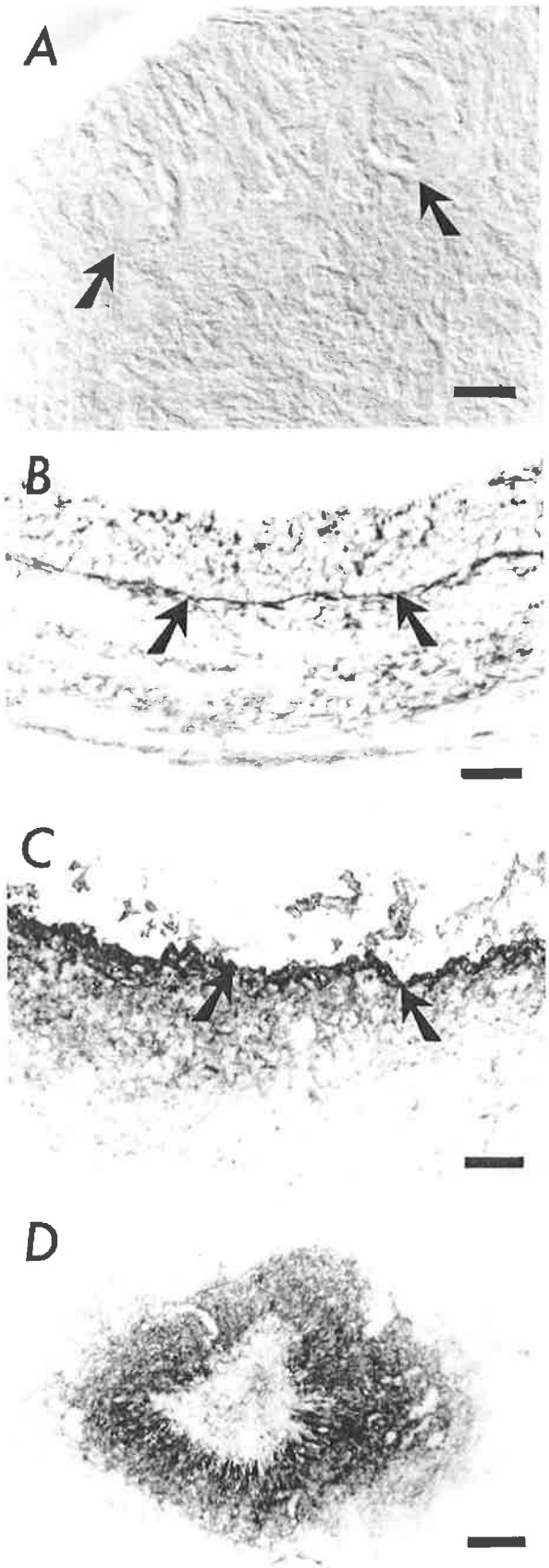
Immunoreactivity to nidogen (n = 6 ovaries) was confined to basal laminas of structures within ovary. Little or undetectable staining was observed in primordial and small-antral follicles (Fig. 8C); however, within antral follicles nidogen was immunolocalized to follicular basal lamina of healthy, atretic, and regressed follicles. Call-Exner bodies present in early and small-antral follicles also stained positively (Fig. 8D). Nidogen was also immunolocalized to the subendothelial basal laminas of the ovarian vasculature (capillaries, venules, and arterioles) and the basal lamina associated with arteriolar smooth muscle cells (Fig. 8D). No staining was observed when normal rabbit serum was substituted for the primary antisera in control sections (Fig. 6, C and D).

No specific staining was observed in the ovary sections incubated with the antibody LF94 to decorin (results not shown). In spite of this antibody reacting with solubilized decorin in the ELISA assays it is suspected that decorin in tissue is in a different confirmation and thus unable to be detected by this particular antibody.

DISCUSSION

This study identified PGs in small bovine ovarian follicles, not just follicular fluid. Radiolabeled sulfate was in-

FIG. 5. Immunolocalization of 4-sulfated CS/DS in the bovine ovary. In many antral follicles (**A**), but not all (**B**), staining was strongest in the theca interna (TI) adjacent to the basal lamina (arrows), and weaker staining was present in the membrana granulosa (G). Staining was associated with fibrillar structures within ovarian stroma tissue or in the theca externa (TE). Staining was absent from primordial (arrows) and preantral follicles (**C**). 4-Sulfated CS/DS was immunolocalized to the connective tissue sheaths surrounding bloods vessels (**D**). Scale bars = 10 μm in **A** and **B**, 20 μm in **C**, and 50 μm in **D**. V, Blood vessel.



corporated into the GAG chains of PGs synthesized during culture of follicles. Radiolabeled PGs were present only in the sodium hydroxide extract, when clearly there was a high yield of GAGs in the other extracts, implying that these GAGs were not from the same cellular pool. Thus we analyzed both PGs with unlabeled GAGs by quantitating GAGs directly and by ELISA assay, and PGs with labeled GAGs by enzymic and chemical digestion of the GAG chains from the PGs. Following column chromatography versican, decorin, and perlecan were identified; aggrecan and biglycan were not detected. Versican and perlecan, and a basal lamina component, nidogen/entactin were then immunolocalized in ovarian follicles.

The GAGs newly synthesized by the follicles in culture in the current study had both CS and DS and less HS. Glycosaminoglycans with DS and CS have previously been documented in the follicular fluid of bovine, porcine, and human ovarian follicles [2, 5–9, 11, 12]. Heparin sulfate has also been observed in rat ovarian slices [13] and in human follicular fluid [10]. The CS/DS GAGs were mostly 4-sulfated. This is also the case in bovine follicular fluid GAGs [6], while porcine follicular fluid contains unsulfated, 4-sulfated, and 6-sulfated CS/DS GAG chains [2], and human follicular fluid contains both unsulfated and 6-sulfated GAGs. Thus there is clearly species variation in the level and type of sulfation of the GAGs in follicles.

The PGs containing CS/DS identified in the current study were a diverse group of PGs. Three peaks of immunoreactivity for 4-sulfated CS/DS were identified by size-exclusion chromatography, corresponding to three peaks of GAGs as measured by uronic acid analyses. This indicated the presence of at least three species of PGs within the follicles that were relatively rich in CS/DS. The first peak (K_{av} 0.24–0.57) indicated that the PGs were of similar size to a CS/DS PG reported in the follicular fluid of bovine follicles by Grimek et al. [2]. Yanagishita et al. [2] also characterized a PG of similar size (K_{av} 0.26) in porcine follicular fluid. This PG was found to have a core protein of approximately 400 000, with an average of 20 DS chains attached. Thus, it appears that some PGs present in both bovine and porcine follicles are of similar sizes. This peak overlapped the peak of immunoreativity of versican, and whether this peak contains only versican remains to be determined.

The second, but broad peak of CS/DS reactivity eluted from a sepharose CL2B column with a K_{av} range of 0.57–0.81. This again confirms the observations of Grimek et al. [6], who documented the majority of GAGs of bovine follicular fluid eluted from a sepharose CL2B column with a K_{av} of 0.65. We were able to show that peak 2 also coincided with immunoreactivity to the antibody GAGβ1, specific to the core proteins of bovine versican. Versican contains CS, but whether there are further CS-containing GAGs in the second broad peak has still to be determined. Cultured rat granulosa cells have also been observed to produce an HS PG in this size range, of K_{av} 0.62 [16, 17]. Perlecan a basal lamina HS PG, produced by human colon carcinoma cells [34] has been characterized by sepharose

FIG. 7. No positive staining for versican was observed in association with primordial or preantral follicles (**A**, arrows). Staining was concentrated at the theca side but toward the follicular basal lamina (arrows in **B** and **C**) in healthy follicles (**B**) but more so in severely atretic (**C**) and regressed (**D**) follicles. Scale bars = 20 μm in **A**, 50 μm in **B** and **C**, and 100 μm in **D**.

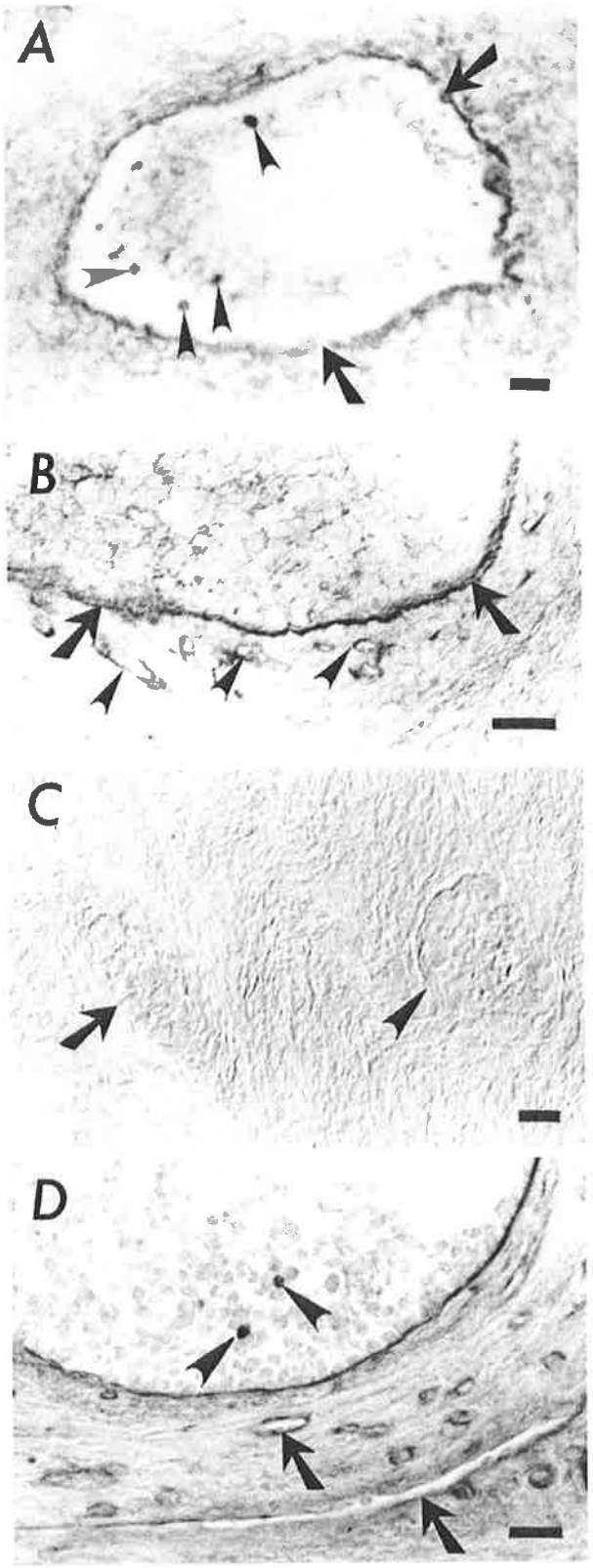


FIG. 8. Immunolocalization of perlecan (**A** and **B**) and nidogen (**C** and **D**). Perlecan localized to the follicular basal lamina (**A** and **B**, arrows), Call-Exner bodies (arrowheads in **A**), and subendothelial basal laminas (arrowheads in **B**). Nidogen was absent from the basal lamina (arrow or arrowhead in **C**) in primordial and preantral follicles but was a component of the follicular basal lamina of antral follicles (**D**), Call-Exner bodies (arrowheads in **D**), and blood vessels (arrows in **D**) within the ovary. Panel **A** shows a 375 × 500-μm follicle, **B** shows a 125 × 250-μm follicle, **C** shows primordial (arrow, 18 × 34-μm) and preantral (arrowhead, 30 × 55-μm) follicles, and **D** shows a 250 × 300-μm follicle. Scale bars = 20 μm in **A**, **B**, and **D**, and 10 μm in **C**.

CL2B chromatography to have a K_{av} of 0.57 [35]. Thus on this basis of reactivity to antibody A76 we were able to identify perlecan in extracts from follicles, eluting with the same elution profile as human perlecan [35]. This overlapped the second peak of GAGs identified.

The third peak of 2B6 reactivity had a K_{av} range of 0.81–1.0 on the CL2B column. A PG of this size has been observed in both bovine [6, 8] and porcine [2] follicular fluid; however, it was not identified by either investigators. The results demonstrate that this peak of 2B6 reactivity corresponds to a peak of reactivity to the antibody LF94, specific to the core protein of the CS/DS-containing PG, decorin. The presence of decorin was further confirmed by chromatographing a purified preparation of bovine decorin on the sepharose CL2B column, showing it to elute in the same position as the decorin immunoreactivity isolated from bovine follicles.

On identifying these PGs they were then immunolocalized in the ovary. The CS/DS sulfate was localized to the follicular basal lamina and the thecal and granulosa cell layers within the antral ovarian follicle. This localization pattern, of course, represents the presence of different PGs containing CS/DS GAGs. Versican is a large CS-containing PG secreted by fibroblasts. It has some domains highly homologous to aggrecan, and it has a hyaluronan-binding domain. Fibroblasts are present in the thecal layers but not in the epithelioid membrana granulosa. In agreement, versican was observed extracellularly in the theca interna of healthy antral follicles and at high concentrations at or near the follicular basal lamina. No staining was observed in Call-Exner bodies of the membrana granulosa, suggesting that if the versican is actually associated with the follicular basal lamina, it is not derived from the granulosa cells, as other components appear to be [36]. Strong immunoreactivity was seen in atretic and even regressed follicles.

Decorin was another CS/DS-containing PG identified here, but it could not be immunolocalized, presumably because the antibody would not recognize decorin by immunohistochemistry methods. However, decorin is a ubiquitous PG that is found associated with type I collagen fibrils throughout the body [37]. Decorin is known to regulate collagen fibril formation and thus is important in the development of the structure of the extracellular matrix [38]. The follicular decorin could be associated with type I collagen that has been shown to be present within the thecal layers of the bovine follicle [39]. In addition, decorin may also influence folliculogenesis by interacting with, and perhaps sequestering, growth factors such as transforming growth factor (TGF)β [40, 41]. Transforming growth factor-β has been identified in the theca of a number of species and effects on granulosa cells demonstrated [42]. Thus, decorin may not only be important in follicles for collagen fibril formation, it may also be involved in regulating the activity of TGFβ.

Perlecan is an HS-containing PG and was localized to the follicular basal lamina of antral ovarian follicles, both healthy and atretic follicles. This indicates that perlecan is not lost from the follicular basal lamina during atresia and

regression. It has previously been observed that the follicular basal lamina contains an unidentified HS-containing PG [43], and the results of this study suggest that this PG may be perlecan. Immunostaining of perlecan in primordial or preantral ovarian follicles was weak or undetectable in the follicular basal lamina. Thus in the follicular basal lamina perlecan, like subunits of laminin [44] and collagen IV [45], appears to be developmentally regulated. Generally the main function of perlecan in the basal lamina is thought to be structural [46]. Perlecan has been shown to interact with other components of the basal lamina, including laminin, [47], type IV collagen [48], and fibronectin [49]. It is likely that perlecan thus stabilizes the follicular basal lamina during folliculogenesis.

The presence of perlecan in the follicular basal lamina may also have an important role in formation of follicular fluid. It has been proposed that the basal lamina acts as a selective filter of serum during fluid accumulation [50]. Perlecan has been implicated in the filtration of serum in the glomerulus of the kidney [51], due to the large anionic charge of the HS GAG chains. Thus, perlecan expression at the time of antrum formation may be a key event in follicular fluid formation.

Perlecan can also interact with growth factors or their binding proteins via its HS side chains. Some of these that are known to be expressed in ovaries that can bind HS include follistatin, a binding protein for activin, insulin-like growth factor binding protein 5, TGFβ, and fibroblast growth factor-2 (FGF2) [52]. Thus, localization patterns showing FGF2 in the follicular basal lamina [53] is probably a reflection of the presence of HS in the basal lamina. These HS PGs are not merely passively acting to sequester growth factors or their binding proteins as perlecan has been shown to increase FGF2-mediated angiogenesis and mitogenesis in vitro [52].

Perlecan was also observed in the subendothelial basal lamina of the thecal vasculature but less in the basal laminas of arteriolar smooth muscle, as we observed previously for the β1 chain of laminin [44]. It confirms the observations of Murdoch et al. [54] who localized perlecan within all vascular basal laminas in the major human tissues such as the kidney, liver, and heart. Perlecan was also localized to Call-Exner bodies that are aberrant basal lamina material located in the membrana granulosa and highly likely to be derived from granulosa cells [36]. Thus, it is likely that perlecan in these different basal laminas that are present in follicles represent different cellular sources of perlecan within the follicle.

Nidogen or entactin is not a PG but a sulfated glycoprotein that is an integral component of basal laminas. It has the capacity to associate with laminin and collagen type IV, and thus is thought to stabilize basal laminas. It was present in the follicular basal lamina in antral follicles, healthy, atretic, and regressed. However, little or undetectable staining was observed in primordial and small-antral follicles. Thus, nidogen is also developmentally regulated during follicular development. Its expression pattern parallels the increasing levels of laminin that occurs in follicles when they reach the antral stage [44]. Call-Exner bodies present in early and small-antral follicles also stained positively for nidogen, indicating that granulosa cells maybe the source of the nidogen in the follicular basal lamina. Nidogen was also immunolocalized to the subendothelial basal laminas of the ovarian vasculature (capillaries, venules, and arterioles) and to the basal lamina associated with arteriolar smooth muscle cells.

Versican probably has a multitude of functions because in different tissues it has a diverse range of expression patterns and it itself has different domains with different functions [55–57]. Even within tissues the expression pattern of versican is variable, sometimes in basal laminas, epithelia, or stroma, and often it is not uniformly expressed. Versican also has the ability to bind to a variety of extracellular matrix components, such as hyaluronin, fibulin 1, and L-selectin, and it is considered to be important in stabalizing extracellular matrix [58, 59]. Versican is also considered to be antiadhesive by the nature of it CS side chains, and it acts to stimulate cell migration and replication [60, 61]. Versican is developmentally regulated, and its expression is important for differentiation. In follicles versican was found to be developmentally expressed, not appearing until the antral stage when theca develops in bovine follicles. The thecal side of the follicular basal lamina was particularly enriched with versican, and the theca externa and membrana granulosa also contained versican. Whether the isoforms of versican in these areas are identical, and whether versican has many roles in follicular development remains to be determined.

Both versican and perlecan have been identified in human follicular fluid aspirated from follicles approaching ovulation following hCG injection [12]. Our studies show perlecan, which is a classical basal lamina component, to be localized to vascular basal laminas of the theca and to the follicular basal lamina. Thus, perlecan is probably not a true component of follicular fluid during follicular growth and development. It was possibly aspirated into the follicular fluid by the sampling procedure in the experiments where it was reported in follicular fluid [12], or alternatively, it only enters the follicular fluid on ovulation when the follicular basal lamina is degraded.

In summary, a number of PGs were identified and localized in the ovary. Their roles in follicle development have been implied from their known functions in other tissues, but these still need to be confirmed. The differential expression patterns of some of these suggest that they may have key roles in follicular development.

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Statement of Authorship

A novel basal lamina matrix of the stratified epithelium of the ovarian follicle

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Statement of Authorship

Remodelling of extracellular matrix at ovulation of the bovine ovarian follicle

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Statement of Authorship

Extracellular matrix of the human cyclic corpus luteum

Irving-Rodgers HF[1], Friden BE[2], Morris SE[1], Mason HD[3], Brannstrom M[2], Sekiguchi K[4], Sanzen N[4], Sorokin LM[5], Sado Y[6], Ninomiya N[7], Rodgers RJ[1]

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Statement of Authorship

Extracellular matrix of the human cyclic corpus luteum

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Statement of Authorship

Atresia revisited: Two basic patterns of atresia of bovine antral follicles

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Composition and morphology of the follicular basal lamina during atresia of bovine antral follicles

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Statement of Authorship

Dynamic changes in the expression of relaxin-like factor (Insl3), cholesterol side chain cleavage cytochrome P450, and 3β-hydroxysteroid dehydrogenase in bovine ovarian follicles during growth and atresia

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Statement of Authorship

Dynamic changes in the expression of relaxin-like factor (Insl3), cholesterol side chain cleavage cytochrome P450, and 3β-hydroxysteroid dehydrogenase in bovine ovarian follicles during growth and atresia.

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Ivell R (co-author)

Roger Domagalski acted under my supervision in my laboratory in Hamburg in raising antibodies, and I sign to this affect.

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Dynamic Changes in the Expression of Relaxin-Like Factor (Insl3), Cholesterol Side-Chain Cleavage Cytochrome P450, and 3β-Hydroxysteroid Dehydrogenase in Bovine Ovarian Follicles During Growth and Atresia[1]

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ABSTRACT

Relaxin-like factor (RLF) is a new member of the insulin-relaxin gene family known to be expressed in the ovarian follicular thecal cells of ruminants. To investigate the pattern of RLF expression in development and atresia of bovine follicles, antisera were raised in rats and rabbits to recombinantly expressed bovine pro-RLF and to chemically synthesized ovine RLF B chain, respectively. On dot blotting analysis, the rat anitserum bound to pro-RLF and less strongly to a synthetic mature ovine RLF lacking the C-domain, whereas the rabbit antiserum bound the mature form of ovine RLF. These antisera were used to immunostain bovine ovarian follicles of differing sizes and stages of health and atresia. 3β-Hydroxysteroid dehydrogenase was colocalized with pro-RLF (n = 86 follicles), and cholesterol side-chain cleavage cytochrome P450 was localized in another section of many of the same follicles (n = 66). Not all follicles expressed pro-RLF in the theca interna, so the results are presented as the proportion of follicles expressing pro-RLF. Both mature and pro-RLF were immunolocalized to steroidogenic thecal cells of healthy follicles. As follicles enlarged to >5 mm, the proportion expressing pro-RLF declined (19/19 for <5 mm and 18/26 for >6 mm). Atresia was divided into antral (antral granulosa cells dying first) or basal (basal cells dying first) and further divided into early, middle, and late. For antral atresia of small follicles (2–5 mm), no decline in the proportion expressing pro-RLF was observed (early 6/6, middle 2/2) until the late stages (1/4). For basal atresia, which only occurs in small follicles (2–5 mm), the proportion expressing pro-RLF declined in the middle (2/5) and late (0/8) stages. In larger follicles (>6 to <10 mm), the proportion expressing pro-RLF also declined with atresia (1/13). These declines in RLF expression with atresia or increasing size were not accompanied by a decline in the expression of steroidogenic enzymes in the theca interna. A significant ($P < 0.001$) inverse relationship in the expression of pro-RLF and 3β-hydroxysteroid dehydrogenase in the membrana granulosa was observed. We conclude that the expression of pro-RLF in the theca interna is switched off as follicles enlarge or enter atresia, whereas the expression of steroidogenic enzymes is maintained in the theca interna.

follicle, follicular development, ovary, relaxin, steroid hormones, thecal cells

INTRODUCTION

A relatively new member of the insulin/insulin-like growth factor/relaxin family, relaxin-like factor (RLF), is the product of the *INSL3* gene. It was initially characterized as the Leydig insulin-like peptide (Ley-I-L), and it was found to be highly expressed in porcine Leydig cells [1]. More recently, the RLF gene was also found to be expressed in the human ovary and trophoblasts [2] and in the mouse testis [3, 4]. Within the ovary, RLF immunoreactivity has been demonstrated in the mouse corpus luteum (CL) [5], the marmoset (*Callithrix jacchus*) [6] and human [7] CL and theca interna layer of antral follicles, and the theca and granulosa layers of the dog ovary [8]. These data together with the similarity in structure of the derived protein sequence between RLF and relaxin, which includes a conserved although shifted relaxin-like receptor binding site, led to the name RLF [9].

In the majority of species, the apparent levels of RLF mRNA and protein found in nontesticular tissues is generally very low, often only detectable by reverse transcription polymerase chain reaction (RT-PCR) or very sensitive immunohistochemistry [3]. An exception is the ovary of cows and sheep, where both thecal cells and CLs are the sites of a very high level of expression, comparable to that in the testicular Leydig cells. The pattern of RLF mRNA expression in the bovine CL is very similar to that of relaxin in some other species [10, 11]. RLF may be expressed as a compensatory reaction to the apparent deletion of the relaxin gene in ruminants [12, 13]. The highest expression of RLF mRNA in the cow is in follicular theca interna [11]. RLF mRNA appears to be expressed in the theca interna at a higher level in small antral follicles than in larger, presumably preovulatory, follicles. As in the Leydig cells of the testis [5], the pattern of RLF gene expression appears to reflect the differentiation state of the ovarian thecal and luteal cells.

At the protein level, specific antibodies raised against either recombinant protein or peptide fragments have detected RLF protein in Leydig cells, CLs, and theca interna of different species [3, 6]. The expression patterns corresponded to the mRNA localization determined by in situ and Northern hybridization and by RT-PCR. Studies of RLF in theca interna have been hampered by the lack of antisera that react with RLF from species that express RLF at high

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levels in the theca interna, such as ruminants. The theca interna is a complex stromal layer that differentiates in bovine follicles at around the time of antrum formation. It has many cell types, including endothelial and smooth muscle cells of the vasculature, steroidogenic differentiated thecal cells, connective tissue fibroblasts, and leukocytes. It is highly plastic and expands during antrum expansion as follicles grow. In addition, the theca interna appears to play a role in one of two types of atresia that occur in bovine follicles [14].

The role of RLF in testis and ovary is not known, but studies using knockout mice, where the *INSL3* gene was partially ablated, suggest that it is important for reproduction [15, 16]. The principal defect in the knockout mice was the failure of the testes to descend into the scrotum. Female homozygous mice were fertile, although litter size appeared to be slightly reduced, and most noticeably the length of the estrous cycles was extended from the usual 4–5 days to >15 days [15]. Given the consistent pattern of RLF expression in follicles and CLs, these results suggest that RLF is probably playing an important role in follicular maturation or luteal function.

We produced antisera against recombinant bovine pro-RLF and synthetic mature ovine RLF. Using both types of antisera, we examined the expression of RLF in bovine follicles during follicular growth and atresia. Atretic follicles were also further classified as undergoing antral or basal atresia. These terms refer to the location in the follicle where death of granulosa cells occurs first; the latter type appears to involve thecal destruction and macrophage infiltration as an early event [14]. The enzymes involved in the synthesis of pregnenolone, cytochrome P450 side chain cleavage (SCC), and in the synthesis of progesterone, 3β-hydroxysteroid dehydrogenase (HSD), were also immunolocalized, thus allowing us to identify precisely when RLF is first expressed, relative to these markers of cell type and maturation, and in which cell types of the theca interna.

MATERIALS AND METHODS

Antisera to Bovine Pro-RLF

The cDNA for the bovine RLF-encoding transcript [12] was recloned into the expression vector pH6Ex5, a derivative of pGEX-5T [17], such that the pro-form, lacking the signal peptide, was directly colinear and in-frame with the N-terminal hexahistidine Tag incorporated into the multiple cloning site of the vector. The N-terminus of the resulting fusion protein was thus MSPIHHHHHHLVPRGSAQEA—, whereby the AQEA residues represent the postulated N-terminus of the RLF propeptide comprising the complete B, C, and A domains in order [11]. Correct plasmid construction was confirmed by sequencing. Competent *Escherichia coli* BL21(DE3) pLysS bacteria (Novagen, Madison, WI) were transformed with this plasmid construct, grown to an A_{575} of 0.5, induced with 0.4 mM isopropyl thiogalactopyranoside, and further incubated for 1–1.5 h. Longer induction times led to a considerable loss of the recombinant product. Bacteria were harvested by centrifugation, and the pellets stored at −70°C. Bacterial pellets were thawed, sonicated (30 sec) in 15 ml of sonication buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 1% Tween-20), and centrifuged for 30 min at 4°C to collect the cell pellet containing inclusion bodies. This pellet was resuspended in 10 ml of buffer A (6 M guanidinium hydrochloride, 0.1 M sodium phosphate, 10 mM Tris-HCl, pH 8.0), sonicated (2 × 45 sec on ice), and then incubated for 1 h at room temperature with gentle mixing. This homogenate was centrifuged again, and the supernatant was incubated with 5 ml of 50% (w/v) Ni-NTA agarose for 1 h at room temperature. Following chelation of the recombinant protein, the agarose was transferred to a column, progressively washed with 35 ml of buffer A, 20 ml of buffer B (8 M urea, 0.1 M sodium phosphate, 10 mM Tris-HCl, pH 8.0), and 20 ml of buffer C (8 M urea, 0.1 M sodium phosphate, 10 mM Tris-HCl, pH 6.3), and eluted with sequential 3-ml aliquots of buffer C with the addition of 0.25 M imidazol. These eluate fractions were monitored by SDS-PAGE and stained with Coomassie blue R250. The recombinant protein was clearly evident as a prominent 14.5-kDa band, and a Western blot using anti-5His antibodies (no. 34660, 1:1000; Qiagen, Hilden, Germany) verified its identity. Because of the presence of other larger nonspecific proteins in these eluate fractions, the fractions were combined and subjected to a second round of Ni-NTA chelation chromatography. The resulting recombinant protein was estimated by SDS-PAGE to be >80% pure. The resulting eluates were then dialyzed overnight at 4°C against PBS (Dulbecco, without Ca^{2+} and Mg^{2+}, no. 14200; Life Technologies, Karlsruhe, Germany), whereby the recombinant protein precipitated out. The precipitate was centrifuged, the pellet was resuspended in 1.5 ml of PBS, and the protein concentration was measured (total yield of 0.5–1.0 mg per 500 ml bacterial culture). Polyclonal antibodies were then generated in rats in our Hamburg laboratory using the suspended recombinant protein as immunogen, as previously described [5]. Of three sera showing specific Leydig cell immunoreactivity in preliminary tests, antiserum R33 indicated the highest specific titer and was used for all further studies. An additional antiserum (W3) was raised in a rabbit against a synthetic peptide (CGGPRWSSEEDG) from the predicted B domain of bovine RLF. This antiserum was used here only to immunopurify recombinantly expressed bovine RLF.

Antisera to Ovine RLF B Chain

Sheep RLF A and B chains were assembled by Fmoc solid-phase peptide synthesis and purified by conventional reverse-phase HPLC. The RLF A/B heterodimer was then produced by combining the chains in solution at high pH as previously described [18]. The purity and identity of the peptides was confirmed by chemical characterization including mass spectrometry [18]. The A/B heterodimer was used for testing the antibody by dot blotting.

Antibodies were raised in three rabbits in our Melbourne laboratory using limpet hemocyanin conjugated to the B chain only [19]. The specificity of the antiserum was tested by dot blotting against the RLF B chain and the complete RLF molecule. For the antibody (designated HFA) with the highest affinity for the RLF peptide, the IgG fraction was isolated using a Protein A column (HiTrap; Amersham Pharmacia, Uppsala, Sweden).

Dot Blot and Western Blot Analyses

For dot blot analyses, peptides or recombinant proteins were blotted onto nitrocellulose membranes (Millipore, Bedford, MA) dampened with distilled water. Membranes were allowed to dry for 30 min, were dampened again with distilled water, and were washed with 20 mM Tris buffer (pH 7.5) containing 0.5 M NaCl (TBS) for 5 min. The membranes were incubated for 1 h in 10% skim milk powder in TBS, washed briefly with TBS, and incubated with the relevant RLF antibodies in 3% skim milk powder in TTBS (TBS containing 0.05% Tween 20) for 1 h at room temperature on an orbital shaker. The membranes were then washed three times with TTBS for 10 min each time and incubated with anti-rabbit horseradish peroxidase-linked secondary antibody (1:2500; Biorad, Sydney, Australia) or anti-rat horseradish peroxidase-linked secondary antibody (1:2500, Chemicon, Temecula, CA) in 3% skim milk powder in TTBS for 1 h. Membranes were washed three times in TTBS for 10 min each time, and antibody-peptide complexes were visualized by chemiluminescence (ECL Western blotting detection kit; Amersham Pharmacia).

Western blot analyses were also carried out on recombinantly expressed pro-RLF that had been purified by immunoprecipitation. Two microliters (~20 ng) and 10 μl (~100 ng) of gel-purified recombinant probovine RLF were incubated with 5 μl of either rabbit preimmune serum or serum W3 in 490 μl of 10 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl and 0.05% Tween 20, for 1.5 h at 4°C on a rotating wheel. Then 50 μl of Protein-A-Sepharose Fast Flow Suspension (50% slurry; Amersham Pharmacia) was added, and the incubation was continued for 1.5 h. Bound immune complexes were pelletted by low-speed centrifugation for 20 sec. Pellets were then washed three times in 10 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl and 0.05% Tween 20 and then once in 50 mM Tris-HCl buffer, pH 7.5, and finally resuspended in 30 μl of 50 mM Tris-HCl buffer, pH 7.5, containing 1% SDS and 100 mM dithiothreitol. After heating to 95°C for 5 min and briefly centrifuging, 20 μl of the supernatant was subjected to SDS-PAGE and Western blotting using antibody R33, following standard procedures as per dot blotting.

Additional Antibodies

Serum from a rabbit immunized against bovine SCC purified from adrenal mitochondria [20] was obtained from OXYgene (Dallas, TX) as a gift from Dr. E. Simpson. Mouse monoclonal antibody FDO66Q was ini-

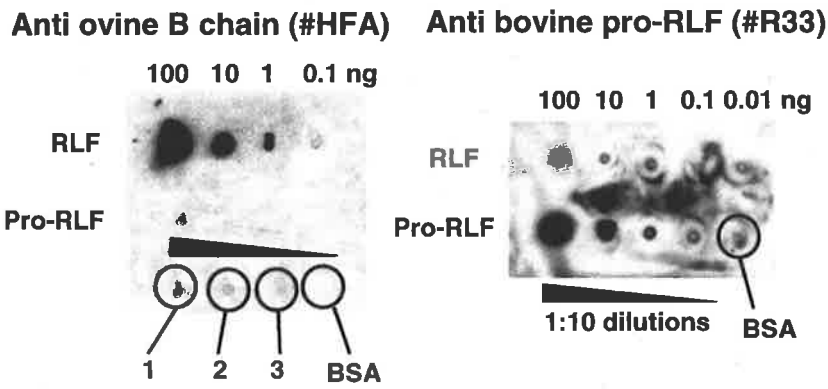

FIG. 1. Dot blot analyses showing antiserum reactivity and specificity. Anti-ovine RLF B chain (HFA, left) and anti-bovine pro-RLF (R33, right) were used to probe blots containing known amounts (0.01–100 ng) of ovine RLF (A and B chains synthesized and cross-linked by disulfide bonds to yield mature RLF), bovine pro-RLF (A, C, and B chains) recombinantly expressed in *E. coli*, and BSA as a control. Anti-ovine RLF was tested against another batch of recombinant bovine pro-RLF (A, C, and B chains) expressed in *E. coli* (1) and against recombinant bovine pro-RLF (A, C, and B chains) containing additional N-terminal amino acids, FLAG, and expressed in mammalian Cos cells (2). Western analysis of this Cos-expressed pro-RLF using antiserum to the FLAG sequence showed that this peptide was not proteolytically processed into mature RLF (A/B and C peptides). Appropriate nontransfected Cos cell controls (3) and BSA are shown.

tially raised against JEG choriocarcinoma cells [21] and IgG purified from mouse ascites fluid. This anitbody recognizes an epitope very similar to FDO161G [22, 23], which recognizes human 3βHSD type I. Type II is expressed in ovaries and is 94% homologous with type I in humans. FDO66Q binds to human ovarian follicles and Leydig cells (B. Kalionis, personal communication), indicating that it cross-reacts with 3βHSD type II.

Tissues and Histology

Antral follicles were harvested from the ovaries of young nonpregnant cows (*Bos taurus*) slaughtered at the local abattoir in South Australia and were transported to the laboratory in transport medium on ice. For small follicles (2–5 mm), 2 were collected per ovary, and for large follicles (6–17 mm), 1, 2, or 3 follicles were collected per ovary from 32, 2, and 1 ovary, respectively. Follicles and adhering stroma were dissected from each ovary and snap-frozen in Tissue Tek OCT compound (Miles Inc., Elkhart, IN). Small (2–5 mm, n = 87) or large (6–17 mm, n = 21) frozen follicles were bisected, and one half was immersed in 2.5% glutaraldehyde, postfixed in osmium tetroxide, and embedded in epoxy resin. Sections were cut at a thickness of 1 μm, stained with methylene blue, and examined by light microscopy. Thirteen large follicles (6–17 mm) were frozen in OCT only. Antral follicles were classified by light microscopy of methylene blue-stained sections where available or of frozen sections if resin-embedded sections were not available.

Immunohistochemistry

Tissue sections were cut from OCT-embedded bovine follicles using a CM 1800 cryostat (Leica Microsystems Pty. Ltd., Victoria, Australia), collected on glass slides treated with 0.01% poly-L-lysine hydrobromide (P-1524; Sigma Chemical Co., St. Louis, MO), and stored at −20°C until use. Unfixed sections of bovine follicles were dried under vacuum for 5 min and then incubated in 10% normal donkey serum (D-9663; Sigma) in antibody diluent containing 0.55 M sodium chloride and 10 mM sodium phosphate (pH 7.1) for 20 min. Sections were then incubated overnight with a combination of primary antibodies (rat anti-bovine pro-RLF, R33, 1:200; mouse anti-human 3β-HSD, FDO66Q, 1:1000) or with rabbit anti-bovine SCC (1 μg/ml IgG) alone. Secondary antibodies used were fluorescein isothiocyanate (FITC)-conjugated AffiniPure donkey anti-rat IgG (cat. 712-096-153, 1:100) and biotin-SP-conjugated AffiniPure donkey anti-mouse IgG (715-066-151, 1:100) or biotin-SP-conjugated AffiniPure donkey anti-rabbit IgG (711-066-152, 1:200), followed by Cy3 conjugated-streptavidin (016-160-084, 1:100), all from Jackson ImmunoResearch Laboratories (West Grove, PA), in antibody diluent. Immunostaining for pro-RLF was colocalized with 3β-HSD (n = 86), and staining for SCC was undertaken on another section (n = 66). Pro-RLF was coimmunolocalized with mature RLF (HFA antiserum, 1:20) as above (n = 11), secondary antibodies employed were Cy3-conjugated AffiniPure donkey anti-rabbit IgG (711-166-152, 1:100) and biotin-SP-conjugated AffiniPure donkey anti-rat IgG (712-066-153, 1:100), followed by FITC-conjugated streptavidin (016-090-084, 1:100). All incubations were carried out at room temperature in a humidified chamber. Following incubation with primary or secondary antibodies or streptavidin-conjugated reagents, sections were washed (3 × 5 min) in hypertonic PBS containing 0.274 M sodium chloride, 5.4 M potassium chloride, and 10 mM sodium phosphate, pH 7.2.

Light Microscopic Observations and Photography

Sections of bovine ovary stained with methylene blue were examined using an BX50 microscope (Olympus Optical Co. Ltd., Tokyo, Japan) and photographed with a SC35 camera attachment (Olympus) and FP-4 125 black-and-white film (Ilford Imaging UK Ltd., Cheshire, U.K.). Sections processed for immunofluorescence staining were observed and photographed with a Vanox AHBT3 fluorescence microscope (Olympus) with a C35AD-4 camera attachment (Olympus) and photographed with T-Max 400 black-and-white film (Kodak, Rochester, NY).

RESULTS

RLF Antibodies and RLF Expression

Polyclonal antisera were raised in rats against bovine pro-RLF recombinantly expressed in *E. coli*. Of these antisera, antiserum R33 had the highest specific titer in immunohistochemistry of bovine testis sections and was used in the present study. In dot blot analysis (Fig. 1) against the same batch of nickel-agarose-purified recombinant pro-RLF used as the immunogen and against the chemically synthesized mature RLF (ovine A and B chains covalently linked), antibody R33 bound to both forms, although more strongly to the pro-form (Fig. 1). Additional recent blots (not shown) observed that this antibody bound to the ovine A chain but not detectably to the ovine B chain. To additionally confirm the specificity of this antiserum, Western blots analyses of *E. coli* recombinantly expressed, gel-purified pro-bovine RLF, which was further purified by immunoprecipitation with another antibody directed against the B chain of bovine RLF (W3), clearly showed that this antiserum detects RLF pro-form at 14 kDa (Fig. 2).

Rabbit antiserum HFA was raised against the ovine B chain. The ovine RLF B chain is identical to the bovine form except for one less Glu residue at position 30, and the ovine A chain is identical to the bovine form except for a

conservative substitution at position 4 of Val for Ile. The antibody clearly bound to the mature form of RLF (ovine A and B chains covalently linked; Fig. 1). Binding to pro-RLF was tested using pro-RLF recombinantly expressed in *E. coli* or Cos cells. In the Cos cells, the pro-RLF was not further proteolytically processed into C and A/B fragments, as determined by Western analysis using antisera to the N-terminal extension containing the FLAG sequence (data not shown). Binding of HFA antibody to either of these bovine pro-RLF preparations was not significantly greater than that to a nonspecific control on dot blotting (Fig. 1).

The anti-pro-RLF antibody R33 bound to bovine Leydig cells and a population of cells in the theca interna in Bouin-fixed and paraffin-embedded tissue (data not shown). This finding was expected, based upon results from other species [3] and identification of RNA in these bovine cells [12]. Staining was cytoplasmic. Colocalization with anti-pro-RLF R33 and anti-RLF B chain (HFA antiserum) using bovine tissues showed that pro-RLF and RLF B chain were colocalized in the theca interna of 11 healthy follicles, 2–5 mm (Fig. 3), and 11 antral atretic follicles (data not shown). Thus, all results obtained suggest that both antibodies clearly were specific for RLF moities and both reacted with bovine RLF.

Follicle Classification

Follicles were classified on the basis of their morphology, using epoxy resin-embedded material where available or frozen sections, as being either healthy (n = 91 for small follicles; n = 28 for large follicles) or atretic. The atretic follicles were further classified as undergoing either antral (n = 29 for small follicles; n = 16 for large follicles) or basal (n = 18 for small follicles) atresia. This classification of atresia has recently been described and characterized at the light and electron microscope level. It simplifies all types of atresia previously reported in bovine follicles [14] into two simple and distinct forms.

Antral atretic follicles had the classic features of atretic follicles (Fig. 4b). They had numerous pyknotic nuclei present in either the layer(s) of the membrana granulosa closest to the antrum or in the antrum itself in close proximity to the membrana granulosa. As atresia progressed, the first cells to die were nearest the antrum, and cell death proceeded progressively towards the follicular basal lamina. Basal atresia, in contrast to antral atresia, exhibited destruction of the most basal layer of granulosa cells (Fig. 4c) while the most antral granulosa cells remained healthy and closely opposed to each other. The granulosa cells close to the antrum contained morphologically normal nuclei. The cells in the most basal layer of the membrana granulosa were separated from each other and often from the basal lamina by large intercellular spaces. Capillaries and macrophages had breached the basal lamina and were present in the basal area [14]. As with previous observations [14], basal atresia was observed only in smaller (<5 mm) follicles.

Follicles were arbitrarily classified as early, middle, or late atretic. Early antral atretic follicles had pyknotic nuclei in the antra, middle atretic follicles had these nuclei in the antral layers, and late atretic follicles had advanced pyknosis, such that only one or two layers of healthy basal cells remained. Basal atresia was classified as middle or late; early stage atretic cells were difficult to differentiate from healthy cells using light microscopy. Cell death in middle basal atretic follicles occurred in the basal layer,

FIG. 2. Western blot analyses of *E. coli* recombinantly expressed, gel-purified pro-bRLF (2 μl; lane 1) and the same material following immunoprecipitation with preimmune serum (lanes 2 and 3) or with immune serum (W3) against the RLF peptide (lanes 4 and 5). Immunopreciptations were derived from 2 μl (lanes 2 and 4) or 10 μl (lanes 3 and 5) of extract. The rat polyclonal antibody R33 recognized a product of the expected size for the bovine RLF pro-form at 14 kDa only where the immunoprecipitation was performed with the W3 antibody (lanes 4 and 5).

with partial expansion of matrix there. In late basal atresia, extensive basal areas of the former membrana granulosa were occupied by fluid, cell debris, and macrophages.

SCC and 3β-HSD Expression

SCC and 3β-HSD were localized to cells of the theca interna (Figs. 5–7). Staining was cytoplasmic and was not evenly distributed in cells. Only a portion of the thecal cells stained positively, as expected given the large number of nonsteroidogenic cells present in the theca, in particular fibroblasts and endothelial cells. Both small and large antral

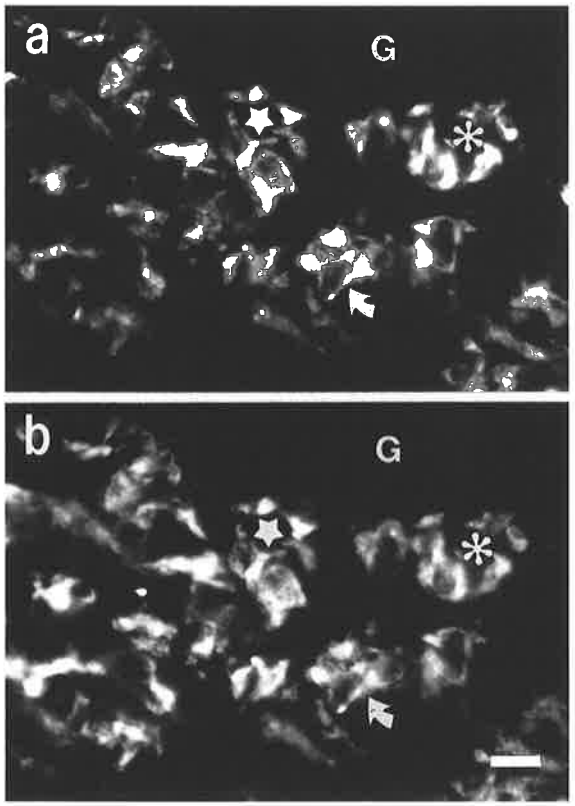

FIG. 3. Colocalization of pro-RFL (**a**) and mature RLF (B chain) (**b**) in a healthy bovine follicle (2.5-mm diameter). Symbols (star, asterisk, arrow) indicate the same location in the same section in both panels, identifying nearby cells containing both pro- and mature RLF immunoreactivity. G, Granulosa. Bar = 20 μm.

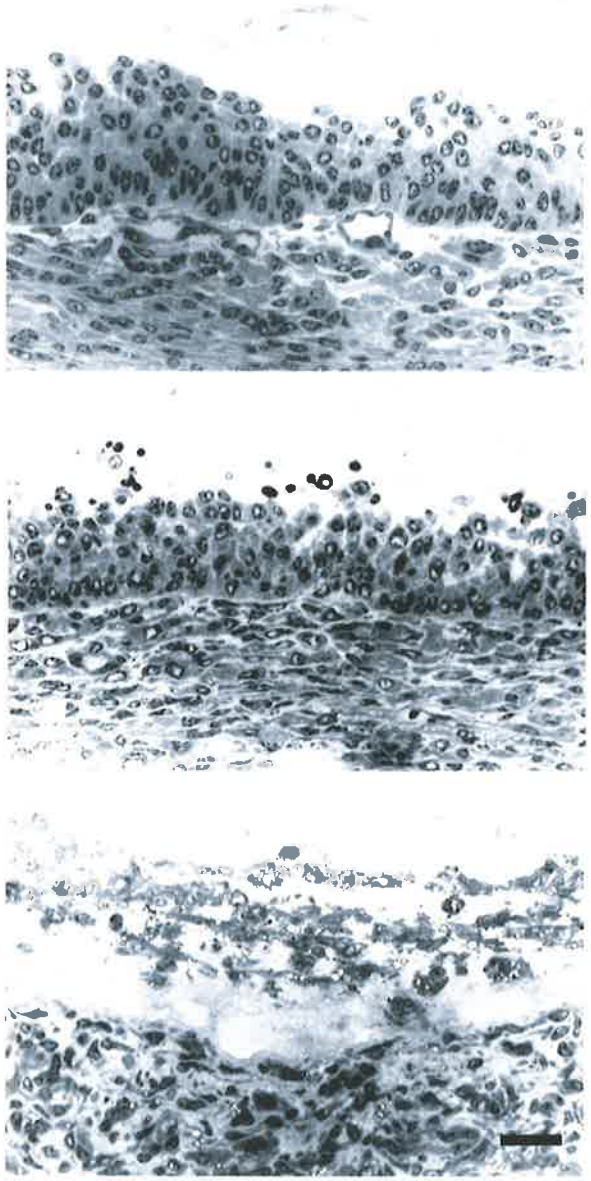

FIG. 4. Light microscopy of healthy follicles (**a**, 4.0 mm) and follicles exhibiting antral (**b**, 3.5 mm) and basal (**c**, 2.5 mm) atresia. Antral atretic follicles (**b**) have the most basal layers of granulosa cells remaining intact, and cells in the antral layers have pyknotic nuclei (arrowheads), indicative of cell death. Cells in the theca lie parallel to the follicular basal lamina (arrows). In basal atretic follicles (**c**), the granulosa cells close to the antrum are tightly arranged, whereas those in the most basal layers are loosely arranged and seperated from the basal lamina by extracellular matrix (asterisk). Many of the cells in the theca are oriented perpendicular to the basal lamina, and there is more extracellular matrix between the cells compared with healthy follicles. Epoxy sections (1 μm thick) stained with methylene blue. Bar = 20 μm.

follicles had thecal cells expressing both of these enzymes (Table 1). With atresia of small follicles, the theca continued to express both enzymes. With atresia of large follicles, many follicles had ceased to express 3β-HSD and about 50% had ceased to express SCC in the theca interna (Table 1). The membrana granulosa cells generally did not express SCC or 3β-HSD. However, on reaching large antral size the membrana granulosa of most follicles expressed 3β-HSD and some expressed SCC. With basal atresia, the remaining antrally located granulosa cells also expressed both SCC and 3β-HSD.

RLF Expression

Colocalization of pro-RLF with 3β-HSD showed that cells staining positive for pro-RLF were steroidogenic thecal cells (Figs. 5–7). In small healthy follicles, a few cells contained 3β-HSD and not pro-RLF (Fig. 5). This finding could indicate a separate population of RLF-negative 3β-HSD-positive cells or could indicate a difference in the expression of the two molecules or differing levels of antibody affinity resulting in detection of one molecule and not the other.

Expression of pro-RLF first occurred coincidently with expression of SCC and 3β-HSD in the theca interna of very small antral follicles when the theca interna first developed (results not shown). Pro-RLF, SCC, and 3β-HSD all continued to be expressed in the theca interna throughout development to the stage of healthy small (2–5 mm) antral follicles (Fig. 5). Then, with atresia the expression pattern in the theca interna diverged between pro-RLF, SCC, and 3β-HSD. With antral atresia of small follicles, pro-RLF, SCC, and 3β-HSD all continued to be expressed in the theca interna until late atresia, when the proportion of follicles expressing pro-RLF declined (Fig. 6 and Table 1). In contrast, with basal atresia the proportion of follicles expressing pro-RLF declined markedly by middle atresia (Fig. 7 and Table 1). These differences in when pro-RLF expression declined in the two types of atresia relate to the degree to which atresia had advanced, as visually assessed. This finding does not necessarily mean that pro-RLF expression declines more quickly in one type of atresia relative to the other, since only antral atretic follicles have been examined at different time points as atresia progressed [14]. As follicles enlarged, expression patterns of pro-RLF, SCC, and 3β-HSD in the theca interna also diverged (Table 1). As healthy follicles enlarged (>10 mm), the proportion of follicles expressing pro-RLF declined to about 50% (Table 1), unlike SCC and 3β-HSD. Upon atresia in follicles >6 mm, which are only of the antral type, the proportion of follicles expressing RLF also declined, unlike SCC and 3β-HSD (Table 1). The decline in RLF expression seen in atresia or during growth of follicles was both qualitative (cells staining less intensely) and quantitative (fewer positive-staining cells) (compare Figs. 5b and 6b).

Expression of pro-RLF in the theca interna was compared with expression of SCC and 3β-HSD in the membrana granulosa (Table 2). A significant ($P < 0.001$, χ^2 test) inverse relationship in the expression of pro-RLF in the theca interna and 3β-HSD in the membrana granulosa was observed. No significant ($P > 0.05$, χ^2 test) relationship was observed with expression of SCC in the membrana granulosa.

DISCUSSION

Pro-RLF or the mature form of RLF or both are present in steroidogenic cells of the bovine theca interna. Exami-

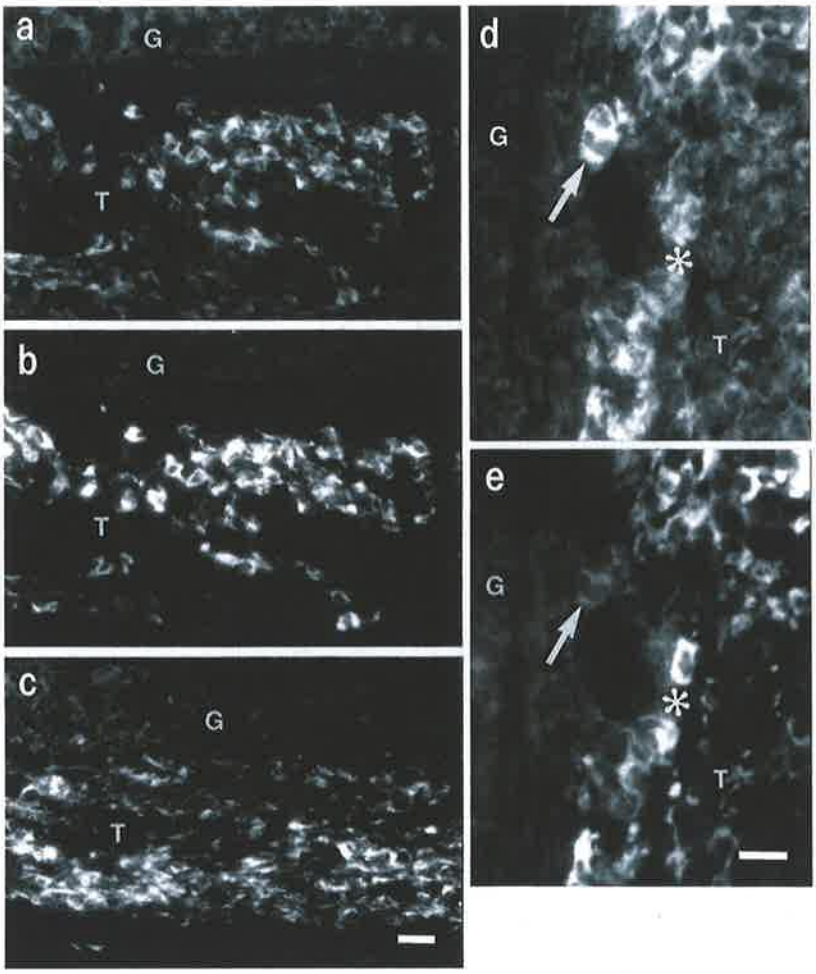

FIG. 5. Immunolocalization of pro-RLF (**b** and **e**), 3β-HSD (**a** and **d**), and SCC (**c**) in healthy 2.5-mm (**a**–**c**) and 3.5-mm (**d** and **e**) follicles. Pro-RLF (**b**) colocalizes with 3β-HSD (**a**) immunopositive cells in the theca of healthy bovine follicles. SCC localization from an adjacent section (**c**). Arrow, 3β-HSD (**d**)-positive and pro-RLF (**e**)-negative cell. Asterisk, 3β-HSD (**d**)-positive and pro-RLF (**e**)-positive cell.

nation of a range of healthy and atretic follicles showed that these follicles could be grouped into those that expressed pro-RLF in the theca interna and those that did not. An analysis of the proportion of follicles expressing pro-RLF, SCC, or 3β-HSD in either the theca interna or membrana granulosa was undertaken. The proportion of follicles expressing pro-RLF declined upon basal atresia of small follicles (2–5 mm) and upon antral atresia in both small (2–5 mm) and large (>6 mm) follicles. Similarly, in large healthy follicles (>10 mm) the proportion expressing pro-RLF also declined. With both atresia or enlargement of the follicles, the decline in the proportion of follicles expressing pro-RLF was not accompanied by a decline in follicles expressing SCC or 3β-HSD in the theca interna. There was, however, a strong significant negative association between the expression of pro-RLF in the theca interna and expression of 3β-HSD in the membrana granulosa.

In the current study follicle status was assess by histological evaluation of health and atresia [14] and by localization of the steroidogenic enzymes SCC and 3β-HSD. Studies of the expression of 3β-HSD [24] and SCC [25, 26] have previously been undertaken in bovine follicles. Although the thecal cells always expressed these enzymes, the granulosa cells in most follicles did not express SCC or 3β-HSD however those in some follicles did [26]. Similar results were obtained in the present study, but with many larger follicles in this study it is clear that the expression in granulosa cells develops as the follicles enlarge to preovulatory size (>10 mm).

Two anti-RLF antisera were studied in detail. One was produced in rat against recombinantly expressed bovine pro-RLF containing the A, B, and C domains but without the predicted signal peptide, which is likely to be removed in vivo. This antibody bound to both pro-RLF and the mature form, although it bound more strongly to the pro-form than did the second antibody. Additional recent blots suggest anti-pro-RLF binds preferentially to the A rather than the B chain. Reactivity to the C chain has not been tested, although binding is also likely to occur. The second antibody was raised against the ovine B chain, which differs in only one amino acid from the bovine B chain. This second antibody clearly bound to the mature form of ovine RLF, but binding to the pro-RLF was not significantly greater than binding to a negative control. The first antiserum reacted with Leydig cells as expected. When used to coimmunostain follicles, both antisera reacted with the same cells; the pro-RLF was colocalized to 3β-HSD-positive cells and thus RLF is expressed in steroidogenic thecal

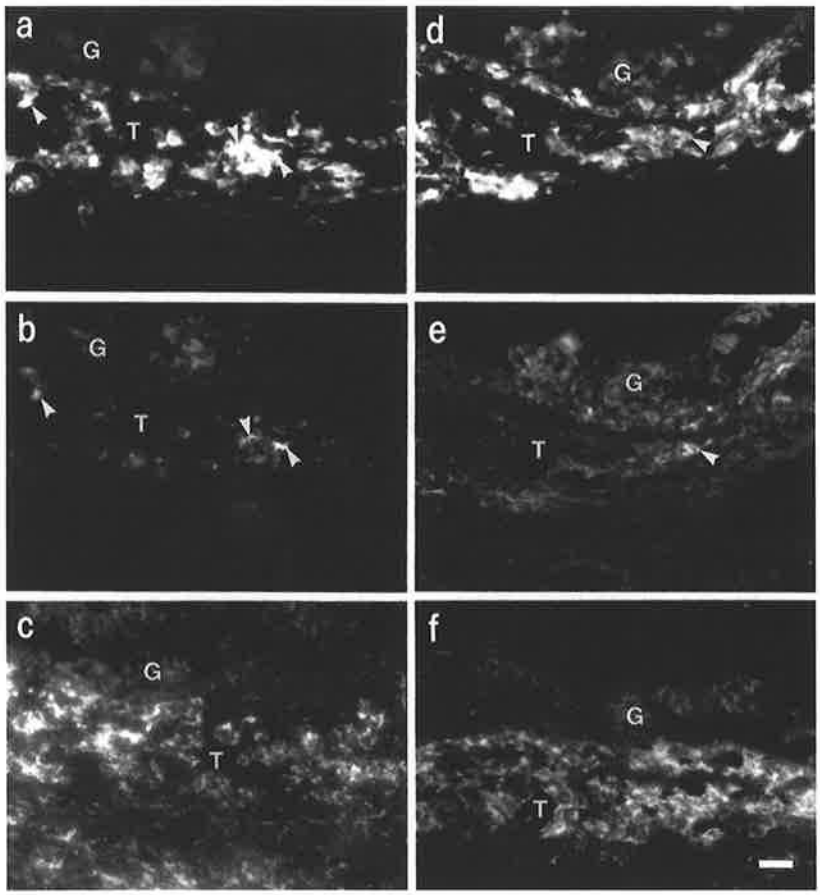

FIG. 6. Immunolocalization of pro-RLF (**b** and **e**), 3β-HSD (**a** and **d**), and SCC (**c** and **f**) in middle (**a–c**, 3.0 mm) and late (**d–f**, 4.5 mm) antral atretic follicles. Some 3β-HSD immunopositive thecal cells (**a** and **d**) contain pro-RLF (**b** and **e**) (arrowheads indicate identical cells). G, Granulosa; T, theca. Bar = 20 μm.

cells, similar to the situation in testis where Leydig cells express RLF [3]. RLF mRNA but not protein has been examined in bovine follicles previously [11, 12]; thus, the current results extend these findings to show that the mRNA is translated into pro-RLF. Very little is known about the processing of pro-RLF in vivo and whether it is processed through to the A/B mature form, as is relaxin. Based upon the differential binding ability of the HFA antibody on dot blots, it is tempting to speculate that pro-RLF is processed into mature RLF in bovine thecal cells. However, additional experiments are required to verify this assumption.

It has been suggested that RLF is a marker for differentiation of the theca interna [11]. In the theca interna of some follicles, not all steroidogenic cells expressed pro-RLF, whereas the converse did not occur. In addition, we observed whole follicles (atretic or large healthy follicles) in which the theca interna failed to express any pro-RLF but continued to express SCC and 3β-HSD. The precursor small healthy follicles expressed pro-RLF, which implies that at some stage of follicle growth pro-RLF expression was switched off. The trigger for this event is not clear. One possibility is that transcription of RLF, SCC, and 3β-HSD all cease simultaneously but that mRNA or protein for both SCC and 3β-HSD have longer cellular half-lives, on the order of days [27], whereas those for pro-RLF have much shorter half-lives. Alternatively, the mechanisms of regulation of the gene promoters may be governed by different factors. Although cloning of the bovine RLF gene promoter (unpublished results) revealed the presence of steroidogenic factor 1 responsive elements as in the promoters of the SCC and 3β-HSD genes, long-term primary cell culture experiments showed that LH has opposite effects on RLF and SCC gene expression. RLF was downregulated and SCC was upregulated by the gonadotropin [11], implicating divergent regulatory mechanisms.

RLF mRNA in bovine ovaries was found previously by in situ hybridization [12] to be present in the theca interna and to be expressed in cultured thecal cells [11]. Northern analysis of follicles and CLs [6] have also been carried out. Although the early CL has minimal RLF mRNA or protein, both are readily detectable later in the luteal phase [11, 28]. Thus, a decline in RLF expression occurs late in follicular development, during ovulation, or during formation of a CL. The results of the present study, examining follicles up to large preovulatory stages, suggest that the decline in RLF expression may precede the LH surge that initiates ovulation and instead may reflect the maturing status of the ovarian follicle. This pattern of expression is not unlike the preovulatory and pre-LH surge expression of SCC, 3β-HSD, cytochrome P450 aromatase, and LH receptor accompanied by a decline in expression of FSH receptors in the membrana granulosa. However, even though these dynamic changes have been recognized in the membrana granulosa, no changes such as those associated with RLF have been previously recognized in the theca interna.

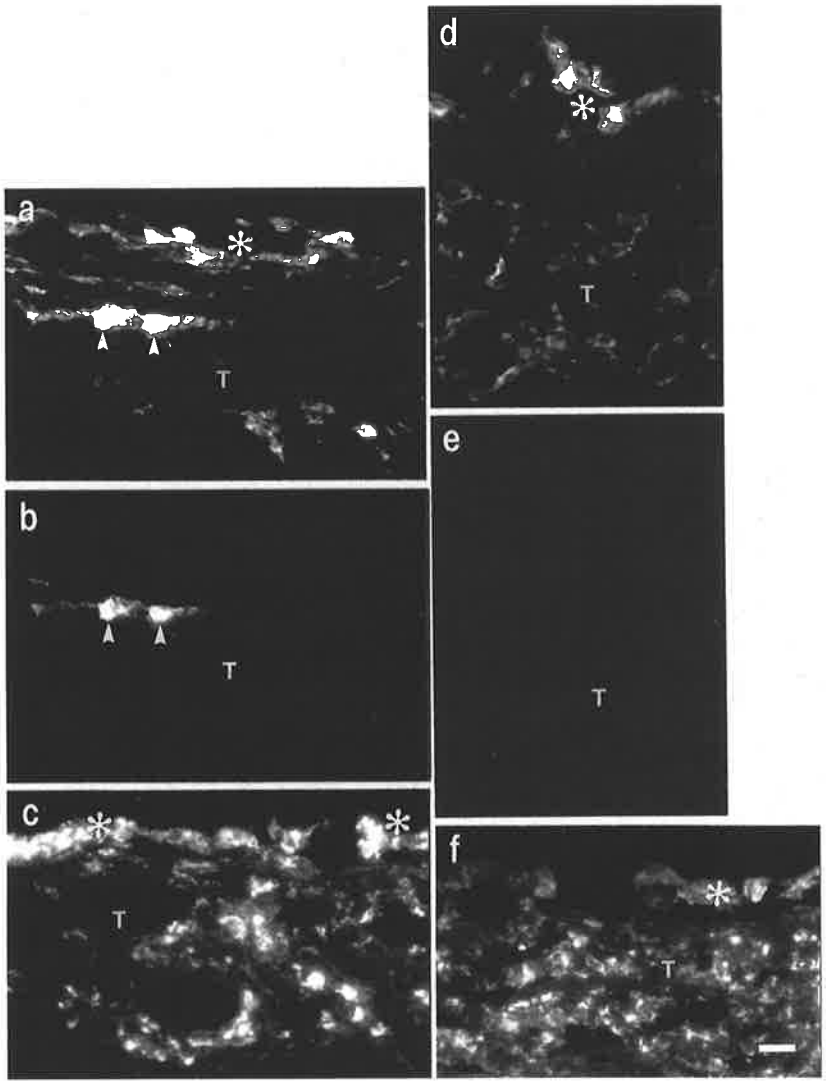

FIG. 7. Immunolocalization of pro-RLF (**b** and **e**), 3β-HSD (**a** and **d**), and SCC (**c** and **f**) in middle (**a–c**, 4.5 mm) and late (**d–f**, 4.0 mm) basal atretic follicles. A few 3β-HSD immunopositive thecal cells contain pro-RLF (**a** and **b**) in middle basal atretic follicles (arrowheads indicate identical cells) but not in late basal atretic follicles (**d** and **e**). Granulosa cells of basal atretic follicles were immunopositive (asterisks) for both 3β-HSD (**a** and **d**) and SCC (**c** and **f**). T, Theca. Bar = 20 μm.

TABLE 1. Proportion of bovine antral follicles (number observed/total number examined) of differing sizes and stages of health or atresia expressing pro-RLF, SCC, or 3β-HSD in the theca interna.

Follicle size (mm)	Follicle status	RLF	SCC	3β-HSD
2–5	Healthy	19/19	11/11	15/15
	Basal atresia			
	Middle	3/7	4/4	6/7
	Late	0/8	8/8	7/8
	Antral atresia			
	Early	8/8		6/6
	Middle	7/9	6/6	5/5
	Late	1/4	5/5	4/4
6–9	Healthy	9/11	7/11	11/11
	Antral atresia	1/13	12/13	13/13
10	Healthy	5/8	6/8	8/8
	Antral atresia	1/3	2/3	3/3
12–17	Healthy	4/7	8/9	8/9

The physiological role of RLF is poorly understood, but RLF knockout mice have been developed [15, 16]. The male phenotype is characterized by failure of the testes to descend. In females, extension of the length of estrous cycle has been observed in one strain of mice [15] but not in

TABLE 2. Proportion of follicles (per total examined), regardless of stage of health or atresia, expressing RLF in the theca.

Granulosa expression*	Theca expression of RLF
3β-HSD positive	9/37
3β-HSD negative	41/49
SCC positive	11/23
SCC negative	26/43

* Follicles subdivided into groups that expressed 3β-HSD or SCC in the membrana granulosa (positive) or did not (negative). Significant negative association ($P < 0.001$; χ^2 test) was observed between expression of RLF in the theca and 3β-HSD in the membrana granulosa. No significant association ($P > 0.05$; χ^2 test) was observed between expression of RLF in the theca and SCC in the membrana granulosa.

another [16]. An accelerated increase in the number of regressed follicles and decreased levels of remaining CLs were observed in ovaries of the knockout mice [29], suggesting that in mice the RLF may have a role related to follicular atresia and luteal regression. In the current study in bovine cells, we found that RLF expression in the theca interna declined upon atresia of the follicles, particularly when atresia was more advanced, suggesting that RLF expression could be related to atresia. The RLF receptor has not been identified or localized, nor have the target cells for RLF been identified. Could the target be or include the granulosa cells, and could RLF play a negative role inhibiting 3β-HSD expression in the membrana granulosa cells? In our study, SCC and 3β-HSD were not coexpressed in the membrana granulosa in many follicles. What was striking was the significant ($P < 0.001$) inverse level of expression of RLF in the theca and of 3β-HSD in the membrana granulosa. RLF was expressed in small and healthy antral follicles, and no expression of 3β-HSD was observed in the granulosa cells. As follicles enlarged or entered atresia, the incidence of RLF expression among follicles declined, and the expression of 3β-HSD in the membrana granulosa increased. This relationship may be a consequence of follicle maturation or may be the result of cause and effect. The answer awaits further investigation.

In the pig [30] and the marmoset [31, 32], the related molecule relaxin is also expressed by the cells of the theca interna and the CL. Relaxin can influence the steroidogenic capacity of thecal cells [32], granulosa cell replication [33], and insulin-like growth factor 1 production [34]. However, the classical role of relaxin is to regulate tissue remodelling by stimulating degradation of structural collagens [10]. Both the thecal layers [35] and the membrana granulosa [36] are extensively remodelled, particularly as the antral cavity of the follicle enlarges and expands the layers laterally [36]. The membrana granulosa offers little resistance for lateral expansion, but the thecal layers in healthy follicles contain structural collagens [26, 37] that must be degraded during the process of lateral expansion. Relaxin secretion in the theca could stimulate collagen breakdown. In support of this hypothsis, injection of anti-relaxin antibodies into the ovarian bursa of the rat blocked ovulation, probably because of relaxin's stimulatory effect on collagenases [38]. In ruminant species, which appear not to have a relaxin gene, RLF may substitute for relaxin, in which case thecal expression of RLF may be important for thecal remodelling during follicular growth.

Expression of RLF occurs in the steroidogenic cells of the theca interna of bovine follicles. Its expression dynamically changes with follicle development, with RLF levels highest at the early antral stages but declining well in advance of SCC or 3β-HSD expression in the theca interna. The results presented here suggest that, as in Leydig cells, RLF in bovine thecal cells is correlated with differentiation of thecal cells but only in the early stages of the differentiation process. The answer to what RLF is doing must await the availability of more new tools, such as peptides, with which to manipulate the follicle in vivo and in vitro.

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Statement of Authorship

Cholesterol side-chain cleavage cytochrome P450 and 3β-hydroxysteroid dehydrogenase expression and the concentrations of steroid hormones in the follicular fluids of different phenotypes of healthy and atretic bovine follicles

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***Biology of Reproduction* 2003 69, 2022-2028**

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Signed Date 12/12/06

Krupa M (co-author)
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Grant permission publication to be included in the PhD submission of Helen Irving-Rodgers.

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Assisted with writing manuscript and acted as corresponding author.

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Signed Date 12/12/06

Statement of Authorship

Theca interna: the other side of bovine follicular atresia

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Theca Interna: The Other Side of Bovine Follicular Atresia[1]

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ABSTRACT

Currently, histological classifications of ovarian follicular atresia are almost exclusively based on the morphology of the membrana granulosa without reference to the theca interna. Atresia in the bovine small antral ovarian follicle has been redefined into antral or basal atresia where cell death commences initially within antral or basal regions of the membrana granulosa, respectively. To examine cell death in the theca interna in the two types of atretic follicles, bovine ovaries were collected and processed for immunohistochemistry and light microscopy. Follicles were classified as healthy, antral atretic, or basal atretic. Follicle diameter was recorded and sections stained with lectin from *Bandeiraea simplicifolia* to identify endothelial cells or with an antibody to cytochrome P450 cholesterol side-chain cleavage to identify steroidogenic cells and combined with TUNEL labeling to identify dead cells. The numerical density of steroidogenic cells within the theca interna was significantly reduced ($P < 0.001$) in basal atretic follicles in comparison with other follicles. Cell death was greater in both endothelial cells ($P < 0.05$) and steroidogenic cells ($P < 0.01$) of the theca interna of basal atretic follicles compared with healthy and antral atretic follicles. Thus, we conclude that the theca interna is susceptible to cell death early in atresia, particularly in basal atretic follicles.

apoptosis, atresia, bovine, follicle, ovary, theca cells, theca interna

INTRODUCTION

At birth, the mammalian ovary contains thousands of primordial follicles. However, in many species, less than 1% of these follicles will ovulate, with the majority of follicles undergoing atresia. Atresia probably evolved to control and limit the number of fertilizable oocytes released from the ovary, and it can also be a critical event in determining the timing of ovulation. Atresia involves death of the follicular cells and reabsorption of the cellular debris.

Generally, there are three pathways through which cells can die; apoptosis, necrosis, or terminal differentiation [1], the last of which is exemplified in normoblasts differentiating into erythrocytes, epidermal cells into keratinocytes, or megakaryocytes into platelets. Apoptosis is an active process within the cell involving DNA cleavage into oligonucleasomal length fragments by an endogenous endonuclease. The chromatin condenses, followed by budding of the nucleus and finally the cell buds to create membrane-bound apoptotic bodies that eventually become phagocytosed by macrophages or, in some epithelia by adjacent cells. In contrast, necrosis occurs as a result of cell injury or trauma and is characterized by rupture of the cell, leading to swelling of the mitochondria and other organelles [1]. The nuclear DNA is then fragmented randomly as it becomes exposed to the external environment.

In the study of atresia, much attention has been paid to how follicular cells die. To date, it has been demonstrated in various species (rat [2–4], horse [5], bovine [6, 7], pig [8] and human [9]) that apoptosis is the underlying process of atresia and that it occurs throughout all stages of follicular development. However, in bovine antral follicles, Van Wezel et al. [7] showed that, whereas granulosa cells in the middle of the membrana granulosa undergo apoptosis (TUNEL positive, classic condensation pattern of chromatin, and phagocytosis by adjacent granulosa cells), those granulosa cells closest to the antrum did not display any of the normal characteristics associated with apoptosis [7]. Hence, whereas these cells had classic pyknotic nuclei, they were essentially nondetectable by TUNEL. Their DNA was randomly nicked as shown by the COMET assay and gel electrophoresis of the DNA aspirated from the follicular antrum, and yet these cells showed no evidence of necrosis by electron microscopic examination. This led the authors to conclude that the cell death observed had more in common with a terminal differentiation mechanism. This observation may only be relevant to bovine follicles, especially in light of the recent description of different types of follicular atresia in the bovine [10].

In bovine antral follicles, atresia can follow one of two processes, and the description of these resolves earlier conflicting descriptions of atresia in this species [10]. Antral atresia, as it is now called, occurs in antral follicles of all sizes and features the initial death of the antrally situated granulosa cells. These cells develop visible pyknotic nuclei. This is the classic form of atresia as observed in other species. The basal granulosa cells stay in close contact with the follicular basal lamina and remain healthy until atresia is well advanced. In contrast, follicles with cell death initially present within the basal layers of the membrana granulosa were described as undergoing basal atresia. Basal atresia is only observed in bovine follicles less than 5 mm in diameter and involves the initial death of the granulosa cells lining the follicular basal lamina. These basal cells undergo classic apoptosis with nuclear and cellular budding and phagocytosis by invading macrophages. Surprisingly,

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the antrally situated cells of basal atretic follicles undergo hypertrophy [10] and differentiate into progesterone-producing cells [11] before succumbing to death as atresia progresses. These two different types of atresia have been considered to arise by the different location of the younger cells either basally or antrally brought about by different rates of follicular antrum expansion during follicular growth [12].

A very important feature of atresia is the susceptibility of the oocyte and granulosa cells to death, which changes during folliculogenesis. In preantral follicles, but not antral follicles, cell death is often initially observed within the oocyte [13, 14]. Conversely, in antral follicles, it is the granulosa cells that die initially. The cumulus-oocyte complex often remains healthy and intact until the late stages of atresia [13, 14]. These observations probably merely reflect the differential rates of growth during folliculogenesis and hence vulnerability to death. Oocyte growth occurs mainly preantrally [15, 16], and the largest expansion of granulosa cells numbers occurs early in antrum formation [15].

The question logically arises of whether there is a stage at which the theca interna is the compartment most susceptible to cell death. Little research has been carried out focusing specifically on the theca interna. Cell death has been shown to occur in the theca interna of several species, including rat [4], pig [17], avian [8], and bovine [10], but is reported to occur at a lower rate than in the membrana granulosa [18, 19]. In an in-depth study of the ultrastructural changes of the theca interna during atresia in the sheep ovary, O'Shea et al. [20] observed cell death in the theca interna at all stages of atresia, but at a much lower frequency than in the membrana granulosa. More recently, Nakayama et al. [19], studying bovine follicles, reported that apoptotic cells were initially detected in the theca interna of bovine follicles in the early stages of atresia when apoptotic cells were visible in the middle layers of the membrana granulosa but concluded that apoptosis within the membrana granulosa is the initial symptom of atresia. Isobe and Yoshimura [21] reported high frequencies of TUNEL-positive cells within the theca interna, which were similar in both early and late atretic bovine follicles.

None of the previous studies on bovine follicles distinguished the two types of atresia. Current evidence would suggest that, at least in bovine basal atretic follicles, the theca interna undergoes significant alterations during atresia. The theca interna of basal atretic follicles is much less cellular and the cells are randomly situated rather than orientated circumferentially, as in healthy or antral atretic follicles [10]. There is also an increased amount of collagen within the theca interna of basal atretic follicles and cellular debris within the capillaries [10]. In addition, the follicular fluid of basal atretic follicles has significantly less of the thecal-derived steroids, testosterone, and androstenedione compared with healthy or antral atretic follicles [11]. Basal atretic follicles also have reduced levels of insulin-like factor 3 (INSL3; relaxin-like factor) expression in theca interna cells [22]. Mice null for INSL3 have increased levels of atresia [23].

Thus, in general, there is a paucity of literature on thecal cell death in atresia. Specifically in the bovine, theca cell death has not been investigated in the two types of follicular atresia. In addition, our studies of bovine atresia found reduced levels of thecal-derived hormones in basal atretic but not antral atretic follicles, suggesting that the theca of the two types of atretic follicles behaves differently. Therefore, our goal was to examine death of the different thecal cell types in both basal and antral atretic bovine follicles.

MATERIALS AND METHODS

Tissues

Bovine ovaries (n = 29) were collected from a local abattoir from cows assessed visually as nonpregnant within 20 min postslaughter. Ovaries were sliced longitudinally into two or three slices approximately 5 to 8 mm thick, producing 66 slices. These were immediately immersed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) and transported to the laboratory on ice. Tissue was transferred into fresh 4% paraformaldehyde and fixed overnight at 4°C before rinsing in 70% ethanol and embedding in paraffin by standard histological methods.

Classification of Follicular Health

One 5-μm-thick section from each paraffin-embedded ovarian slice was cut and stained with hematoxylin and eosin. Sections were viewed on an Olympus BX50 microscope (Olympus Australia Pty. Ltd.) and each antral follicle identified. Analysis was restricted to the examination of antral follicles ≤5 mm in order to provide sized-matched controls for basal atretic follicles, which only occur in this size range. The cross-sectional diameter from the follicular basal lamina of follicles (1–5 mm) was measured using an occular micrometer, and the follicles were classified into one of three categories: healthy, antral atretic, or basal atretic, as previously described [10]. The condition of the membrana granulosa was used to ascertain follicular health. Cell death was identified as intensely stained round or crescent-shaped pyknotic nuclei [7] or as apoptotic nuclei. Follicles at the very late stages of atresia, which had no remaining membrana granulosa, were excluded because the type of atresia could not be determined.

Immunohistochemistry

Sections (5 μm thick) were cut from each portion of paraffin-embedded ovary and deparaffinized in xylene. Sections were rehydrated in decreasing concentrations of ethanol (100%, 95%, 70%, 50%) and immersed in H_2O before treatment with blocking solution (10% normal donkey solution [Sigma Chemical Co., St. Louis, MO]) in antibody diluent containing 0.55 M sodium chloride and 10 mM sodium phosphate (pH 7.2), for 30 min at room temperature. Rabbit anti-bovine cholesterol side-chain cleavage cytochrome P450 (*CPY11A*; $P450_{SCC}$) antiserum (OXYgene, Dallas, TX) was used to identify steroidogenic cells. Incubation with primary antibody (1:200) was conducted for 48 h at room temperature before incubating with biotin-donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 2 h. This was followed by incubation with a 1:100 dilution of Cy3-conjugated streptavidin (Jackson ImmunoResearch Laboratories Inc.) for 1 h at room temperature. Sections were washed 3 × 5 min in phosphate-buffered saline (PBS) containing 0.274 M sodium chloride, 5.4 mM potassium chloride, and 10 mM sodium phosphate between incubations, and mounted in fluorescence mounting medium (DAKO, Carpinteria, CA). Lectin from *Bandeiraea simplicifolia* (Sigma-Aldrich) was used as a marker for endothelial cells within the theca interna [24, 25]. Sections were incubated with 1:200 dilution of FITC-labeled lectin in antibody diluent for 1 h in a humidified chamber at room temperature.

TUNEL Labeling

A terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) method was used to identify degraded DNA. Following rehydration, sections (5 μm thick) were immersed in phosphate-buffered solution for TUNEL (PBST; 10 mM sodium/potassium phosphate in 0.317 M sodium chloride and 5 mM potassium chloride solution) for 10 min. Sections were treated with 5 μg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany) in proteinase K buffer (50 mM Tris-hydrogen chloride, 5 mM EDTA) for 45 min at 37°C in a humidified chamber. Following washing in PBST (4 × 5 min), sections were treated with TUNEL reagents, 0.5 nM biotin-16-2′-deoxy-uridine-5′-triphosphate (biotin-dUTP; Roche Diagnostics GmbH, Mannheim, Germany), 50 U/ml terminal transferase (Roche Diagnostics GmbH) and 1.5 mM cobalt chloride in reaction buffer (30 mM Tris-chloride, pH 7.2, and 140 mM sodium cacodylate) for 1 h at 37°C. Sections were washed in PBST (3 × 5 min) before incubation with a 1:100 dilution of Cy3-conjugated streptavidin

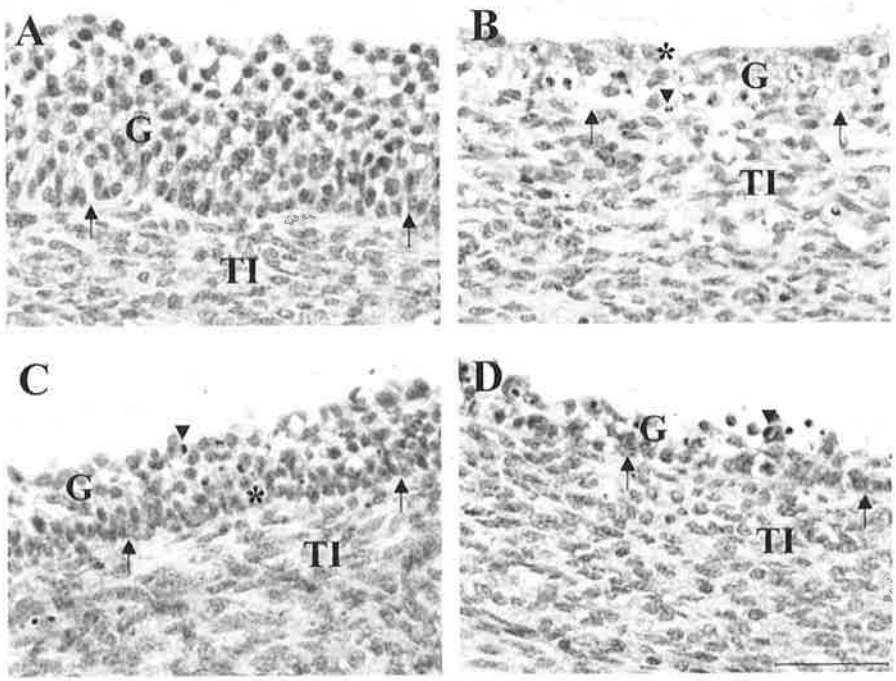

FIG. 1. Morphological features of bovine follicles. A) Healthy 2-mm follicle. B) Basal atretic 2.5-mm follicle. Granulosa cells lining the follicular basal lamina have apoptotic nuclei (arrowhead), indicating cell death, while antrally situated granulosa cells show signs of hypertrophy (asterisk) when compared with granulosa cells of healthy follicles (A). C) A 2.5-mm early–midantral atretic follicle. Healthy granulosa cells align the follicular basal lamina (asterisk). Cells in the middle and antral layers of the membrana granulosa contain pyknotic nuclei (arrowhead). D) A 3-mm late antral atretic follicle. The number of granulosa cell layers is reduced in comparison with early–midantral atretic follicles. TI, theca interna; G, membrana granulosa; arrow, follicular basal lamina. Scale bar = 50 μm.

(Jackson ImmunoResearch Laboratories Inc.) for 1 h at room temperature in a humidified chamber.

Following immunohistochemistry or TUNEL labeling, sections were counterstained by incubation with the nuclear stain 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) solution (Molecular Probes, Eugene, OR) for 30 min in a humidified chamber at room temperature.

Multiple Labeling

TUNEL labeling, lectin binding, and DAPI staining were combined to identify cell death in the vasculature of the theca interna. Immunohistochemical colocalization of $P450_{SCC}$ was combined with TUNEL labeling and DAPI staining in order to identify degraded DNA in steroidogenic cells. In this experiment, biotin-dUTP was replaced with a direct-labeled fluorescein-12-deoxy-uridine-5′-triphosphate (fluorescein-dUTP) (Molecular Probes). Terminal transferase was omitted in negative-control sections. When immunohistochemistry was combined with TUNEL labeling, sections were treated with proteinase K prior to incubating with primary antibody. The TUNEL reaction was conducted after completion of immunohistochemistry.

Image Analyses

Follicles for examination were chosen from those previously classified on hematoxylin and eosin-stained sections. On serial sections processed for immunohistochemistry, TUNEL labeling, or multiple labeling, selected follicles were identified. To ensure unbiased sampling of each follicle, the first field of view containing the membrana granulosa and the theca interna of stained sections was selected randomly and photographed using an ultraviolet filter and filters suitable for viewing FITC and Cy3 on an Olympus BX50 microscope with SPOT camera attachments (Diagnostic Instruments Inc., Sterling Heights, MI) and 40× and 60× magnification. Fields of view directly south, east, and west of the initial view were also photographed. Images were merged to identify colocalization of staining, and densitometry was performed on selected reference areas using analySIS software (Soft Imaging System GmbH, Münster, Germany).

Statistical Analyses

One-way analysis of variance (one-way ANOVA) and post hoc Student-Newman-Keuls and Duncan multiple range tests were performed using Statistical Package for the Social Sciences 11.5 for Windows (SPSS Inc., Chicago, IL).

RESULTS

The 66 sections from the 29 ovaries were examined and all follicles within each section were mapped and classified as healthy or atretic. Healthy follicles (n = 305) were classified as those with an intact membrana granulosa with only occasional or no pyknotic nuclei (Fig. 1A). Antral atretic follicles (n = 131) were identified by numerous pyknotic nuclei within the layers of the membrana granulosa closest to the antrum. In these follicles, the number of granulosa cell layers was reduced, compared with healthy follicles, and cellular debris was often observed within the follicular antrum. Antral atretic follicles were further classified according to the degree of atresia as either early–midantral atresia or late antral atresia (Fig. 1, C and D, respectively). Early–midantral atretic follicles were those with a decreased number of granulosa cell layers and several pyknotic nuclei within the layers closest to the antrum. Follicles classified as late antral atretic were those with numerous pyknotic nuclei and very few healthy granulosa cells. Follicles assessed as undergoing basal atresia (n = 135) had apoptotic nuclei predominately within the layers of the membrana granulosa closest to the basal lamina (Fig. 1B). The membrana granulosa was detached from the follicular basal lamina, and death of granulosa cells resulted in the appearance of spaces within the basal granulosa cell layers. Remaining antrally situated granulosa cells showed signs of hypertrophy. From the 29 ovaries classified, 16 sections from 12 ovaries were selected on the basis of their follicle distribution, and 21 healthy, 41 antral atretic, and 26 basal atretic follicles were examined further. The numbers of blocks chosen were based on a preliminary experiment and a power analysis. Follicles did not differ significantly in their cross-sectional diameter ($P > 0.05$).

Composition of the Theca Interna

From 10 sections from 9 ovaries immunohistochemically stained for $P450_{SCC}$ to identify steroidogenic cells, 11

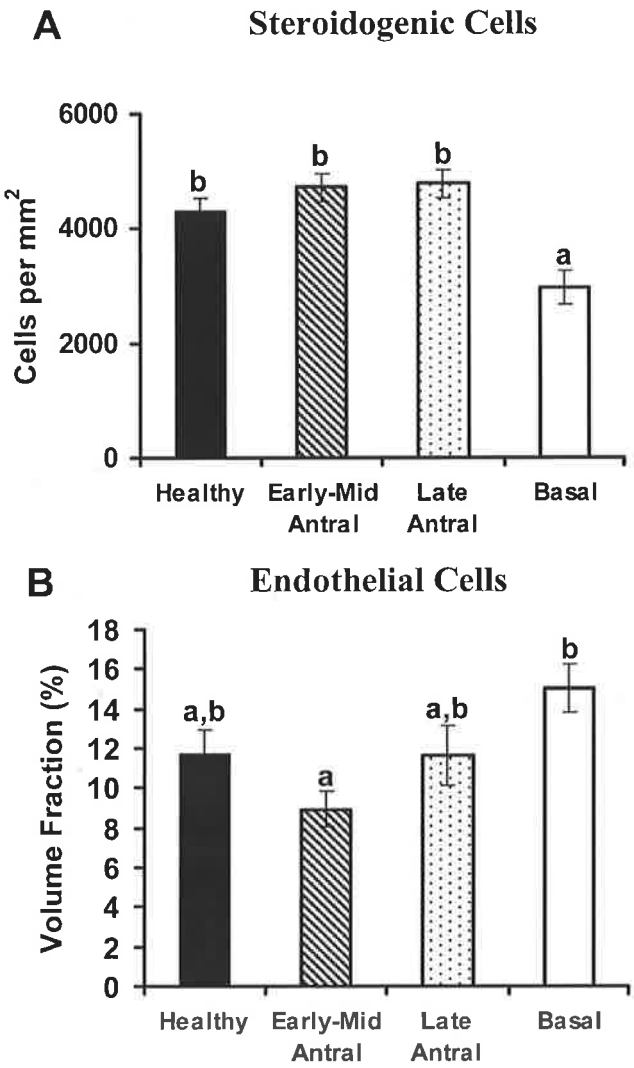

FIG. 2. Quantitation of the cellular components of the theca interna of healthy and atretic follicles. **A**) Mean (±SEM) numbers of steroidogenic cells per cross-sectional area of theca interna. Bars with different superscripts are significantly different ($P < 0.001$). **B**) Mean (±SEM) volume fraction of lectin binding within the theca interna. Bars with different superscripts are significantly different ($P < 0.05$).

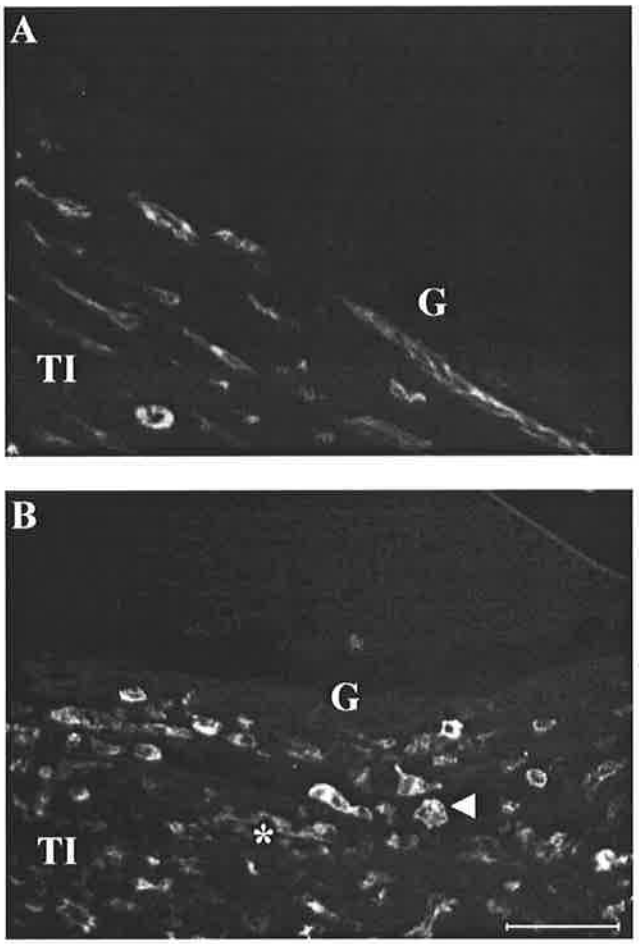

FIG. 3. Identification of vasculature in the theca interna of bovine follicles. **A**) Micrograph of lectin binding in a 2.5-mm follicle undergoing antral atresia. Both healthy (not shown) and antral atretic follicles displayed intense and uniform lectin binding associated with the vasculature orientated essentially parallel with the follicular basal lamina. **B**) Micrograph of lectin binding in a 2-mm follicle undergoing basal atresia. Some binding did not appear to be associated with any vasculature (arrowhead). Basal atretic follicles display a pattern of lectin binding that is mottled and uneven (asterisk) in comparison with healthy and antral atretic follicles. TI, theca interna; G, membrana granulosa. Scale bar = 50 μm.

healthy, 14 early–midantral atretic follicles, 7 late antral atretic follicles, and 9 basal atretic follicles were examined. Positive immunostaining for $P450_{SCC}$ was localized exclusively to the cytoplasm of a population of cells within the theca interna. The area of the theca interna was measured, and all cells positively staining for $P450_{SCC}$ and identified by colocalization of DAPI were counted per cross-sectional area of theca interna. Healthy follicles and both early–midantral atretic and late antral atretic follicles did not differ in the numerical density of steroidogenic cells in the theca interna (Fig. 2A). In comparison, the number of steroidogenic cells in the theca interna of follicles undergoing basal atresia was significantly reduced ($P < 0.001$; Fig. 2A).

In sections in which lectin binding was used to identify the vasculature, healthy follicles and follicles in all stages of antral atresia showed uniform staining of cells (Fig. 3A). The vasculature within the theca interna of healthy and antral atretic follicles was situated in close proximity to the follicular basal lamina and vessels were predominantly oriented circumferentially around the follicle. In contrast, in basal atretic follicles, the lectin binding to the vasculature was uneven in appearance and randomly dispersed throughout the theca interna and, hence, the capillaries often appeared to be orientated toward the center of the follicle (Fig. 3B). Also, in basal atretic follicles, there was some additional labeling of individual cells. These did not appear to be associated with the vasculature, which was recognizable as closely associated cells both in basal atretic follicles and in healthy and antral atretic follicles (Fig. 3B).

From 10 sections from 8 ovaries, 7 healthy, 13 early–midantral atretic follicles, 6 late antral atretic follicles, and 18 basal atretic follicles were examined. Densitometric measures (Fig. 2B) showed the volume fraction of lectin binding per cross-sectional area of theca interna to be the same for healthy, early–midantral atretic follicles and late antral atretic follicles (Fig. 2B). Basal atretic follicles did not differ from healthy and late antral atretic follicles but did contain a significantly higher volume fraction of lectin

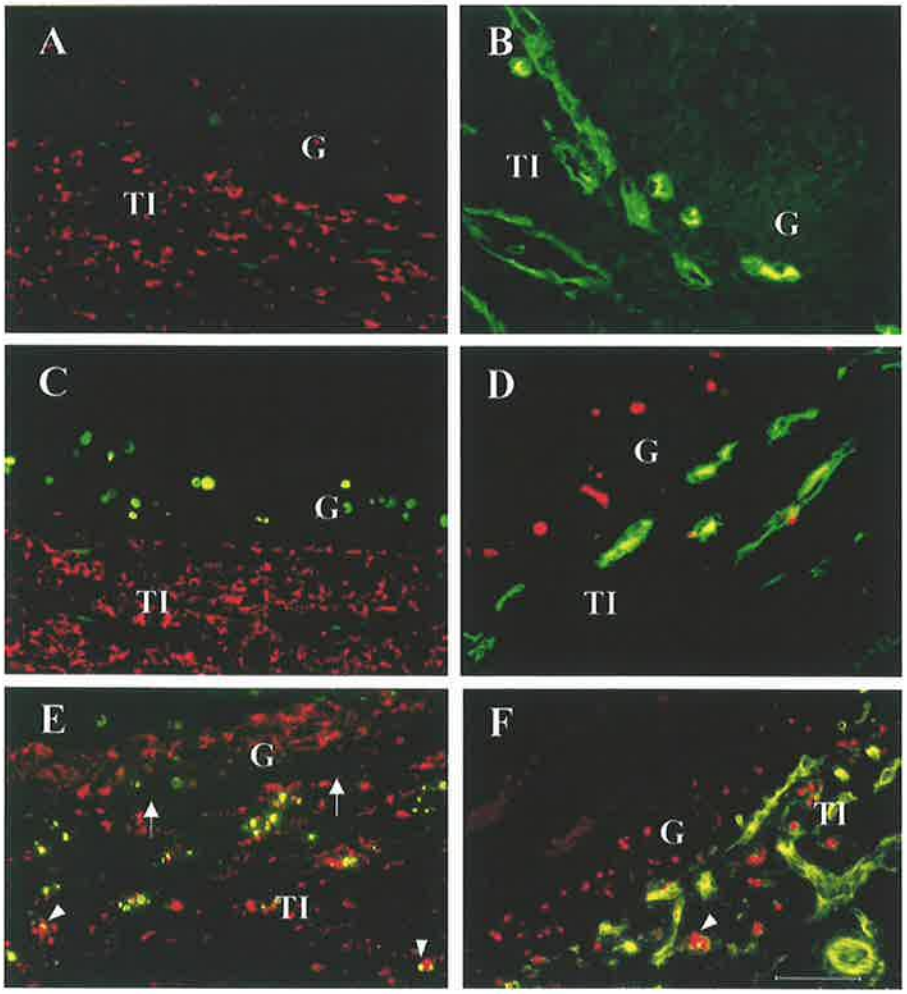

FIG. 4. Dual staining of bovine follicles identifying death of steroidogenic cells and endothelium using TUNEL combined with immunohistochemistry. **A**, **C**, **E**) Healthy, antral atretic and basal atretic follicles, respectively, stained with TUNEL (green) and $P450_{SCC}$ (red). Yellow-green staining indicates TUNEL and $P450_{SCC}$ colocalization within steroidogenic cells undergoing either apoptosis or necrosis (arrowhead). Colocalization is rarely seen within healthy and antral atretic follicles. **B**, **D**, **F**) Healthy, antral atretic and basal atretic follicles, respectively, with lectin binding (green) and TUNEL (red). Yellow-orange staining indicates the TUNEL and lectin colocalization within endothelial cells undergoing cell death (arrowhead). Arrow, follicular basal lamina; TI, theca interna; G, membrana granulosa. Scale bar = 33 μm (**A–D**), = 25 μm (**E**), = 50 μm (**F**).

binding within the theca interna in comparison with early–midantral atretic follicles ($P < 0.05$; Fig. 2B).

Cell Death in the Theca Interna

From 10 sections from 8 ovaries, 7 healthy, 13 early–midantral atretic follicles, 6 late antral atretic follicles, and 18 basal atretic follicles were examined. Labeling was observed within all sections treated by the TUNEL protocol and absent in negative-control sections when terminal transferase was excluded (results not shown). TUNEL labeling was rarely observed within the theca interna of healthy follicles (Fig. 4A). Those undergoing antral atresia had an increased amount of cell death in the theca interna compared with healthy follicles (Fig. 5A). Antral atretic follicles at the late stage had significantly more TUNEL-positive cells than those in the early to mid stages of antral atresia ($P < 0.05$; Fig. 5A). Follicles undergoing basal atresia had significantly more TUNEL-positive cells within the theca interna compared with all other follicles ($P < 0.05$; Fig. 5A). This TUNEL-positive staining was often seen in clusters of cells in close association with one another (Fig. 4, E and F).

TUNEL-Positive Steroidogenic Cells in the Theca Interna

From 10 sections from 9 ovaries immunohistochemically stained for $P450_{SCC}$ and labeled with TUNEL to identify cell death of steroidogenic cells, 11 healthy, 14 early–midantral atretic follicles, 7 late antral atretic follicles, and 9 basal atretic follicles were examined. Healthy and early–midantral atretic follicles did not differ significantly in numerical density of steroidogenic cell death per cross-sectional area of theca interna ($P > 0.05$; Fig. 5B). Both late antral and basal atretic follicles had significantly more steroidogenic cell death than healthy follicles ($P < 0.001$). Basal atretic follicles had significantly more steroidogenic cell death than all other follicle classifications ($P < 0.01$; Fig. 5B).

TUNEL-Positive Endothelial Cells in the Theca Interna

From 10 sections from 8 ovaries, 7 healthy, 13 early–midantral atretic follicles, 6 late antral atretic follicles, and 18 basal atretic follicles were examined by dual lectin binding and TUNEL labeling to identify death of endothelial cells (Fig. 4B, D and F). The number of TUNEL-positive endothelial cells per cross-sectional area of theca interna is shown in Figure 5C. Follicles undergoing atresia had significantly more TUNEL-positive endothelial cells than healthy follicles ($P < 0.05$; Fig. 5C). There was significantly more endothelial cell death occurring in follicles undergoing basal atresia than antral atresia ($P < 0.001$; Fig. 5C).

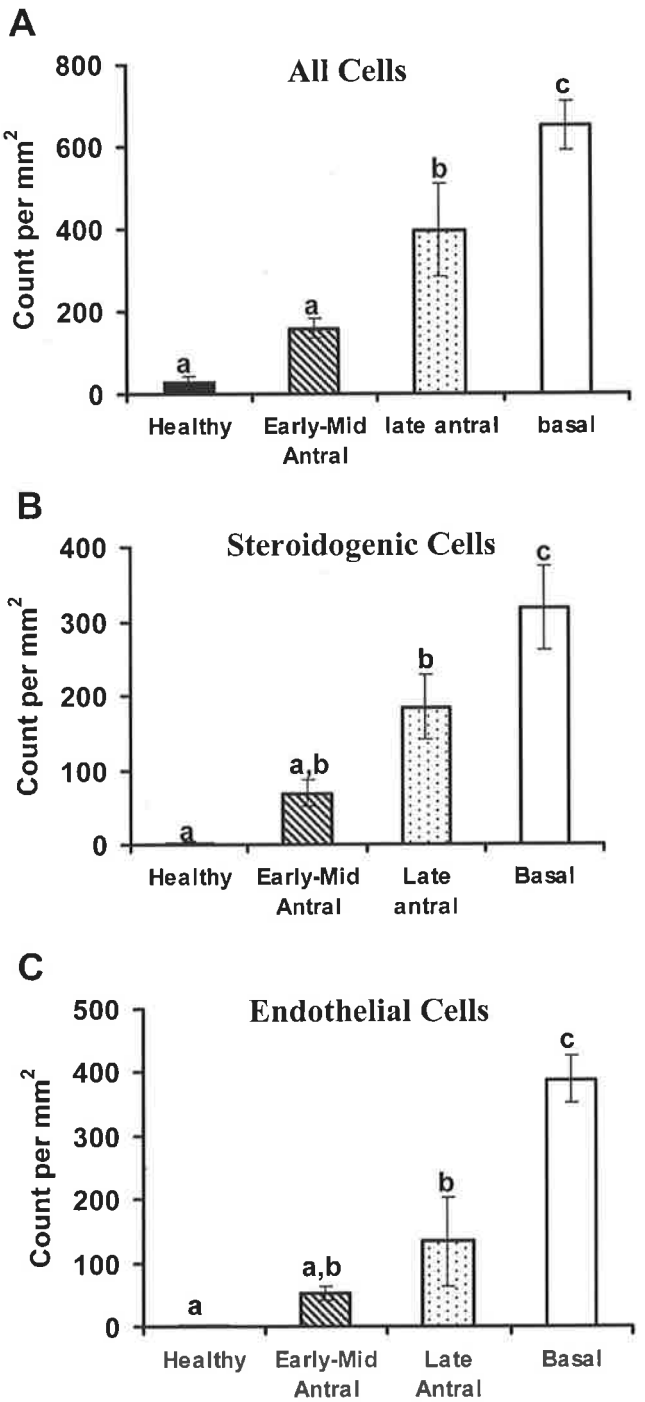

FIG. 5. Mean (±SEM) number of TUNEL-positive cells per cross-sectional area of theca interna of healthy and atretic follicles. **A**) All cells. **B**) Steroidogenic cells. **C**) Endothelial cells. Bars with different alphabetical superscripts are significantly different ($P < 0.05$).

Cell Death in the Membrana Granulosa

From 10 sections from 8 ovaries, 8 healthy, 5 early–midantral atretic follicles, 6 late antral atretic follicles, and 14 basal atretic follicles were examined. Cell death in the membrana granulosa was calculated as a percentage of granulosa cells that were TUNEL-labeled (Fig. 6). All follicles undergoing atresia had significantly more cell death

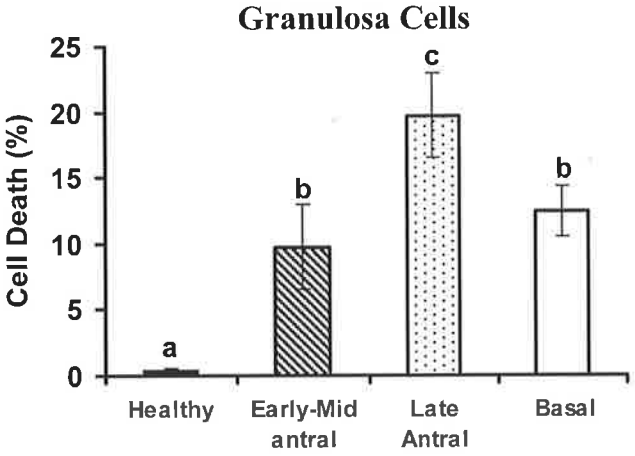

FIG. 6. Cell death within the membrana granulosa. The mean (±SEM) percentage of granulosa cells within the membrana granulosa that were TUNEL-positive. Different alphabetical superscripts are significantly different ($P < 0.01$).

in the membrana granulosa than healthy follicles ($P < 0.001$). Those follicles in the late stages of antral atresia had a significantly greater proportion of granulosa cells that were TUNEL-positive than those in the early–midstages and those undergoing basal atresia ($P < 0.01$; Fig. 6). Follicles undergoing basal atresia and early–midantral atresia did not differ in their proportion of granulosa cells that were TUNEL-positive ($P > 0.05$; Fig. 6).

DISCUSSION

The current study focuses on the theca interna and its different cell types. Using multilabeling and quantitative analyses, it has been shown that basal atretic follicles have fewer $P450_{SCC}$-positive thecal cells. The endothelial cells were orientated randomly, unlike antral atretic or healthy follicles, where the vasculature was orientated parallel to the follicular basal lamina. The total number of cells and the number of steroidogenic cells and endothelial cells that were TUNEL-positive was significantly greater in basal atretic follicles than either antral atretic or healthy follicles, whereas the proportion of TUNEL-positive granulosa cells was similar in all the atretic follicles.

The results confirm and extend observations on the basal atretic follicles. The reduced number of $P450_{SCC}$-positive theca cells could have arisen by either enhanced death of these cells as observed here and/or reduced expression of $P450_{SCC}$. Either way, it would be predicted that the synthesis and secretion of steroid hormones from the theca in these follicles would be reduced. In agreement with this, reduced levels of thecal-derived testosterone and androstenedione have been observed in the follicular fluid of basal atretic follicles [11]. The basal atretic follicles also have reduced levels of INSL3 [22] and INSL3 mRNA (unpublished results). INSL3 is expressed in steroidogenic cells of the theca interna [22]. Whereas not all roles of INSL3 have been discovered, INSL3 null mice have an increased rate of follicular atresia and luteal regression [23], suggesting that INSL3 in some way maintains cells or prevents apoptosis of cells. Consistent with these data is the suggestion that maybe INSL3 maintains the theca interna and that its reduced expression in basal atresia initiates death of cells in the theca interna. Of course an equally consistent hypothesis is that death of theca interna cells leads to a loss

of expression of INSL3 or of cells expressing INSL3 in basal atretic follicles. Additional experiments will be required to address this issue.

The study by O'Shea et al. [20] on the theca interna of atretic follicles in the sheep ovary suggests that vasculature changes in the theca interna play a major role in the atretic process. In the current study, lectin from *Bandeirea simplicifolia* was used to identify endothelial cells [24, 25]. This lectin has been shown to colocalize with an antibody recognizing von Willebrand factor in endothelial cells of bovine follicles (unpublished results). Histological observations showed basal atretic follicles contained some binding, which was uneven in appearance compared with the intense and clear binding to the endothelial cells seen within healthy or antral atretic follicles. Confocal microscopy suggested that this binding occurred within individual cells not associated with vasculature structures (unpublished results). The cause of this is currently unknown. These cells could represent endothelial cells not associated with vasculature that are most likely to have arisen before the progression into atresia or the acquisition of lectin binding domains by nonendothelial cells as observed previously [26, 27]. Irrespective of this additional set of cells in basal atretic follicles that bind lectin, the vasculature in the theca interna clearly is oriented differently in these follicles. It appears to radiate toward the antrum, whereas in healthy and antral atretic follicles, it is orientated essentially parallel to the follicular basal lamina. Given the increased endothelial cell death observed here and the increased cell debris observed previously in the capillaries of the theca of basal atretic follicles [10], it can be speculated that the blood flow through these capillaries is reduced. Whether this is a cause or effect of basal atresia is not known at this stage.

The TUNEL method of labeling degraded DNA is a valid method for identifying cell death [21, 28–30], even if it does not definitively distinguish between apoptosis and necrosis. Apoptosis is reported to be the major cause of cell death in atresia [3, 8]; however, these reports are limited to studies of the membrana granulosa, and such studies do not account for other possible causes of cell death [7]. TUNEL labeling in this study showed a greater proportion of cell death within the theca interna of follicles undergoing basal atresia compared with healthy and antral atretic follicles. In addition, there was significantly more endothelial and steroidogenic cell death in basal atretic follicles compared with healthy follicles and even in the later stages of antral atresia. However, the amount of cell death observed in the membrana granulosa of basal atretic follicles was equal to that of follicles in the early–midstages and less that that of the late stage of antral atresia. Taken together, these results suggest that cell death is more prominent in the membrana granulosa in antral atretic follicles and more so in the theca interna in the basal atretic follicles.

We conclude that the theca interna can be very susceptible to cell death and may even be a site of initiation of atresia, at least in basal atretic follicles. This is a major finding because basal atresia involves a significant proportion (50%) of all atretic follicles <5 mm in diameter [10] in the bovine. Whereas antral atresia largely involves the membrana granulosa, it can occur at these and larger sizes of follicles. Thus, we suggest that, during follicle growth and development, the order of susceptibility to death is the oocyte, then both the theca interna and membrana granulosa, and finally the membrana granulosa. The logical corollary of this is that there will be many real and inducible causes of follicular atresia.

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Statement of Authorship

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Extracellular Matrix of the Developing Ovarian Follicle

Helen F. Irving-Rodgers, B.Sc.,[1] and Raymond J. Rodgers, Ph.D.[1]

ABSTRACT

There are many different types of extracellular matrices in the different follicle compartments. These have different roles in follicle development and atresia, and they change in composition during these processes. This review focuses on basal lamina matrix in particular, and considers follicular fluid, the newly identified focimatrix, and thecal matrices. When follicles commence growing, the follicular basal lamina changes in its composition from containing all six α chains of type IV collagen to only α1 and α2. Perlecan and nidogen-1 and -2 subsequently become components of the follicular basal lamina, and there is an increase in the amount of laminin chains α1, β2, and γ1, in the bovine at least. Late in follicular development and on atresia some follicles contain laminin α2. On atresia the follicular basal lamina is not degraded, as occurs in ovulation, but can be breached by cells from the thecal layer when it is not aligned by granulosa cells. A novel type of basal lamina-like matrix, called focimatrix (abbreviated from focal intraepithelial matrix), develops between the cells of the membrana granulosa as aggregates of basal lamina material. It does not envelop cells and so cannot perform functions of basal lamina as currently understood. It is hypothesized that focimatrix assists or initiates depolarization of the membrana granulosa necessary for the transformation into luteal cells. The largest osmotically active molecules in follicular fluid are hyaluronan and chondroitin sulfate proteoglycans, including versican and inter-α trypsin inhibitor. It has been suggested that these might be responsible for the formation of follicular fluid by creating an osmotic gradient across the follicular wall. The formation, development, and then either ovulation or regression of follicles requires considerable tissue remodeling, cellular replication, and specialization. The expectation of researchers is that extracellular matrix will be intimately involved in many of these processes. Much research has focused in identifying the components of extracellular matrix and associated developmental changes. We review the components of extracellular matrix associated with follicular development, including the basal lamina, focimatrix, follicular fluid, and matrix of the thecal layers.

KEYWORDS: Ovary, follicle, extracellular matrix, basal lamina, focimatrix, laminin, collagen type IV, nidogen, collagen type XVIII, usherin

THE FOLLICULAR BASAL LAMINA

Basal laminas are specialized sheets of extracellular matrix that in epithelia underlie the epithelial cells and separate them from adjoining stroma. Several general reviews have been written about basal laminas.[1,2] Basal laminas influence epithelial cell proliferation and

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differentiation, and can retard the passage of cells and molecules selectively. Basal laminas are composed of a lattice-type network of type IV collagen intertwined with a network of laminin. This structure is stabilized by the binding of entactin/nidogen-1 or -2[3] to the collagen and laminin. The heparan sulfate proteoglycans perlecan and collagen XVIII and other molecules such as the glycoprotein usherin are associated with basal laminas. Usherin has been shown to interact with both collagen type IV[4] and fibronectin,[5] and has been localized to basal laminas of mouse and human ovary.[6]

Importantly, basal laminas in different regions of the body differ in the ratio of all these components. Furthermore, some components are really a class of several molecules. For example, each molecule of type IV collagen comprises three α chains. However, there are six different chains of type IV collagen α (α1 to α6, each encoded by a separate gene).[7] Given that only three of these α chains are required to make one collagen molecule, many potential different combinations of type IV collagen could exist. Some of these different combinations have been observed in nature (e.g., α1α1α2, α3α4α5), and each can be regarded as different or unique.[8] Similarly, each laminin molecule is composed of three chains; one α, one β, and one γ chain.[9] There are five different α chains, three β chains, and three γ chains (all encoded by separate genes), potentially giving rise to 45 ($5 \times 3 \times 3$) different molecular combinations, with alternative splicing resulting in more combinations.[9] Thus, much complexity in basal laminas can be generated by the composition of laminin and type IV collagen alone. It is now recognized that the unique composition of each basal lamina contributes to its specific functional properties.

Based upon the roles of basal laminas in other tissues and organs, it is speculated that the follicular basal lamina is important for maintaining the polarity and the degree of specialization of granulosa cells aligning it.[10,11] Unfortunately polarity studies of the membrana granulosa have not been undertaken, unlike in many other epithelia. Studies on the changing nature of the follicular basal lamina and follicle phenotype have been conducted, and are discussed. However, there are very few studies attempting to show that changes in the basal lamina are responsible for changes in cell and tissue behavior during follicle development.

The follicular basal lamina probably has a role in retarding the entry of larger molecular weight plasma proteins and molecules (e.g., low-density lipoproteins) into the follicular antrum. Conversely, the follicular basal lamina may result in entrapment in the follicular fluid of those large molecules (e.g., some proteoglycans) synthesized by granulosa cells and oocytes. The molecular mass cut-off of the follicular barrier is calculated to be 100 to 500 kd, based upon comparisons of the composition of follicular fluid with that of plasma.[12] This is not necessarily just the limit imposed by the follicular basal lamina, given that plasma proteins also have to traverse the vascular subendothelial basal lamina before reaching the follicular antrum.

In addition, molecular size may not be the only determinant of movement across the follicular basal lamina. It has been shown that the blood–follicle barrier to movement of inter-α trypsin inhibitor is due to its negative charge,[13] suggesting that the follicular basal lamina may likewise exclude this material. The follicular basal lamina also contains the heparan sulfate proteoglycan perlecan.[14] Heparan sulfate proteoglycans can bind several growth factors (e.g., fibroblast growth factor [FGF-2]), or their binding proteins (e.g., follistatin, and IGF-BP2 and -5). Thus it is possible that the follicular basal lamina may also restrict the movement of growth factors. Certainly the molecular exclusion capability of the follicular basal lamina allows it, in part, to determine the milieu of factors to which granulosa cells and the oocyte are exposed.

During follicular development the follicular basal lamina expands in surface area, and also changes in composition.[11,15,16] In bovine follicles, expression of collagen type IV α3 to α6 declines during growth, whereas α1 and α2 continue to be expressed.[15] Studies in the rat also found a decline in collagen type IV α3, but detected collagen type IV α4 and α5 in large follicles.[17] In the bovine, laminin β1 is transiently expressed at the preantral stage, and α1, β2, and γ1 appear to be more highly expressed in preantal and antral follicles.[18] Expression of laminin α1 has been detected in the rat. The expression of α1, β2, and γ1 in the bovine suggests that laminin 121 (α1β2γ1 is laminin 3 in the older nomenclature[9]) is present in the follicular basal lamina. Assembled laminin 121 has only been isolated from placenta,[19] and hence it is tempting to speculate a causal association between laminin 121 expression and steroid hormone synthesis. The expression of laminin α2 is unusual, and is only expressed in a few healthy antral follicles and proportionately more atretic antral follicles.[18,20] However, it has not yet been shown that the identified α1, β2, and γ1 are assembled together into a laminin molecule in the follicular basal lamina, and in the absence of studies of other laminin chains (namely α3, α4, α5, β3, γ2, or γ3, capable of complexing with α1, β2, or γ1, and hence giving rise to different laminin isoforms), it remains to be confirmed which laminin molecule(s) are present in the follicular basal lamina. In addition, neither nidogen-1 nor perlecan are detectable in primordial follicles but are expressed later in follicular development,[14] whereas the heparan sulfate proteoglycan collagen type XVIII[21,22] is present in the follicular basal lamina throughout development[23] (Fig. 1).

From these studies it is clear that during follicle development, the follicular basal lamina becomes less

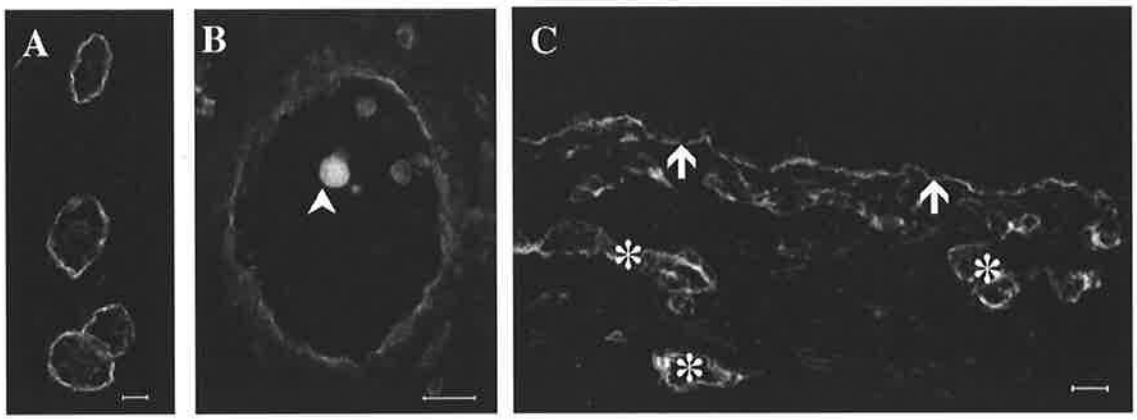

Figure 1 Localization of collagen type XVIII in bovine ovarian follicles. (A) Collagen type XVIII is localized to the follicular basal lamina of primordial/primary follicles. (B) Collagen type XVIII is localized to the follicular basal lamina and Call-Exner bodies (arrowhead) of a preantral follicle. (C) Collagen type XVIII is localized to the follicular basal lamina (arrows) and subendothelial basal laminas of an antral follicle (asterisks). Scale bars are A = 20 μm, B = 10 μm, C = 50 μm.

collagenous and more laminin rich. By the nature of the different bonds between laminins and collagens, such basal laminas are more expandable, which is a property required for follicle enlargement. In is not clear what the effects of loss of collagens type IV α3 to α6 may have on the function of the follicular basal lamina. The lack of expression of nidogen-1[14] or -2[24] (Fig. 2) in primordial follicles might at first appear perplexing because its role is to cross-link collagens and laminins, both of which are present in primordial follicles. However, recent double knockout of both nidogen-1 and -2 show that they are not necessary for basal lamina assembly.[25] Upregulation of the heparan sulfate proteoglycan perlecan may be one of the more significant changes occurring during early follicular development because of its ability to bind growth factors.[26,27] Thus, certain growth factors could more freely cross the follicular basal lamina of primordial follicles in the absence of perlecan, than in growing follicles when perlecan is present. It should be emphasized that these comments relating to follicles are speculative at this stage.

Although immunolocalization of matrix components has been extremely useful in localizing matrix components to their specific site in the follicle wall, it is not a suitable technique for identifying which cells synthesized them. Evidence suggests many components of the follicular basal lamina are produced by granulosa cells. Granulosa cells cultured under anchorage-independent conditions also synthesize a basal lamina,[28] which contains at least collagen type IV and fibronectin.[28,29] Fibronectin,[29–31] laminin γ1 chain,[31] nidogen-1,[32] and perlecan[32] have all been shown to be either produced or expressed by granulosa cells. Hence it is likely that most of the components of the follicular basal lamina are synthesized by the granulosa cells. Whether there is a contribution to the follicular basal lamina from the theca in larger follicles, or indeed the stroma surrounding preantral follicles, is not known. One candidate component is laminin α2. In other tissues, where it is a component of an epithelial basal lamina, it appears to originate from the stroma.[33,34] In bovine follicles laminin α2 is only expressed in a very small proportion of healthy follicles but more commonly in atretic follicles.[20] The expression pattern of this laminin chain is also different from that of the other molecules in the follicular basal lamina and it is not expressed in Call-Exner bodies[35] or focimatrix,[36] and these observations could be explained by a different cellular origin.

The event of follicular atresia is unusual in the body because it eventually involves complete destruction of the epithelial layer by death of all the epithelial cells. In other epithelia, such as luminal gut epithelium or involuting glands, cells undergo apoptosis as they age, but this is regional and only involves a few cells at any one time, and not normally the entire epithelial layer. Histochemical observations of bovine and ovine follicles undergoing atresia have identified the presence of laminin chains, perlecan, nidogen, and collagen type IV chains.[20,37] In these studies the components identified in atretic follicles were the same as those present in the class of follicles from which they were derived, except for laminin α2, the expression of which did not correlate with the type or severity of atresia[20]. Resorption of follicular fluid during atresia of antral follicles causes shrinkage and folding of the basal lamina. This is very different from what happens to the follicular basal lamina upon ovulation, when it is extensively degraded. Even though the follicular basal lamina remains intact during the early events of atresia, macrophages, endothelial cells, and fibroblast can breach the follicular basal lamina as they migrate into the follicle from the theca interna.[38] However, movement of cells from the thecal side across the basal lamina appears to be restricted when the basal lamina is aligned with healthy granulosa cells.[38]

FOLLICULAR FLUID

Granulosa cells of antral follicles are bathed in follicular fluid containing proteins and soluble extracellular matrix molecules including proteoglycans. Proteoglycans consist of a core protein with attached glycosaminoglycans. Over decades many authors have analyzed follicular fluid to identify its glycosaminoglycans composition and synthesis by granulosa cells. The proteoglycans identified in follicular fluids include verscian[14,36,39] and inter-α trypsin inhibitor.[40,41] Perlecan was also identified in human follicular fluid aspirated from patients undergoing oocyte retrieval in an in vitro fertilization (IVF) program[39] and in homogenates of whole bovine follicles.[14] Perlecan is probably not a component of follicular fluid because it is a component of the follicular wall,[14] and may have been present as a contaminant in the follicular fluid in the study by Eriksen et al.[39]

Inter-α trypsin inhibitor contains two of three different heavy chains linked by a chondroitin sulfate chain to bikunin produced by the liver, and is found abundantly in serum. In mice, inter-α trypsin inhibitor appears to be sequestered from the blood stream because it appears within the follicle fluid within minutes of the luteinizing hormone (LH) surge[42]. On entering the fluid, it associates with hyaluronan being synthesized by the cumulus cells, liberating free bikunin and producing a covalent bond between the heavy chains and hyaluronan.[43] However, more recently inter-α trypsin inhibitor,[40,41] inter-α-like trypsin inhibitor, and pre-α trypsin inhibitor[41] were identified in porcine and bovine follicular fluids, well in advance of the LH surge and additionally in follicles too small to ovulate. Follicular fluid from ovulating follicles of patients undergoing IVF contained inter-α trypsin inhibitor at a concentration 70% of that found in serum and only 6% of the follicular fluid inter-α trypsin inhibitor was bound to hyaluronan.[44] These results from human, bovine, and porcine suggest that there are additional roles for inter-α trypsin inhibitor and related molecules in follicular fluid. In addition to its localization to the cumulus oocyte complex and granulosa cells and follicular fluid, inter-α trypsin inhibitor has been localized to the ovarian stroma [45,46] and connective tissue in the bovine corpus luteum, suggesting further roles for this molecule.

Versican is a chondroitin sulfate proteoglycan that can be synthesized by the membrana granulosa.[47] At its N-terminal it contains a G1 domain that can bind hyaluronan and may participate in cell–matrix and cell–cell interactions. The G3 domain at the C-terminal end contains several motifs (lectin-like, epidermal growth factor-like, and compliment binding). Between these two domains are two alternatively spliced regions where the glycosaminoglycan chains are attached. Splicing in this region produces four isoforms (V0, V1, V2, V3), having different degrees of glycosaminoglycan substitution. The V0, V1, and V3 isoforms have been detected in mouse, rat, and bovine ovaries.[23,41,47]

Hyaluronan is a glycosaminoglycan that lacks a core protein. It has been found in human follicular fluid at 50.0 ± 2.6 ng/mL and it has been localized previously adjacent to and including the spaces between antral granulosa cells.[48] It is produced by granulosa and cumulus cells,[49,50] and bovine follicular fluid hyaluronan is estimated to be ~0.4 to 2×10^6 d.[41] Hyaluronan is also a component of the ovarian stroma and theca throughout follicular development,[45] and in the bovine corpus luteum.

In a recent study of the removal from follicular fluid of hyaluronan and chondroitin sulfate/dermatan sulfate and their aggregates with associated molecules by enzymatic digestion followed by dialysis, a substantial reduction in osmotic potential at molecular weight cut-offs of 100 and 300 kd was observed.[41] From these results it was concluded that hyaluronan, versican, and inter-α trypsin inhibitor and their related molecules or aggregates are sufficiently large enough to be trapped in the follicular fluid and thus to contribute to its osmotic potential.[41] It was hypothesized that this osmotic potential is part of the mechanism by which follicular fluid is recruited to the follicular antrum.[41] If this is the case, then regulation of the synthesis of these components could be a mechanism by which follicular growth and follicle dominance is attained.

FOCIMATRIX

A novel type of basal lamina-like matrix, called focimatrix (abbreviated from focal intra-epithelial matrix), has been discovered recently in the membrana granulosa of bovine follicles (Fig. 3A).[36] It develops as aggregates of basal lamina material deposited between the granulosa cells.[36] Focimatrix (Fig. 3A) contains α1 and α2 chains of collagen type IV; laminin α1, β2 (Fig. 3D), and γ1 chains; nidogen-1 and -2; perlecan; collagen type XVIII; and usherin (Fig. 3C); but not versican.[36] These components are similar to those found in the follicular basal lamina at the stage of follicular development when focimatrix is observed.[14,15,18] Focimatrix first appears in bovine follicles > 5 mm in diameter, and the amount of focimatrix increases with increasing follicular size and probably exists in other species. Laminin subunits[51] and collagen type IV[51,52] have been detected previously between granulosa cells isolated from human preovulatory follicles and also in bovine cumulus cells.[53] In addition, expression patterns of extracellular matrix in the membrana granulosa of sheep[37] and rat ovaries (see Fig. 17 in Frojdman et al[17]) suggest the presence of focimatrix in these species.

The ultrastructure of focimatrix is similar to that of basal lamina and it contains typical basal lamina

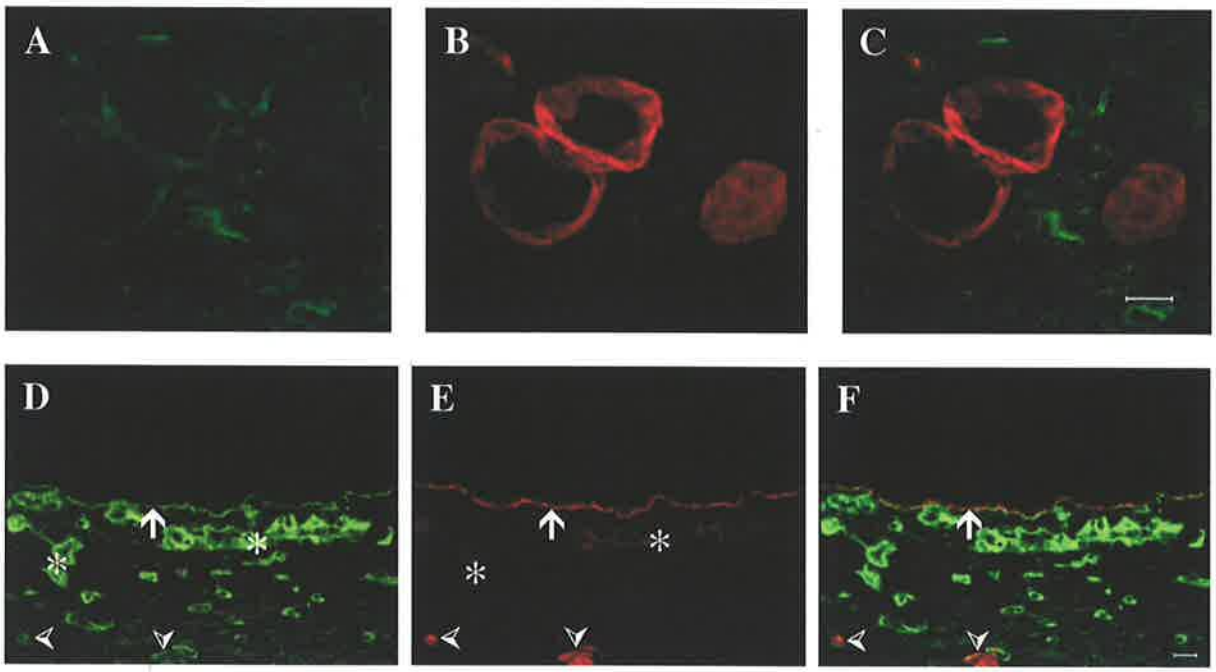

Figure 2 Colocalization of nidogen-2 (green in A and D) and laminin β2 (red in B and E). Nidogen-2 is absent from the follicular basal lamina of primordial follicles (A), but present in the follicular basal lamina of antral follicles (D) (arrow), subendothelial basal lamina (asterisks), and arterioles (arrowheads). Laminin β2 is localized to the follicular basal lamina of primordial follicles (B), and in antral follicles (E) (arrow) and the basal lamina surrounding arteriolar smooth muscle (arrowheads), but not capillary subendothelial basal lamina (asterisk). (C) and (F) are merged images. Scale bars = 20 μm.

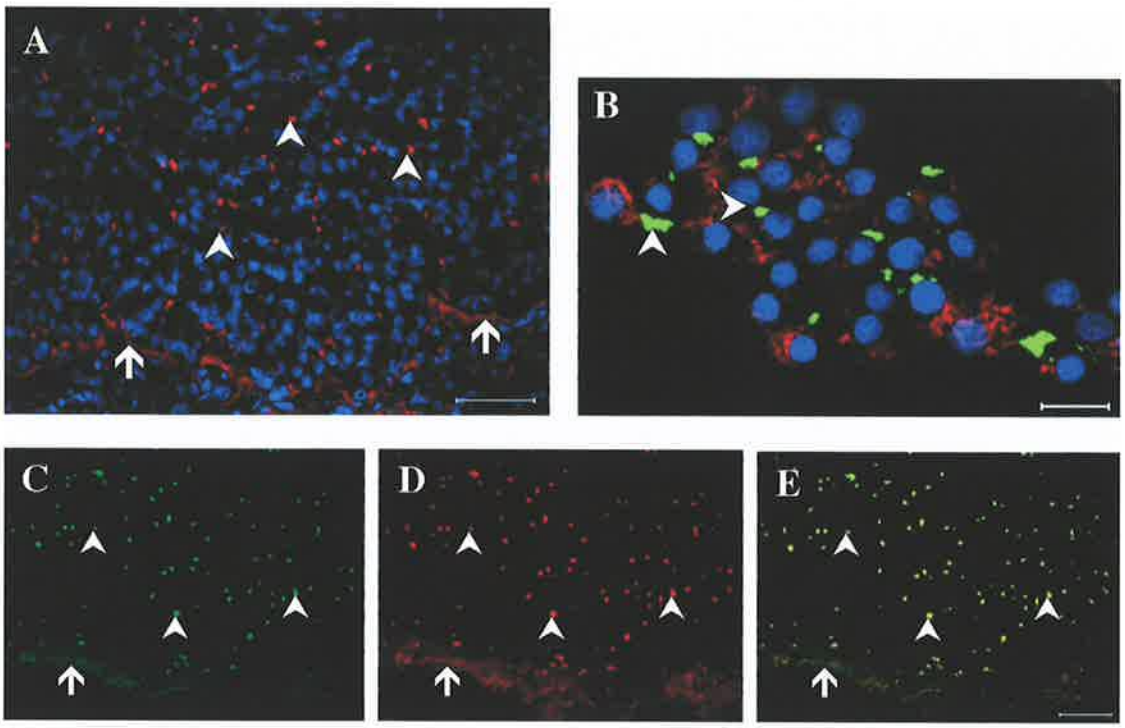

Figure 3 Localization of focimatrix components to the membrana granulosa of bovine large antral follicles. Laminin 111 components (red in A and green in B) localize to the follicular basal lamina (arrows in A) and focimatix within the membrana granulosa (arrowheads in A and B). (B) Granulosa cells are also labeled with an antibody to vimentin (red in B). Nuclei are staining blue with 4,6, diamidino-2-phenylindole, 2 HCl. (A) 14-mm-diameter follicle; (B) 17-mm-diameter follicle. Usherin (green in C) and laminin β2 chain (red in D) colocalize to the follicular basal lamina (arrow) and focimatrix (arrowheads) within the membrana granulosa of a follicle 11 mm in diameter. (E) Merged images of (C) and (D). Scale bars, A = 50 μm; B = 20 μm; C, D, E = 50 μm.

components; however, it is neither basal nor laminate. Based on the morphology of focimatrix (Fig. 3B), it does not appear to be in a position to filter material or create microenvironments for enclosed cells, as do true basal laminas, because it does not enclose individual cells or groups of cells. In the bovine it is estimated that focimatrix is first expressed ~10 days prior to ovulation. Prior to this, the only basal lamina matrix that the granulosa cells come in contact with is the underlying follicular basal lamina, or the Call-Exner bodies in preantral follicles.[35] The follicular basal lamina presumably dictates the polarity of the granulosa cells, enabling directional secretion, uptake of molecules, and other functions of polarized cells. After expression of focimatrix, the granulosa cells have basal lamina material deposited on many sides. It is hypothesized that this reduces the polarization cue from the follicular basal lamina[36] and commences a process with similarities to an epithelial-to-mesenchymal transition postulated to be part of the process called luteinization.[54,55] Loss of polarization is a key feature of the epithelial-to-mesenchymal transition and granulosa cells are polarized, whereas luteal cells are not.

CALL-EXNER BODIES

The membrana granulosa of ovarian follicles of many species and granulosa cell tumors[56,57] contain Call-Exner bodies that have been described as "a ring of granulosa cells disposed radially around a central cavity,"[58] and are distinguished by the presence of a basal lamina-like structure.[59–61] In bovine they are predominant in preantral follicles and contain laminin α1, β2, and γ1; collagens type IV α1 to α6; nidogen-1 and -2; perlecan[14,24,35]; collagen type XVIII (Fig. 1B); and usherin. Thus, although the composition is different from that of focimatrix, both focimatrix and Call-Exner bodies contain the same components as the follicular basal lamina has at the time focimatrix or Call-Exner bodies are formed. For instance, collagens type IV α1 to α6 are detected in the follicular basal lamina bodies of preantral follicles, and the Call-Exner bodies in these follicles also have collagens type IV α1 to α6. In larger antral follicles, the follicular basal lamina only has collagens type IV α1 and α2, and focimatrix in these follicles only has collagens type IV α1 and α2.[35] The exception is laminin α2, which is absent from both Call-Exner bodies and focimatrix, but is found in the follicular basal lamina in a proportion of antral follicles.

Although Call-Exner bodies occur in most preantral follicles, they are far less abundant than the focimatrix of large antral follicles. The focal accumulation of matrix in Call-Exner bodies may cause the polarization of immature granulosa cells, resulting in the identifiable rosette formation. Focimatrix does not appear to cause polarization of adjacent granulosa cells, however, possibly because there is a substantial amount of focimatrix dispersed throughout the stratified layers of the membrana granulosa, or because the granulosa cells are more mature when it is produced.

EXTRACELLULAR MATRIX OF THE THECAL LAYERS

In the thecal layers basal laminas underlie endothelial cells (laminin β1 and β2 chains, and collagen type IV α1 and α2) and surround the smooth muscle cells of the arterioles (laminin β2 chain and collagen type IV α1 and α2).[15,17,18] In addition to these basal lamina components, the laminin γ1 chain, laminin 1 components (α1, β1, or γ1),[18] and collagen type IV chains α1 and α2,[15] are present in bovine follicles throughout the theca interna and not in association with any identifiable basal laminas, such as those of blood vessels. At the electron microscope level, fragments of basal lamina-like, electron-dense material have been observed, and this matrix has been named the thecal matrix.[11] In the theca and the interstitial tissue of developing gonads of other species such as mouse and rat, a similar matrix has been observed by localizing laminin 111 (formerly laminin 1) components. The origins and functions of the thecal matrix are not known. Structural collagens have been observed in the theca externa of many species, and collagen type I has been identified.[31,62] Versican is also present in the theca of bovine,[14,36] mouse, and rat follicles.[47] In bovine there is a particularly large amount of versican localized to the thecal side of the follicular basal lamina.[14,36] The role of versican in the theca has not been investigated. Hyaiuronan and inter-α trypsin have also been localized to the theca of the mouse[45] and bovine follicle.[46]

Collagen type VI, involved in the extracellular organization of fibronectin, has also been identified in the theca interna.[63] Fibronectin is commonly a matrix of stroma and it is important for cell migration, which clearly must occur during expansion of the theca during follicular development. In humans, at least 20 different isoforms of fibronectin exist, due to alternative splicing of mRNA at three separate sites (extra domain A [EDA], EDB, the variable [V] region or IIICS domain). Fibronectin exists as a homo- or heterodimer of these splice variants. Several of the splice variants are present or expressed in follicles,[37,64] and the EDA domain of fibronectin has been shown to be mitogenic for granulosa cells in vitro,[65] while fibronectin synthesis by granulosa cells can be upregulated by FGF-2[29]. There are a considerable number of publications showing the ovarian localization of fibronectin, often using antibodies that recognize all forms of fibronectin. There does not appear to be a consistent localization pattern across species, with reports of fibronectin, usually of type undefined, being localized to different follicular compartments. Although these differing reports may be

accurate, there is also a circulating form of fibronectin lacking EDB and EDA domains. In many of the ovarian studies, the antibodies used would react with all fibronectin isoforms. Thus, considerably more effort is required to identify the precise expression patterns of fibronectin isoforms during follicle development if we are to identify its roles during follicle development.

CONCLUSIONS

In recent years, there has been a major effort to identify, localize, and correlate matrix components with different stages of follicular development. More importantly, the precise isoforms are now being identified in ovaries. Future research will no doubt focus on the origins and regulation of synthesis of the matrix components in the ovary. Studies of the effects of the molecules on cell or tissue behavior have also commenced. Although these studies are useful in identifying behaviors that matrix may control, ultimate proof of their roles will only come by using or mimicing the native matrix molecules. This will require more sophisticated cell culture conditions or systems. At present, many of these methods were established with only endocrine parameters in mind. Other approaches, using genomic mutations, such as successfully being used to study the zona pellucida, may independently shed light upon the roles of matrix in ovarian function. In summary, recent years have seen a considerable increase in our knowledge on this subject and the next few promise to produce results that are just as exciting.

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Statement of Authorship

Extracellular Matrix of the Corpus Luteum

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Extracellular Matrix of the Corpus Luteum

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ABSTRACT

The potential importance of the extracellular matrix to luteal formation and development, additional development in response to pregnancy hormones in some species, and luteal function and regression is possibly under-appreciated. Collagens I and III and fibronectin change dynamically during the formation of the corpus luteum and probably reflect the necessity for directional migration of cells in the establishment of a vascularized corpus luteum. Extracellular proteins may also be essential for the maintenance of luteal cell phenotype. Laminins, collagens type IV, and nidogen-1 have been localized to varying degrees of completeness in different species. Each capillary has a subendothelial basal lamina that changes in composition during luteal formation. These subendothelial basal laminas are often adjacent to luteal cells. The high vascularity of corpora lutea may have led to the assumption that luteal cells are surrounded by basal laminas. However, in rat, bovine, and human corpora lutea, there is no evidence of basal laminas surrounding luteal cells. Instead there are fibers or aggregates of basal lamina material rich in laminins interspersed throughout the luteal tissue. Versican appears to be localized to the capsule in human corpora lutea but is widely dispersed in the bovine corpus luteum, similar to the distribution of thecal derived cells, and is not associated with capillaries. Hyaluronan is also present in the luteal parenchyma. Clearly more studies of corpora lutea are required for a fuller understanding of the roles of extracellular matrix in luteal function.

KEYWORDS: Corpus luteum, luteal cell, endothelium, laminin, collagen, fibronectin, hyaluronan, versican

The mammalian corpus luteum develops as a postovulatory structure by reorganization of the cells and tissues of the ruptured follicle. During formation of the corpus luteum, the previously discrete theca and granulosa layers of the follicle wall become less distinguishable. The capillary network, previously confined to the theca, penetrates the membrana granulosa and follicular antrum, and subsequently vascularizes the entire corpus luteum. A major event in this transition is the loss of integrity of the follicular basal lamina, allowing capillary penetration but also permitting cells from the follicle wall to intermingle. Other elements of the follicular matrix also undergo reorganization, and the whole tissue becomes remodeled via processes similar to those occurring during fetal tissue development and wound repair. At the same time, the endocrine cells of the luteinizing follicle undergo major phenotypic changes, with the result that the ovary progresses to a different phase of the reproductive cycle. Following ovulation, the immediate development of the corpus luteum involves extremely rapid growth, followed by a period of mature function, which may or may not be extended by preg-

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nancy, and ultimately by biochemical decline and tissue regression. Although the external initiation and regulation of the ovarian cycle are brought about by the hormones and growth factors of the reproductive axis, many cellular and tissue events occur in response to changes in the identity, abundance, and organization of local regulatory factors. Key among these are the components of the follicular[1] and luteal extracellular matrices.

Although the study of ovarian extracellular matrix has focused predominantly on follicles, the potential importance of the matrix to luteal formation and development, additional development in response to pregnancy hormones in some species, and luteal function and regression is possibly under-appreciated. Extracellular matrices in general have many different roles,[2] including effects on cell adhesion, cell shape, migration, division, differentiation, and cell death. All of these cellular processes occur during the development and regression of the corpus luteum, as they also do during the follicular cycle. The extracellular matrix defines specialized microenvironments that both directly and indirectly influence cell and tissue functions. Several matrix components have been shown to be important for filtering soluble materials and can therefore affect the fluid dynamics of a tissue. Other molecular components, such as heparan sulfate proteoglycans and laminins, have the ability to bind growth factors, whereas others interact directly with integrin receptors on cell surfaces. Thus, the specialized composition of defined matrices is responsible for regulating cellular function, as well as for providing mechanical support for cells. The maintenance of the luteal cell phenotype is crucial to the proper function of the corpus luteum, and therefore to the maintenance of cyclicity and pregnancy. The cyclic development of the corpus luteum and the appropriate remodeling of tissue structures over the lifespan of the organ are likely to be heavily dependent on the correct extracellular environment.

FOLLICULAR ORIGIN OF LUTEAL TISSUES AND EXTRACELLULAR MATRIX

Given that the corpus luteum develops from the ovulating follicle, the arrangement of tissues in the follicle provides a starting point for understanding subsequent structures in the corpus luteum. Excluding the specialized matrix of the cumulus oocyte complex, key components are the basal lamina, the acellular elements of the membrana granulosa and antral cavity, and the highly complex matrix of the theca interna and externa. The follicular basal lamina is crucial to follicular integrity; it separates the theca and granulosa layers and also defines a boundary to capillary endothelial penetration of the follicle interior. It has a conventional basal lamina composition (comprising collagen type IV, laminin, nidogen/entactin, and perlecan) but its molecular identity changes considerably during follicle development.[1] In the bovine, for example, synthesis commences at the primordial stage with expression of all six collagen type IV α genes. During subsequent follicle growth, the α3 to α6 chains are lost, but nidogen 1 and perlecan appear, and there is an increased expression of laminin α1, β2, and γ1 (the components of the laminin 3 isoform).[1] The most abundant matrix of the antrum is follicular fluid, which contains hyaluronan, versican, and inter-α trypsin inhibitor.[3] In addition, as the follicle approaches preovulatory size, aggregates of basal lamina-like material appear between the granulosa cells. This material, named focimatrix, has the same composition as the contemporaneous follicular basal lamina but it is not organized as a discrete sheet.[4] The endocrine and other cells of the theca are immersed in a complex thecal matrix comprising collagen type IV α1 and α2, and a laminin isoform containing the laminin γ1 chain.[5] The vasculature (endothelium and vascular smooth muscle) has its own associated basal laminas.[5,6] The origin and role of the thecal matrix are poorly understood, as is the exact demarcation between vascular and other elements.

At ovulation, the follicular basal lamina, focimatrix, and the external follicle wall are degraded.[7] As the follicular fluid and cumulus-oocyte complex are released, the follicle wall folds inward, forming protrusions from the underlying theca interna. This tissue includes connective tissue and endothelial/vascular elements that are later represented in the mature corpus luteum by septa rich in larger blood vessels and connective tissue.[8,9] Cells from the theca infiltrate the membrana granulosa and form a distinct population of small steroidogenic luteal cells. In ruminants, these are interspersed between the granulosa-derived large luteal cells, but in humans they more or less retain their location at the periphery of the corpus luteum.[10,11] The resultant structure is therefore vascularized mesenchymal tissue rather than a stratified epithelium, and the membrana granulosa can be described as undergoing an epithelial-mesenchymal transition[12–14] with the cells differentiating into granulosa lutein (primates) or large luteal (other species) cells. Cellular luteinization, which can be easily modeled in culture,[15] is characterized by hypertrophy, a greatly increased capacity for progesterone synthesis, and altered patterns of peptides and proteins secretion. Luteinization appears to be promoted by exposing the cells to extracellular matrix, and the luteinizing cells themselves contribute to the deposition and remodeling of matrix material.[16–19] Importantly, the follicular extracellular matrix is remodeled completely during the follicular-luteal transition.[20,21] We review what little literature exists on the extracellular matrix of the corpus luteum. Different types of extracellular matrix and different classes of molecules have been identified and are

reviewed elsewhere.[1,22–25] We provide a summary of the principal elements relevant to the corpus luteum.

COLLAGENS

Collagen comprises up to about one sixth of the dry matter (3% of wet weight) of the bovine corpus luteum.[26] Synthesis of collagen continues throughout corpus luteum formation and growth, and the highest amounts and densities occur in the mature tissue. It also remains as a major component of the tissue while cellular material is lost during luteolysis. The predominant luteal collagen is the fibrillar type I,[27,28] which is found throughout the parenchyma and in the capsule.[29] Collagen type I is a major fibrillar collagen that provides tensile strength in several tissues. Specific expression of subunits α1 and α2 of collagen type I can be detected throughout the life of the bovine corpus luteum but especially during the early formation phase.[28] The expression of α1(I) and α2(I) genes is increased during luteolysis.[30] Although the precise cellular origin of collagen in the corpus luteum remains uncertain, the increasing expression of collagen α1(I) and α3(IV) by bovine granulosa cells luteinizing in culture[31] suggests that large luteal cells are a likely source of these materials. Lower levels of expression of subunits α2 and α3 of collagen type IV can also be detected at all stages of luteal life[28] (this collagen is discussed later in the article).

BASAL LAMINAS

Basal laminas are specialized sheets of extracellular matrix that either underlie or envelop groups of cells such as in endothelium or epithelia, separating them from adjoining stroma, or alternatively envelop entire cells such as muscle cells, nerve cells, and adipocytes, and hence partition cells or groups of cells from the surrounding tissue.[32,33] Basal laminas therefore create compartments within tissues and can selectively retard the passage of molecules, including growth factors. Basal laminas have been shown to influence epithelial cell proliferation and differentiation and dictate cellular polarity.[34,35] Basal laminas are composed of a lattice-type network of type IV collagen intertwined with a network of laminin. This structure is stabilized by the binding of entactin/nidogen-1 or -2[36] to the collagen and laminin. The heparan sulfate proteoglycans, perlecan and collagen XVIII, and other molecules are associated with the collagen type IV-laminin backbone. Importantly, basal laminas in different regions of the body differ in the ratios of these components. Furthermore, each component is really a class or family of several molecular isoforms. For example, each molecule of type IV collagen comprises three α chains. However, there are six different chains of type IV collagen α (α1 to α6, each encoded by a separate gene).[1,2] Given that only three of these α chains are required to make one collagen molecule, many different combinations of collagen type IV may exist.[37] Some of these have been observed in nature (e.g., α1α1α2, α3α4α5), and each one can be regarded as different.[38] Similarly, each laminin molecule is composed of three chains; one α, one β, and one γ chain.[39] Because there are five different α chains, three β chains, and three γ chains (all encoded by separate genes), a total of 45 possible molecular combinations exists.[40,41] In addition, alternative splicing produces more combinations.[39] Varying the composition of laminin and collagen type IV alone can generate much complexity in basal lamina composition. It is now recognized that the unique composition of each basal lamina contributes to its specific functional properties.

There has been speculation on the presence or absence of a basal lamina associated with luteal cells.[42–45] In an electron microscopic study of ovine corpus luteum, O'Shea et al[46] reported "Those areas of the surface of small luteal cells which did not make close contact with neighboring cells.... basal laminae were occasionally seen on such areas." When referring to large luteal cells they reported "basal lamina formation on surfaces lacking cytoplasmic processes was more marked." This would indicate that basal laminas do not completely envelope individual luteal cells in sheep. In rats, immunological electron microscopic studies identified "laminin specifically within perisinusoidal areas (capillaries) and in basement membrane-like plaques between lutein cells."[47] This suggests that basal laminas do not envelop or completely surround individual luteal cells of rat corpus luteum. Light microscopic immunological studies described laminin around luteal cells of mice.[48] However, on examination of the immunostaining results presented in this study of mice corpora lutea,[48] we suggest that much of this staining is associated with numerous capillaries, and there are certainly many instances where cells that are sufficiently large to be luteal cells are not enveloped by laminin-positive staining. Our observations[29] are that only a tiny percentage of bovine and ovine large luteal cells possess an associated layer of collagen type IV extending more than halfway around the cell.

In more recent studies of bovine[4] and human[49] corpora lutea, we have been unable to detect any uniform basal lamina surrounding luteal cells. In bovine, a reticular network of fibers throughout the corpus luteum was observed by staining with antibodies to either laminin γ1 or components of laminin 1 (α1β1γ1; there was no laminin α1, α2, β1, β2, or collagen type IV α1, α2, or α3; Table 1).[4] In human corpora lutea,[49] a parenchymal matrix located as aggregates at irregular intervals between the nonvascular cells was observed, and perhaps these aggregates are similar to the plaques of immunostaining for laminin observed in rat corpora lutea.[47]

Table 1 Localization of Basal Lamina Components in Bovine Corpora Lutea

Matrix	Chain	Endothelium	Vascular Smooth Muscle	Reticular Fibers
Laminin	α1 or β1 or γ1	+	+	+
	α1	–	–	–
	α2	–	–	–
	β1*	+/–	–	–
	β2	+	+	–
	γ1	+	–	+
Collagen type IV	α1	+	–	–
	α2	+	–	–
	α3	–	–	–

*Only 50% of corpora lutea had laminin β1-positive subendothelial basal laminas.
(From Irving-Rodgers HF, Harland ML, Rodgers RJ. A novel basal lamina matrix of the stratified epithelium of the ovarian follicle. Matrix Biol 2004;23(4):207–217.)

Collagen type IV α1 and laminins α2, α5, β1, and β2 were localized in this parenchymal matrix in human corpora lutea (Table 2). Laminin α4 appeared only in the parenchyma in early corpora lutea and later only in subendothelial basal lamina, suggesting that this early pool was destined for incorporation into the subendothelial basal lamina at a later stage. Laminin α1 and α3 chains were not detected in human corpora lutea, and hence the luteal parenchymal matrix does not contain laminins 1, 3, 5, 6, or 7.[49] We therefore conclude that in several species, individual luteal cells are not completely enveloped by basal laminas. Although there may be some organization of cells into groups, as occurs in the adrenal cortex,[50] this is highly unlikely, at least in bovine and human, given that no consistent pattern of staining with this organizational arrangement is observed.[50]

The origin of these basal lamina components in corpora lutea also remains to be identified. Studies on the bovine follicle wall using a combination of immunocytochemistry, laser capture, and reverse transcriptase polymerase chain reaction (RT-PCR) techniques (Roger and Luck, unpublished observations, 2006) showed that mRNAs encoding laminin and collagen type IV are expressed by basal and antral granulosa cells as well as by steroidogenic, endothelial, and other interstitial cells of the theca. If these expression patterns were to continue within the developing corpus luteum, they would indicate a multicellular origin for luteal basal lamina components.

Table 2 Summary of Localization of Laminin Chains in Human Corpora Lutea at Different Stages of Development[49]

Luteal Development Stage	Subendothelial Basal Lamina	Parenchymal Matrix
Early	α5, β2, γ1	α2, α4, α5, β1, β2
Mid	α4, α5, β1, β2, γ1	α2, α5, β1, β2
Late	α4, α5, β1, β2, γ1	α2, α5, β1, β2
Regressing	α4, α5, β1, β2, γ1	α2, α5, β1, β2

No staining was observed with either laminin α1 or α3.

BASAL LAMINA OF THE VASCULATURE

In corpora lutea as in other tissues, basal laminas underlie endothelial cells and surround the smooth muscle cells associated with arterioles. Tables 1 and 2 summarize the compositions of these basal laminas in bovine and human corpora lutea, respectively. The studies of laminins in bovine corpora lutea[4] are not comprehensive due to a lack of antibodies for some of the bovine laminin chains. However, subendothelial basal lamina in the bovine contained laminin β2 and γ1 and only a proportion contained β1.[4] Similarly, human corpora lutea subendothelial basal laminas contained the same composition of laminin chains and laminin β1 expression changed developmentally. It was present in mature but not early corpora lutea.[49] The significance of a change in the expression of laminin β1 is unknown, but in other organs (such as the renal glomerulus[51]) there is a switching from one laminin β chain to another during development. The subendothelial basal lamina in corpora lutea does not contain laminin α1 or α2 in bovine or human, and laminin α3 was also absent in the human. Laminin α4 followed a similar developmental pattern to laminin β1 in human subendothelial basal lamina.[49] Therefore, human luteal subendothelial basal laminas potentially contain laminin 11 during early luteal development and, additionally, laminins 8, 9, and 10 at the mid-luteal phase. Collagen type IV was present in subendothelial basal laminas (α1 and α2 identified in bovine[4] and α1 identified in human[49]).

VERSICAN

Versican is a chondroitin sulfate proteoglycan highly expressed in tissues where cells are metabolically active and undergoing proliferation, such as during wound repair and tumor growth.[52] In the ovary, versican can be synthesized by the membrana granulosa and contributes to the matrix expansion process of the cumulus-oocyte complex.[53] At its N terminus, versican contains a G1 domain that can bind hyaluronan, and it may participate in cell–matrix and cell–cell interactions. The G3 domain at the C-terminal end contains several motifs (lectin-like, epidermal growth factor-like, and complement binding) and has been implicated in promoting angiogenesis associated with tumor growth.[52] Between the G1 and G3 domains are two alternatively spliced regions where the glycosaminoglycan chains are

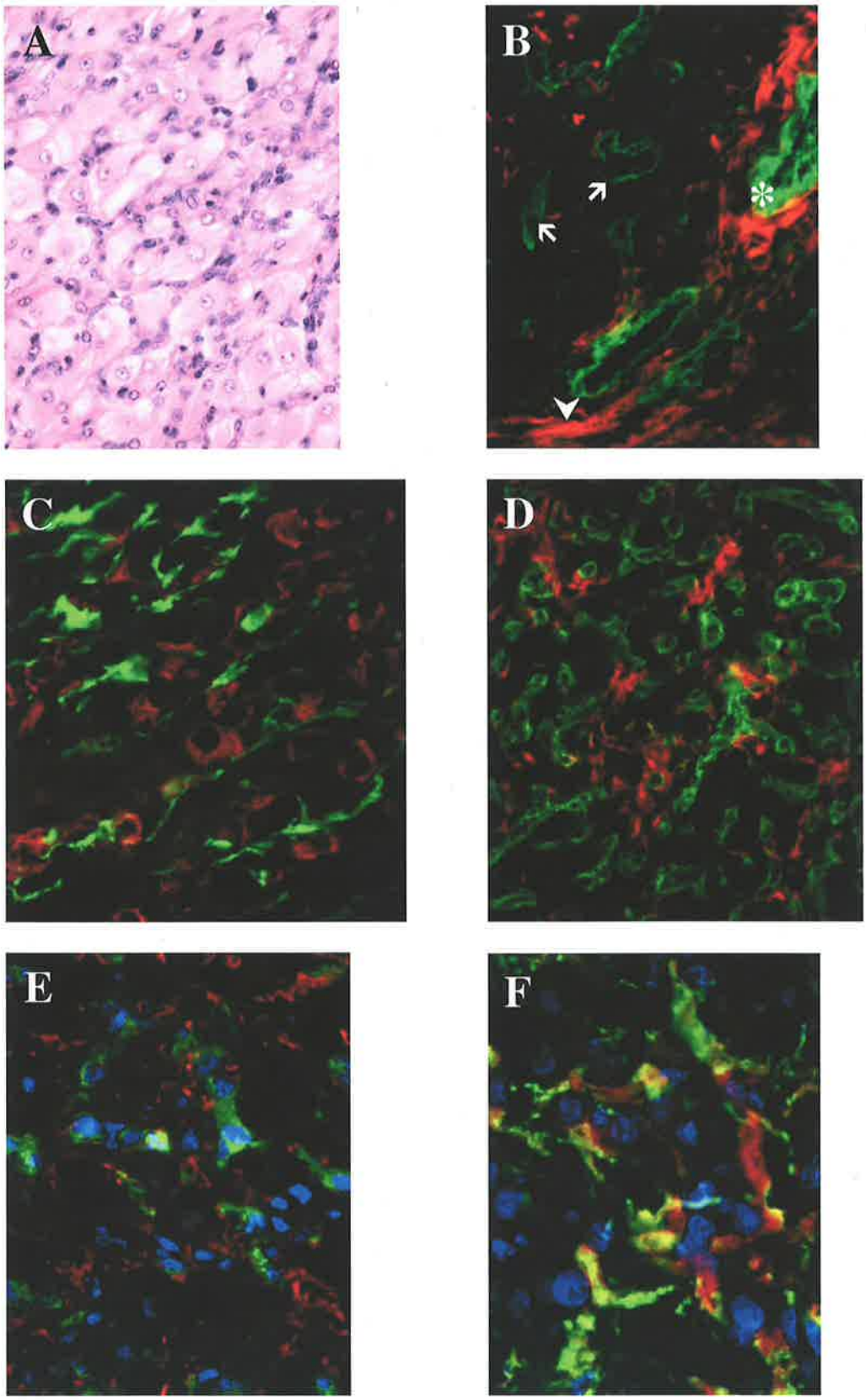

Figure 1 Localization of extracellular matrix components in bovine corpora lutea. (A) Hematoxylin and esosin staining. (B) Collagen type XVIII is localized to the basal laminas of arteriolar smooth muscle cells surrounding an arteriole (asterisk) and capillary subendothelial basal laminas (arrows). Versican (antibody 12C5; red) is localized to the capsular connective tissue layer (arrowhead) and connective tissue surrounding an arteriole (asterisk). (C) Versican (green) localizes to connective tissue stroma between luteal cells

attached. Splicing in this region produces four isoforms (V0, V1, V2, V3), with different degrees of glycosaminoglycan substitution. Ectopic expression of the V1 isoform has been shown to induce a mesenchymal-epithelial transition in NIH3T3 cells.[54] The V0, V1, and V3 isoforms have been detected in bovine corpora lutea by RT-PCR.[7] Versican was localized to the capsule in human corpora lutea, using an antibody that detects the hyaluronan binding region of the N-terminal end.[49] However, versican is more widely distributed throughout the parenchyma of the bovine corpus luteum (Fig. 1), probably reflecting the distribution of the thecal-derived cells in the bovine compared with the human corpus luteum where thecal-derived cells are located near the periphery.

HYALURONAN

Hyaluronan is a high molecular weight linear polysaccharide found in the extracellular matrix, on the cell surface, and within cells.[55] The physicochemical properties of hyaluronan result in highly viscose and elastic solutions with space filling, lubricating, and filtering capabilities.[56] Hyaluronan is synthesized at the cell surface by one of three hyaluronan synthases (HAS1, 2, or 3) producing a repeating glycosaminoglycan chain of hyaluronan polymer extending into the extracellular space. Synthesis of hyaluronan is stimulated by growth factors and cytokines.[57] Following synthesis, hyaluronan is either released into the extracellular space or remains attached to the cell surface via cell surface proteins such as CD44. Retention of hyaluronan as a pericellular coat permits incorporation of extracellular hyaluronan-binding proteins such as aggrecan, versican, and inter-α trypsin inhibitor.[55] Hyaluronan is present in follicular fluid[3] and localizes to the ovarian stroma and theca of follicles at all stages,[58] and to the cumulus oocyte complex and antrally situated granulosa cells 5 hours after luteinizing hormone stimulation in the mouse, whereas newly formed corpora lutea have reduced hyaluronan.[59] Hyaluronan production by cumulus cells has been studied extensively.[60–64] In the bovine corpus luteum, hyaluronan is localized to the connective tissue of the luteal parenchyma (Fig. 1E and 1F).

FIBRONECTIN

Fibronectin is well known as a circulating glycoprotein synthesized in the liver,[65] but it is also an abundant and ubiquitous extracellular matrix glycoprotein. It is organized into a fibrillar network via interactions with transmembrane integrin receptors.[66] Fibronectin is important for cell adhesion, migration, and growth, and has a critical role in embrogenesis.[67,68] Fibronectin is also part of an important matrix formed following tissue injury.[68] There are many different isoforms of fibronectin with different roles in tissue development. In several species the fibronectin gene is composed of several conserved domains present in all fibronectin isoforms. Alternative splicing of the fibronectin gene occurs in three main regions, called the ED-A (extra domain A), ED-B (extra domain B), and V (variable or IIICS) region.[69] Splicing within the ED-A region results in the production of two different isoforms, fibronectin containing the ED-A (ED-A+) or fibronectin lacking the ED-A (ED-A–). Splicing in the ED-B region occurs in the same manner.[70] Fibronectin lacking both ED-A and ED-B regions is the circulating isoform produced by the liver.[71] Alternative splicing in the V region has the potential to produce at least two isoforms in cattle, three isoforms in mice and rats, and up to five isoforms in humans.[65] Hence, there is the potential for at least 12 isoforms of fibronectin mRNA in cattle and rodents, and up to 20 isoforms in humans. Fibronectin has been immunolocalized to corpora lutea,[8,29] and northern analyses have shown that fibronectin mRNA is expressed by corpora lutea.[28] Bovine corpora lutea (cyclic, early to late mid-luteal phase) were shown to express ED-A+, ED-A–, ED-B+, ED-B–, V+ and V– fibronectin isoforms,[72] which were similar to fetal, but generally not similar to adult tissues.

MATRIX IN LUTEAL DEVELOPMENT

The overall formation and development of the corpus luteum has been compared with other physiological events, including inflammation,[73] wound repair,[29,74,75] and epithelial-mesenchymal transition.[13,14] These comparisons are instructive and illuminative for identifying the roles of extracellular matrix. It is reasonable to expect that wound repair processes are involved in corpus luteum formation. Wound repair conventionally involves re-epithelialization and the formation of granulation tissue; the latter process encompassing macrophage accumulation, fibroblast in-growth, matrix formation, and angiogenesis.[76] However, re-epithelization does not occur during formation of the corpus luteum, whereas the luteinization event has been compared with an epithelial-mesenchymal transition,[13,14] in which polarization of granulosa cells is lost. The loss of polarization

(red), identified with an antibody to bovine CYP11A1 (cytochrome P450 cholesterol side-chain cleavage). (D) Nidogen-1 (green) localizes to subendothelial basal laminas of capillaries. Versican (red) localizes to the connective tissue stroma within luteal parenchyma, but does not specifically localize to capillaries. (E) Hyaluronan (identified by binding with hyaluronan-binding protein; red) is localized to the connective tissue stroma within luteal parenchyma. Green; *Bandeiraea simplicifolia* lectin binding to endothelial cells. Nuclei stained with 4,6-diamidino-2-phenylindole, 2HCl (blue). (F) Versican (red) and hyaluronan (green) are not colocalized within the luteal parenchyma.

may be due to the production of focimatrix countering the polarizing action of the follicular basal lamina.[4]

The idea that granulation occurs as in wound healing is supported by an analysis of the density profiles of fibronectin and collagen type I in various regions of bovine and ovine luteal tissue at key developmental stages,[29] and a comparison of the observed time sequence of matrix deposition with that described for classical granulation tissue.[77–79] The key observations are (1) that the center of the early corpus hemorrhagicum is a blood clot, rich in platelets and endothelial cells; (2) that newly formed luteal tissue is rich in fibronectin and relatively low in collagen I; and (3) that as the tissue becomes more organized and grows, the abundance ratio of collagen to fibronectin reverses to produce a firm tissue with a well-developed matrix. The significance of the clot is that platelet and endothelial activation in this region will provide the serine proteinase and metalloproteinase activities necessary for subsequent tissue turnover and remodeling.[21,80] The destruction of follicular basal lamina and focimatrix[7] may also liberate growth factors potentially associated with these matrices. It is likely that the role of fibronectin is to define the early structural arrangement of the tissue, as it appears to do in a repairing wound, and to provide a guiding scaffold for subsequent collagen deposition. Our observations suggest that this fibronectin–collagen time sequence is initiated principally at the interface between the clot and the blocks of radially expanding parenchyma, and that it continues in an outward direction as the main tissue grows and develops. By this analysis, outer luteal tissue is older than inner tissue.

Interpreting luteal development in this way provides several testable hypotheses and directions for future research. First, if the remodeling sequence is exactly the same as in granulation tissue, it should be possible to detect a transient appearance of collagen type III in between the early deposition of the fibronectin scaffold and the later establishment of the collagen type I matrix.[77,78] In support of this, levels of collagen III mRNA in whole mouse ovary are elevated transiently at estrus.[81] Second, a close analysis of matrix gene expression in different regions of tissue should identify the cells responsible for the sequence of deposition and provide a basis for understanding its local control. Third, parallels with a repairing wound and epithelial-mesenchymal transition may help to understand how the vascular supply becomes established and the extent to which a local or paracrine association between endothelial, endocrine cells, and matrix is necessary for the establishment and maintenance of luteal activity.[82] Indeed, it might well be possible to exploit the corpus luteum as a model for the vascular matrix and other interactions occurring in the growth of embryological tissues or tumors.

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