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

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The fate of the follicular basal lamina during atresia was investigated using bovine follicles, in which different follicle phenotypes have been observed. These phenotypes include: healthy follicles with rounded basal granulosa cells with an aligned basal lamina or follicles with columnar basal granulosa cells with a basal lamina of many loops (loopy), and atretic follicles in which either the antral granulosa cells (antral atresia) or the basal cells (basal atresia) die first. Loopy lamina and basal atresia occur only in small antral follicles < 5 mm in diameter. Follicles were collected from cattle of unknown reproductive history and processed for immunohistochemistry and electron microscopy, and from animals in which follicle growth had been monitored by daily measurements of follicle diameter by ultrasonography. Electron microscopic observations of dominant follicles during the growth phase, plateau and regression showed that the basal lamina was still visible

and intact upon atresia. These follicles had a conventional aligned basal lamina, which they retained, except for some degree of folding, as they progressed into antral atresia. In small follicles (2–5 mm in diameter), the basal cell shape (rounded or columnar) and appearance of the basal lamina (aligned or of many loops) did not appear to be related to the type of atresia. On atresia the follicular basal laminae retained immunoreactive laminin  $\alpha$ 1 and  $\beta$ 2, type IV collagen  $\alpha$ 1 and nidogen. Laminin  $\alpha$ 2, which may come from the theca, was present in the follicular basal lamina of only 22% of healthy follicles, but was expressed very commonly in 71% of the atretic follicles. Laminin  $\alpha$ 2 expression was found in both phenotypes of healthy follicles, antral and basal atretic follicles, and follicles with aligned or loopy basal laminae. It is concluded that the basal lamina is not degraded upon atresia, but does undergo a variety of other changes.

## Introduction

The mammalian ovary contains a pool of inactive primordial follicles. Each follicle is composed of a small inactive oocyte and a single layer of non-replicating granulosa cells enveloped by a basal lamina. In adults, a few primordial follicles become activated each day, the oocyte begins to enlarge and the granulosa cells divide. Layers of granulosa cells form and eventually a fluid-filled antrum develops in the centre of the follicle. Only large healthy antral follicles ovulate. Most of the follicles never reach this size and instead become atretic and regress.

Studies in many mammalian species have led to the concepts of follicle 'recruitment' and follicle 'dominance'. In bovine ovaries, follicle recruitment refers to a group of

follicles growing at the same time and at a similar rate, as if in a wave. Follicle dominance refers to one follicle growing at a faster rate than other 'subordinate' follicles. These subordinate follicles eventually become atretic. If the dominant follicle is eliminated after atresia of the subordinate follicles has commenced, another cohort of growing follicles is recruited from the pool of smaller follicles. Two or three waves of follicles emerge in each oestrous cycle of cows. Follicles in each wave grow to about 5 mm in diameter before one dominant follicle emerges and enlarges to > 10 mm in diameter. Follicles of this diameter are sufficiently large to ovulate if simulated by a surge release of LH.

Non-ovulating follicles, including dominant follicles, undergo atresia. In our studies of atretic follicles, we have found that there are two forms of atresia: one in which cell death occurs initially in the basal region and spreads towards the antrum (basal atresia) and another in which the converse occurs (antral atresia) (Irving-Rodgers *et al.*, 2001). Basal atresia occurs in antral follicles < 5 mm in diameter only. The structure of the membrana granulosa also varies among healthy follicles of this size (van Wezel *et al.*, 1999).

In each follicle, the epithelial membrana granulosa is

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enveloped by a follicular basal lamina, which separates it from the surrounding stromal elements in primordial and preantral follicles, or from the specialized theca in antral follicles (van Wezel and Rodgers, 1996; Irving-Rodgers and Rodgers, 2000). There are two ultrastructural phenotypes of basal lamina in healthy bovine antral follicles < 5 mm in diameter. One phenotype is a conventional single layer, aligned closely to the base of the basal granulosa cells (Irving-Rodgers and Rodgers, 2000). The other phenotype is composed of many additional loops of basal lamina with associated matrix vesicles (Irving-Rodgers and Rodgers, 2000). Larger follicles (> 5 mm in diameter) have the former type only. The 'loopy' basal lamina occurs in healthy follicles with columnar basal cells and, conversely, the conventional aligned basal lamina occurs in follicles with rounded basal cells (Irving-Rodgers and Rodgers, 2000).

Basal laminae are often composed of type IV collagen, laminin and other matrix components. Importantly, basal laminae vary in composition from one epithelium to another, and each basal lamina will change in composition as the cells aligning it alter their behaviour. In follicles, the follicular basal lamina doubles 19-fold in surface area during follicular development (van Wezel and Rodgers, 1996) and changes in composition as it does so (Rodgers *et al.*, 2000). During follicle growth, expression of type IV collagen  $\alpha 3$  to  $\alpha 6$  decreases, whereas  $\alpha 1$  and  $\alpha 2$  expression continues (Rodgers *et al.*, 1998). Laminin  $\beta 1$  is expressed transiently at the preantral stage and expression of laminin  $\alpha 1$ ,  $\beta 2$  and  $\gamma 1$  increases (van Wezel *et al.*, 1998). Nidogen and perlecan are not present in primordial follicles but are expressed later in follicular development (McArthur *et al.*, 2000). Laminin  $\alpha 2$  is very unusual; it is expressed in only a few healthy antral follicles and commonly in atretic follicles (van Wezel *et al.*, 1998).

Conflicting observations and comments have been made about the effects of atresia on the follicular basal lamina. These range from suggestions that it is absent (Grimes *et al.*, 1987) to enlarged to > 50  $\mu\text{m}$  (Singh and Adams, 2000). When the ultrastructure of the basal lamina has been observed during atresia it has not been the major focus of any study. Studies on the composition of the bovine follicular basal lamina have shown that some laminin chains ( $\alpha 1$ ,  $\alpha 2$ ,  $\beta 2$  and  $\gamma 1$ ) (van Wezel *et al.*, 1998), the heparan sulphate proteoglycan, perlecan, and nidogen (McArthur *et al.*, 2000) are present in atretic follicles. In the ovine follicular basal lamina of atretic follicles, Huet *et al.* (1997) also observed at least one of the components of laminin 1 ( $\alpha 1$ ,  $\beta 1$  or  $\gamma 1$ ), type IV collagen and heparan sulphate-containing proteoglycans. In the present study, an examination of the ultrastructure and composition of the follicular basal lamina during atresia was undertaken to advance our knowledge of the basal lamina and follicular atresia. Large dominant follicles were monitored by ultrasonography before harvesting and the smaller follicles, which cannot be monitored satisfactorily by this means in cattle, were collected from animals of unknown reproductive history.

## Materials and Methods

### Tissues

Bovine ovaries were collected from two different sources (see below) and handled and processed by a variety of methods (see below), thus increasing the probability of identifying possible artefacts produced by handling and processing. As the features observed were consistent between these different methods, the morphological features observed and described below were not artefacts of processing. The ovaries in groups 1 (small follicles) and 2 (large follicles) were harvested in such a way as to enable both immunohistochemical and electron microscopic examinations to be conducted on the same follicles, whereas ovaries in groups 3 and 4 were prepared for light and electron microscopy only.

*Group 1.* Bovine ovaries were collected at a local abattoir in South Australia, within 20 min of death, from mixed breeds of *Bos taurus* cows, assessed visually as not being pregnant. Ovaries were transported to the laboratory on ice ( $n = 87$ ). Two follicles and adhering stroma (2–5 mm) were dissected from each ovary; their diameter was measured with the aid of an ocular micrometer in the eyepiece of a dissecting microscope and they were snap frozen in Tissue-Tek OCT embedding compound (Miles Inc., Elkhart, IN). The frozen follicles were bisected and one half was immersed in 2.5% (w/v) glutaraldehyde, post-fixed in osmium tetroxide and embedded in epoxy resin (as described below). This portion was for subsequent light and electron microscopic assessment, and the remaining tissue in OCT compound was retained for subsequent immunohistochemical examination.

*Group 2.* Large follicles (6–17 mm in diameter, one per ovary,  $n = 34$ ) were collected from the same source as group 1 and processed similarly. An additional 13 ovaries (two follicles were processed from two ovaries, and one follicle per ovary for the remainder) were snap frozen in OCT embedding compound. A total of 49 follicles was collected.

*Group 3.* Nine reproductive tracts were collected and one ovary per tract was perfusion fixed for electron microscopy, as described below. A total of 23 follicles (2–5 mm in diameter) was examined in this manner. Follicle diameters were measured on tissue sections by averaging the maximum and minimum diameters from the position of the basal lamina.

*Group 4.* In the USA, cows aged 2.5 years, nulliparous and of composite breeding (one quarter Angus, one quarter Hereford, one quarter Pinzgauer and one quarter Red Poll) were used. Oestrus was synchronized by two i.m. injections of PGF<sub>2 $\alpha$</sub>  (25 mg; Lutalyse; Pharmacia and Upjohn, Kalamazoo, MI) 10 days apart. During the first follicular wave of the next oestrous cycle (between day 7 and day 10,

day 0 = oestrus) and once follicular dominance had been identified, all follicles  $\geq 5$  mm in diameter were aspirated using an Aloka 500V ultrasound machine attached to a 5 MHz trans-vaginal aspiration probe. Aspiration initiated the synchronous emergence of a new wave of growing follicles, which was monitored by daily ultrasonography using a 7.5 MHz trans-rectal probe attached to the Aloka 500V machine. Ovaries were collected when the dominant follicle of the ensuing wave was in one of three development stages: (i) growing ( $n = 6$ ): had reached dominance, was  $> 8$  mm in diameter and had not reached plateau stage; (ii) plateau ( $n = 4$ ): had reached dominance, was  $> 8$  mm in diameter and had no growth for at least the 24 h before ovary collection, but was still functionally dominant as determined by the absence of new growth of follicular development; (iii) regression ( $n = 7$ ): had reached dominance, but was starting to regress in size, and emergence of a new wave of follicular development was evident. Ovariectomies were performed by laparotomy via para-lumbar incision and both ovaries were collected when possible.

#### Processing of tissues for microscopy

Portions of follicles from groups 1 and 2 (those fixed in glutaraldehyde only) were processed for electron microscopy. For groups 3 and 4, ovarian arteries were cannulated and the ovaries were flushed with 20 ml medium (Earle's balanced salt solution for group 3 and Hank's solution for group 4), before flushing with 50 ml 2.5% (w/v) glutaraldehyde in buffer (0.1 mol morpholinopropanesulphonic acid  $\text{I}^-$ , pH 7.3, for group 3 or 0.1 mol phosphate buffer  $\text{I}^-$ , pH 7.3, for group 4) delivered over 5–10 min. The ovaries were placed in fixative at 4°C overnight. Follicles were dissected from each ovary, cut in half and the cross-sectional diameter was measured with the aid of an eyepiece ocular micrometer. For all groups, small pieces of the follicle wall were taken and placed in fixative, and stored at 4°C. After several rinses with buffer to remove excess fixative, samples were post-fixed in 2% (v/v) aqueous osmium tetroxide for 1 h at 4°C, rinsed three times with distilled water (5 min each) and dehydrated by successive washes with acetone of increasing concentration to 100%, on ice. After overnight infiltration with epoxy resin at room temperature, the specimens were embedded in fresh resin and polymerized at 60°C overnight. Sections (1  $\mu\text{m}$  thickness) were stained with 1% (v/v) aqueous methylene blue for light microscopic examination and 100 nm thick sections were stained with uranyl acetate and Reynold's lead citrate, and observed and photographed with a JEOL (Peabody, MA) CS1200 transmission electron microscope.

#### Antibodies

The primary antibodies for matrix molecules used were rabbit polyclonal antisera raised against human placental laminin  $\alpha 2$  chain (M4; at 1:50 dilution; Lindblom *et al.*, 1994), laminin 1 isolated from Englebreth Holm-Swarm (EHS) tumours (L9393; Sigma Chemical Co., St Louis, MO;

at 1:100 dilution), purified nidogen isolated from mouse EHS tumour (913 or 914; 1:200 dilution; Dziadek *et al.*, 1985) or recombinantly expressed mouse laminin  $\alpha 1$  chain (317; Durbeej *et al.*, 1996; at 1:100 dilution). Additional antibodies used were mouse monoclonal antibodies raised against bovine laminin  $\beta 2$  chain (C4; at 1:100 dilution; Sanes and Chiu, 1983; Hunter *et al.*, 1989), human laminin  $\beta 1$  chain (clone 4E10; at 1:500 dilution) and laminin  $\gamma 1$  chain (clone 2E8; at 1:500 dilution), both from Gibco BRL (Gaithersburg, MD). Rat monoclonal antibodies were raised against the non-collagenous 1 domain of each of the type IV collagen  $\alpha 1$  to  $\alpha 5$  chains (at 1:100 dilution; Sado *et al.*, 1995) and  $\alpha 6$  (at 1:100 dilution; Lees *et al.*, 1998).

#### Immunohistochemistry

Portions of ovaries embedded in OCT compound were used for localization or co-localization using an indirect immunofluorescence method. Tissue sections (10  $\mu\text{m}$  thickness) were cut from each of the frozen ovaries using a CM1800 Leica cryostat, and collected on glass slides treated with 0.01% (w/v) poly L-ornithine hydrobromide (P-4638; Sigma Chemical Co.) and stored at  $-20^\circ\text{C}$  until use. Unfixed sections were dried under vacuum for 5 min followed by fixation in either 100% ethanol (in conjunction with anti-laminin  $\alpha 2$  chain or anti-laminin 1) or 100% acetone (in conjunction with anti-laminin  $\beta 2$  chain, anti-laminin  $\beta 1$  chain, anti-laminin  $\gamma 1$  chain, anti-perlecan and anti-type IV collagen  $\alpha 1$  to  $\alpha 6$  chains), or unfixed (in conjunction with anti-nidogen). After fixation, sections were rinsed in three  $\times 5$  min changes of hypertonic PBS (10 mmol sodium/potassium phosphate  $\text{I}^-$  with 0.274 mol NaCl  $\text{I}^-$ , 5 mmol KCl  $\text{I}^-$ ; pH 7.2; hPBS) before treatment with blocking solution (10% normal donkey serum (D-9663; Sigma Chemical Co.) in antibody diluent containing 0.55 mol NaCl  $\text{I}^-$  and 10 mmol sodium phosphate  $\text{I}^-$ , pH 7.1) for 20 min at room temperature. Incubation in primary antisera was carried out overnight at room temperature. The secondary antibodies used were fluorescein (DTAF)-conjugated AffiniPure donkey anti-rabbit IgG (1:100; 711-015-152) for the detection of nidogen, biotin-SP-conjugated AffiniPure donkey anti-mouse IgG (1:100; 715-066-151) for the detection of laminin  $\gamma 1$ ,  $\beta 1$ ,  $\beta 2$  chains and perlecan, biotin-SP-conjugated AffiniPure donkey anti-rat IgG (1:100; 712-066-153) for the detection of type IV collagen chains, and biotin-SP-conjugated AffiniPure donkey anti-rabbit IgG (1:200; 711-066-152) for the detection of laminin  $\alpha 1$  chains, and laminin 1 (EHS laminin), followed by Cy3-conjugated streptavidin (1:100; 016-160-084), all from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA) in hypertonic PBS. Sections were mounted in mounting medium for fluorescence (S3023; Dako, Botany).

#### Light microscopic observations and photography

Sections of bovine ovary stained with methylene blue were examined using an Olympus BX50 microscope, with or without Nomarski optics, and photographed with an

**Table 1.** Proportion of group 1 (2–5 mm in diameter) bovine follicles (per number examined) classified as healthy with rounded or columnar basal cells, or antral or basal atretic at early, mid- or late stages of atresia that contained matrix components

Matrix component	Healthy		Basal atresia		Antral atresia		
	Rounded	Columnar	Mid-	Advanced	Early	Mid-	Advanced
Type IV collagen							
$\alpha 1$	1/1	4/4	8/8	4/4	4/4	2/2	3/3
$\alpha 3$	0/0	0/3	0/4	0/2	0/5	0/3	0/5
$\alpha 4$	0/1	0/4	0/4	0/4	0/5	0/3	0/5
$\alpha 5$	0/0	0/3	0/4	0/4	0/5	0/4	0/5
$\alpha 6$	–	–	1/6	0/3	0/5	0/3	0/4
Laminin							
$\alpha 1$	0/0	4/4	0/2	–	–	0/1	1/1
$\alpha 2$	1/11	3/7	6/10	5/7	6/6	7/12	6/7
$\beta 2$	1/1	4/4	8/8	4/4	5/5	2/3	5/5
Nidogen	11/11	7/7	4/4	7/7	8/8	11/11	8/8

Olympus SC35 camera attachment and Ilford FP-4 125 black and white film. Sections stained immunohistochemically were observed and photographed with an Olympus Vanox AHBT3 epifluorescence microscope with Olympus C35AD-4 camera attachment and photographed with Kodak T-Max 400 black and white film.

#### Classification of follicles by light microscopy

Follicles were assessed as healthy or atretic. Atretic follicles were assessed as undergoing basal or antral atresia (Irving-Rodgers *et al.*, 2001), and divided further into early, mid- or late stages on the basis of the degree of cellular degeneration during atresia.

## Results

#### Classification of follicles

Follicles were classified as either healthy or atretic on the basis of their morphology, using epoxy resin-embedded material where available or frozen sections. Healthy follicles were classified as having either columnar or rounded basal cells (Irving-Rodgers and Rodgers, 2000).

The atretic follicles were classified further as undergoing either antral or basal atresia (Irving-Rodgers *et al.*, 2001). Atretic follicles were further classified arbitrarily as early, mid- or late atretic. Early antral atretic follicles had pyknotic nuclei in the antra, mid-atretic follicles had pyknotic nuclei in the antral layers and late atretic follicles had advanced pyknosis, such that only 1–2 layers of basal healthy cells remained. Basal atresia was classified as mid- or late; early stages were difficult to differentiate from healthy follicles using light microscopy. Cell death in mid-basal atretic follicles occurred in the basal layer 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.

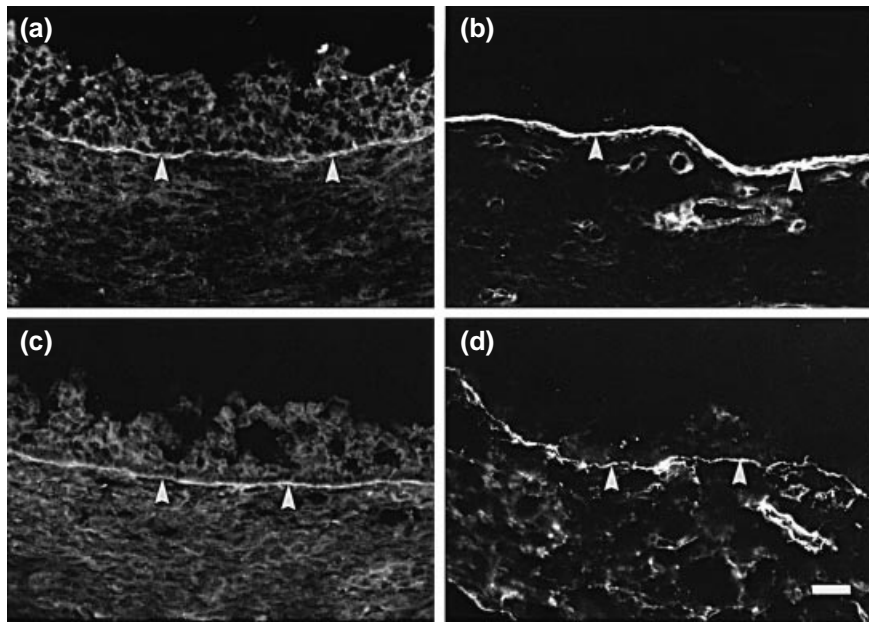
#### Classification of follicles in groups 1 and 2

In group 1, 139 of 174 follicles were examined: 91 follicles were healthy (65%), 29 were undergoing antral atresia (21%) and 19 follicles were undergoing basal atresia (14%). Of these 139 follicles, 68 were taken for immunohistochemistry, and the mean  $\pm$  SEM sizes of the follicles were similar among the healthy ( $3.5 \pm 0.2$  mm;  $n = 20$ ), basal atretic ( $3.5 \pm 0.2$  mm;  $n = 19$ ) and antral atretic ( $3.8 \pm 0.1$  mm;  $n = 29$ ) follicles. Classification of healthy follicles used for immunohistochemistry into columnar or rounded basal granulosa cells is shown (Table 1). Of the early, mid- and late antral atretic follicles, 3/6, 10/12 and 6/6 had rounded basal cells, respectively. The other follicles had columnar cells, except one of the early follicles, which was intermediate in appearance. In group 2, 44 follicles were examined: 28 were healthy ( $n = 19$  follicles in 6–10 mm range, mean  $8.6 \pm 0.4$  mm in diameter; and  $n = 9$  follicles in 12–17 mm range, mean  $14.7 \pm 1.7$  mm in diameter); 16 were undergoing antral atresia (all 6–10 mm in diameter, mean  $7.8 \pm 0.4$  mm in diameter) and none were undergoing basal atresia.

#### Composition of the basal lamina in groups 1 and 2

Immunostaining for nidogen was used extensively for localizing the follicular basal lamina. The follicular basal lamina was present in all follicles examined in group 1, regardless of whether they were healthy with rounded or columnar basal cells (Table 1) or were undergoing antral or basal atresia (Table 1). Similarly, in group 2, nidogen was present in the follicular basal lamina of all the healthy (13/13 in 6–10 mm and 9/9 in 12–17 mm follicles) and antral atretic (10/10 for 6–10 mm follicles) follicles examined by immunostaining for nidogen.

As shown by Rodgers *et al.* (1998), the follicular basal laminae of healthy antral follicles of the sizes examined in the present study contained type IV collagen  $\alpha 1$ , but not  $\alpha 3$



**Fig. 1.** Immunolocalization of laminin  $\beta 2$  in (a) healthy (2 mm), (b) late basal (5 mm) and (c) early antral (4 mm) atretic bovine follicles, and (d) type IV collagen  $\alpha 1$  in a late basal atretic follicle (4.5 mm). Follicular antrum is towards the top of each panel. Arrowheads indicate basal lamina. Scale bar represents 20  $\mu\text{m}$ .

to  $\alpha 6$  (Table 1);  $\alpha 2$  was not examined in the present study. The composition of collagens in the basal lamina did not change during either antral or basal atresia (Table 1 and Fig. 1).

Laminin  $\alpha 1$  and  $\beta 2$  (Fig. 1) were present in both healthy and atretic follicles (van Wezel *et al.*, 1998) of the sizes examined in the present study. The composition did not change with respect to these laminin chains during either antral or basal atresia. In agreement with previous observations, where laminin  $\alpha 2$  was expressed more highly in atretic follicles (van Wezel *et al.*, 1998), laminin  $\alpha 2$  was detected in 4/18 healthy follicles examined only (Table 1) compared with 27/41 atretic follicles. Laminin  $\alpha 2$  was found in both basal and antral atretic follicles (Table 1 and Fig. 2b,d,e). As there was some diversity in the expression of laminin chains in both healthy and atretic follicles, the follicular basal laminae were examined by electron microscopy. The pattern of expression was independent of whether the follicular basal lamina was a conventional aligned basal lamina or a 'loopy' basal lamina (Table 2).

#### Morphology of the basal lamina in groups 1, 2 and 3

The follicular basal lamina was observed as either a single layer of electron-dense material or with additional loops of basal lamina as described by Irving-Rodgers and Rodgers (2000). Follicles undergoing antral atresia (Figs 3 and 4) had either an aligned (Figs 3 and 4b) or loopy basal lamina (Fig. 4a), irrespective of whether the tissue was fixed by perfusion (Figs 3a and 4b) or immersion (Fig. 3b).

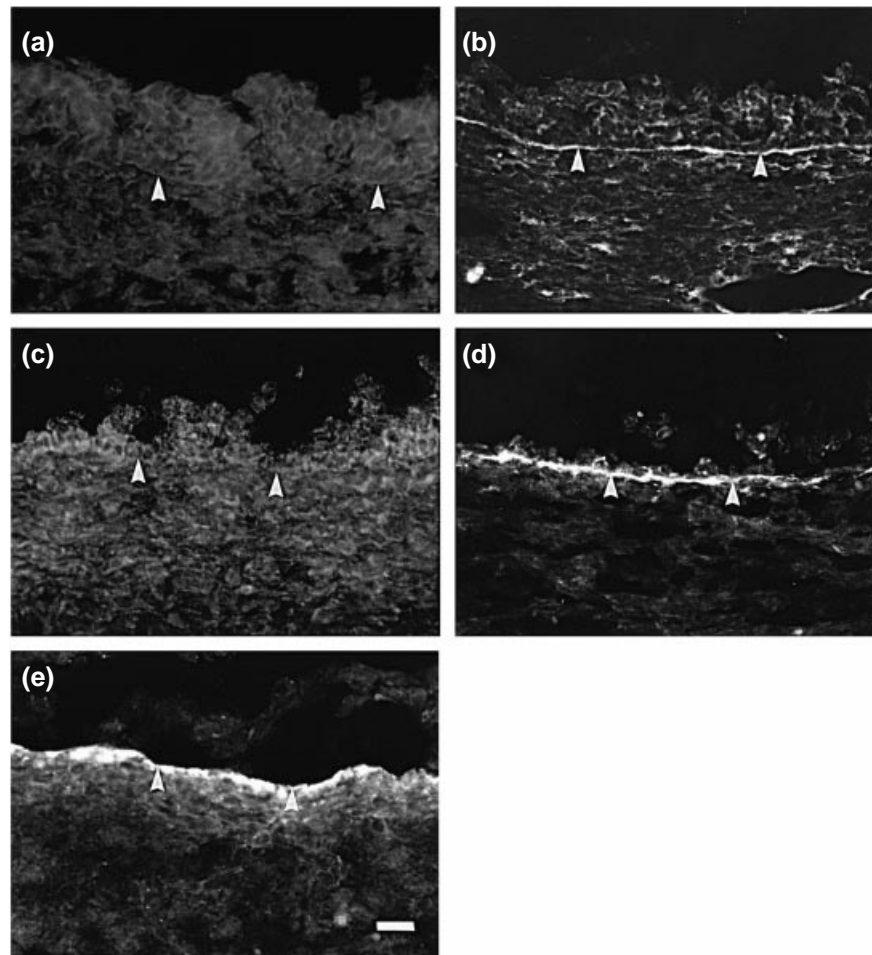
**Table 2.** Numbers of healthy or antral or basal atretic bovine follicles in group 1 with (positive) or without (negative) laminin  $\alpha 2$  in the follicular basal lamina which were either of 'loopy' or aligned phenotype

Follicle type		Laminin $\alpha 2$	Loopy	Aligned
Healthy		Positive	1	1
		Negative	3	1
Basal atresia	Mid-	Positive	3	2
	Late	Positive	2	3
Antral atresia	Mid-	Positive	2	2
		Negative	3	3
	Late	Positive	2	2
		Negative	3	0

Similarly, basal atretic follicles had either an aligned (Fig. 5a) or loopy basal lamina (Fig. 5b). The basal lamina often appeared convoluted late in antral atresia (Fig. 4b). Regressing follicles had an extensively folded basal lamina, which was often traversed by macrophages, endothelial cells and fibroblasts (Fig. 6).

#### Growth parameters and classification of group 4 follicles

Full details of the growth parameters of these follicles have been reported by Irving-Rodgers *et al.* (2001). In brief, follicles in each of the three groups were harvested at different intervals during the follicle wave. Follicles were harvested at  $3.7 \pm 0.7$ ,  $7.5 \pm 1.7$  and  $8.3 \pm 0.9$  days after



**Fig. 2.** Immunolocalization of laminin  $\alpha 2$  in (a) healthy (2 mm) or (c) antral atretic (4.5 mm) bovine follicles, which do not have detectable staining, and (b) early antral (4 mm), (d) late antral (3.5 mm) or (e) late basal (3 mm) atretic follicles, which do stain positively. Follicular antrum is towards the top of each panel. Arrowheads indicate basal lamina. Scale bar represents 20  $\mu\text{m}$ .

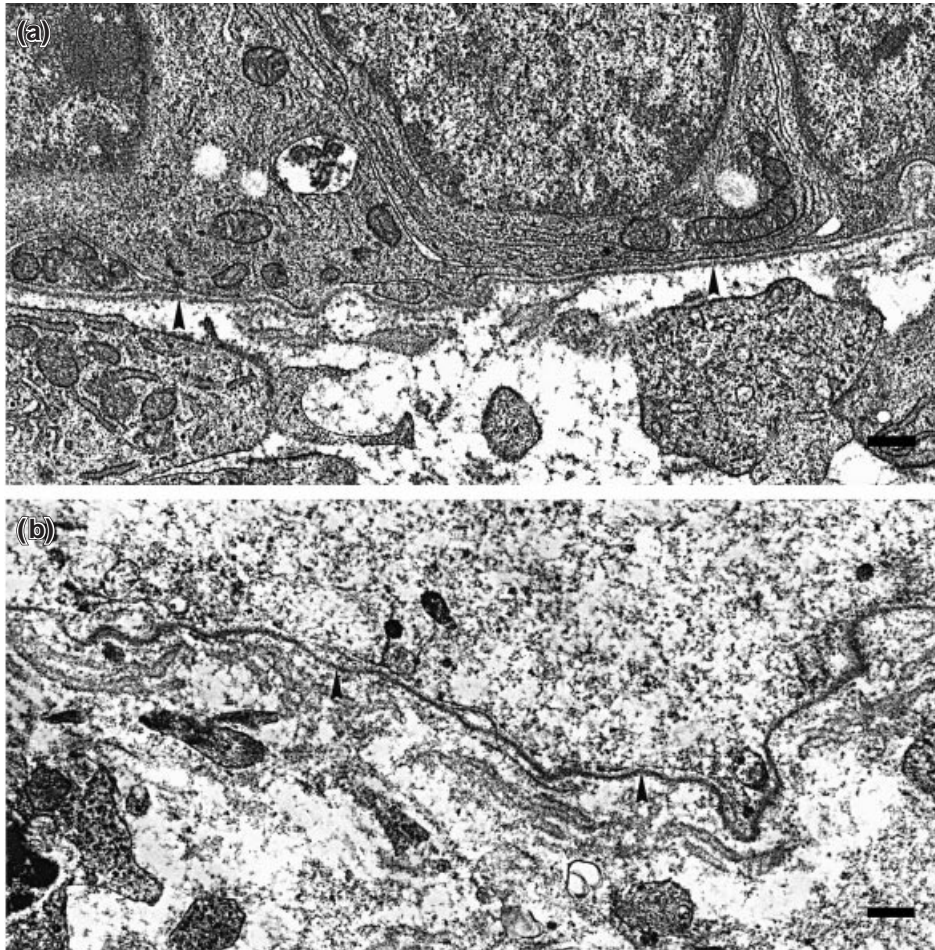
reaching a size of 5 mm for the growing, plateau and regressing groups, respectively. Maximal growth rates attained before harvesting (growing subgroup) or on reaching dominance (plateau and regressing subgroups) were similar for the three subgroups ( $1.2 \pm 0.4$ ,  $1.0 \pm 0.6$  and  $1.5 \pm 0.5$  mm day<sup>-1</sup>, respectively), indicating that the three subgroups of follicles followed similar growth trajectories as they assumed dominance. The sizes of the follicles at harvesting were similar between the subgroups ( $9.1 \pm 0.6$ ,  $9.9 \pm 0.5$  and  $9.0 \pm 0.9$  mm for growing, plateau and regressing follicles, respectively), but clearly the growth just before collection was significantly different ( $P < 0.05$ ; ANOVA and SNK tests;  $1.2 \pm 0.4$ ,  $0.0 \pm 0.0$  and  $-0.6 \pm 0.1$  mm day<sup>-1</sup>, respectively). Thus, the follicles in each of the three groups were similar except they were at different stages of dominance or regression.

Of the growing dominant follicles, five follicles had a healthy granulosa layer and no or very few pyknotic nuclei;

basal granulosa cells were rounded in shape. One follicle had cells with pyknotic nuclei located near the antrum. All of the plateau dominant follicles had rounded granulosa cells; pyknotic nuclei were observed on the antral aspect of the membrana granulosa (2/4 follicles) or among basal cells (one follicle). The regressing dominant follicles were atretic, five follicles had rounded granulosa cells; the membrana granulosa of the other two follicles consisted of flattened cells. All atretic follicles fit the description of antral atretic follicles except that the granulosa cells of dominant atretic follicles were flattened in comparison to small follicles and even follicles 10 mm in diameter.

#### *Ultrastructure of the follicular basal lamina in group 4*

All follicles examined, irrespective of their stage of growth or atresia, had a basal lamina that was a single layer and aligned closely to the membrana granulosa. As atresia progressed, granulosa cells became detached from the basal



**Fig. 3.** Electron micrograph of basal lamina from antral atretic bovine follicles. (a) Early antral atresia, perfusion fixed, aligned basal lamina, follicle 6.5 mm in diameter. (b) Late antral atresia, immersion fixed, aligned basal lamina, follicle 4 mm in diameter. Follicular antrum is towards the top of each panel. Arrowheads indicate basal lamina. Scale bar represents 200 nm.

lamina (Fig. 4b), which was ‘concertina-like’ in appearance and exposed to the follicular fluid. However, the basal lamina remained intact and there was no evidence of degradation as either thinning of the basal lamina or breaches in the basal lamina.

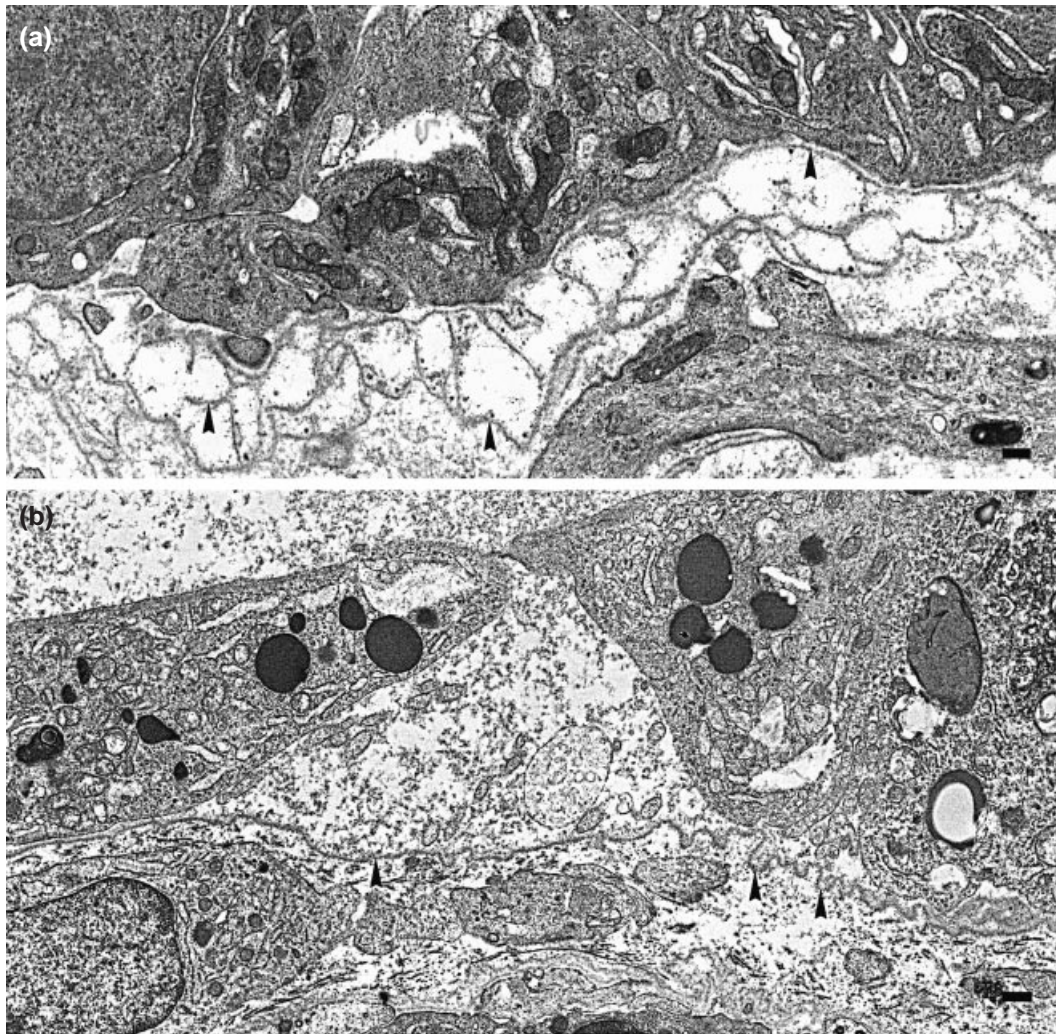
### Discussion

The results of the present study show that on atresia of follicles, regardless of the type of atresia, the basal lamina is not degraded. Electron microscopic observations found that the basal lamina is still visible and intact, and retains immunoreactive laminin  $\alpha 1$  and  $\beta 2$ , and type IV collagen  $\alpha 1$  and nidogen. Laminin  $\alpha 2$  is expressed more commonly in atretic follicles than in healthy follicles, but its expression does not appear to be related to either type of atresia or the degree of atresia. Although large healthy follicles have a conventional aligned basal lamina which they retain as they

progress into antral atresia, the smaller follicles on atresia have either the aligned or loopy type, irrespective of whether the atresia process first involves death of antral or basal cells.

In general, basal laminae can undergo many changes, not just in composition as occurs with follicle development (Rodgers *et al.*, 1998; van Wezel *et al.*, 1998; McArthur *et al.*, 2000). For example, diabetics often have one thickened basal lamina or one of many layers (Vracko, 1974; Martinez-Hernandez and Amenta, 1983; Abrahamson, 1986). Basal laminae are often degraded on epithelial–mesenchymal transition, as occurs to granulosa cells on ovulation. The event of atresia as it occurs in follicles is unusual, as it 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 involves only a few cells at any one time. Certainly the stem





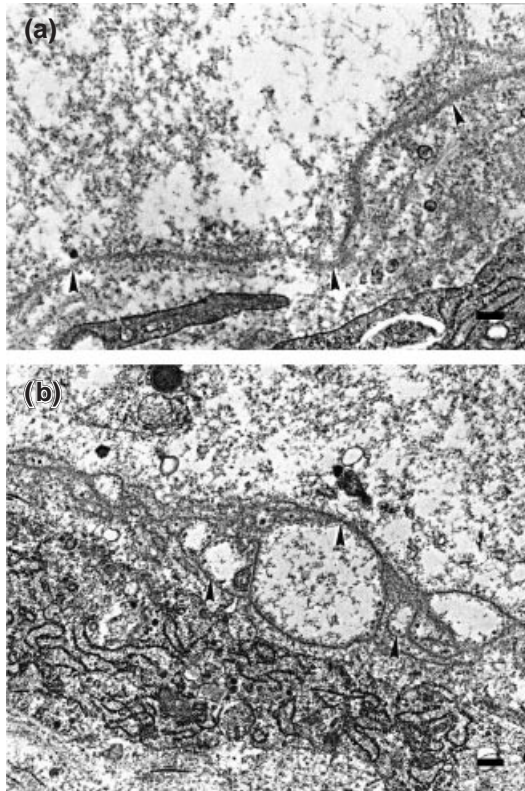
**Fig. 4.** Electron micrograph of basal lamina from antral atretic bovine follicles. (a) Early antral atresia, immersion fixed, loopy basal lamina, follicle 3 mm in diameter. (b) Antral regressing follicle, perfusion fixed, aligned basal lamina, 7 mm in diameter. Follicular antrum is towards the top of each panel. Arrowheads indicate basal lamina. Scale bar represents 500 nm.

cells survive to regenerate the populations of cells. However, the stem cells of the membrana granulosa (Lavranos *et al.*, 1999; Rodgers *et al.*, 1999, 2001) do not survive follicle atresia. Thus, the study of follicular atresia and the changes in the follicular basal lamina pose unique questions.

Very little information has been published on the follicular basal lamina during atresia, but there have been passing comments. For example, Grimes *et al.* (1987) described atretic follicles as having '...a sparse or almost absent granulosa layer, a few squamous cells among the theca which varies in thickness and loosely arranged and disorganized and absent basement membrane...'. Singh and Adams (2000) used light microscopy to show that the basal lamina was enlarged to up to 50  $\mu\text{m}$ . As this is 1000-fold thicker than conventional basal laminae, they may in fact have been referring to the region visible between the

theca and the basal layers of granulosa cells. From the present study it is clear that the basal lamina is not degraded or thickened. Electron microscope studies show that as the follicle shrinks, folding of the basal lamina does occur, but this is not thickening as can occur in diabetic basal laminae. This folding becomes more extensive as follicles continue to regress. Thus, complete destruction of the follicular basal lamina is not one of the early events of atresia.

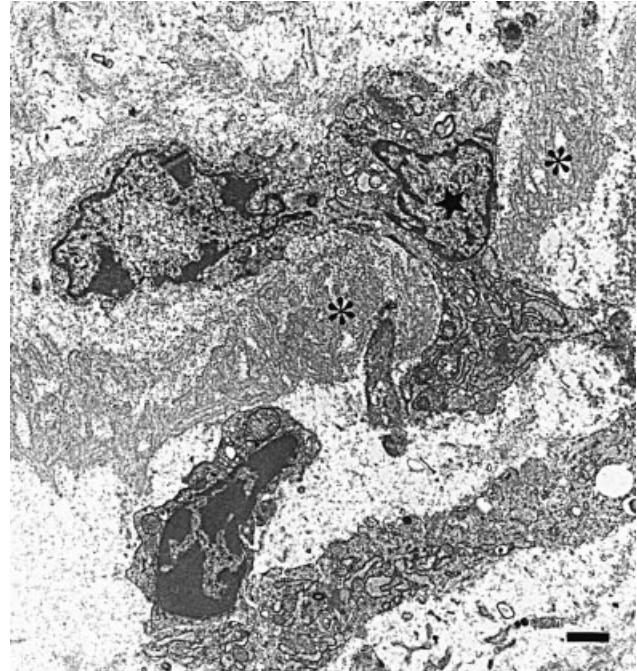
Even if the basal lamina is not destroyed completely early in atresia, it is clear that cells from the theca can breach it (Bagavandoss *et al.*, 1983). Macrophages, endothelial cells and fibroblasts have been observed breaching the follicular basal lamina as they migrate from the thecal layer of atretic follicles. In a more detailed study, Irving-Rodgers *et al.* (2001) found that macrophages were not present in the membrana granulosa of healthy follicles or in antral atretic



**Fig. 5.** Electron micrograph of basal lamina from basal atretic bovine follicles. (a) Late basal atresia, immersion fixed, aligned basal lamina, follicle 5 mm in diameter. (b) Mid-basal atresia, immersion fixed, loopy basal lamina, follicle 3.5 mm in diameter. Follicular antrum is towards the top of each panel. Arrowheads indicate basal lamina. Scale bars represent (a) 250 and (b) 200 nm.

follicles until late in atresia, when most of the cells aligning the basal lamina had died. In basal atresia, where cells aligning the basal lamina die first, macrophages often breach the follicular basal lamina (Irving-Rodgers *et al.*, 2001). Thus, movement of cells from the theca is restrained while the follicular basal lamina is aligned with healthy granulosa cells. However, wholesale degradation of the basal lamina does not occur.

As reported in the present study and by van Wezel *et al.* (1998), laminin  $\alpha 2$  is expressed in some healthy follicles and in a high proportion of, but not all, atretic follicles. In the present study it was found that laminin  $\alpha 2$  expression was not related to the different phenotypes of basal granulosa cells (rounded or columnar), basal lamina (aligned or loopy) or atresia (basal or antral). Of the laminin  $\alpha$  isoforms,  $\alpha 2$  is often associated with non-epithelial basal laminae, such as are found in smooth, cardiac or skeletal muscles, or in capillaries or nerves (Jucker *et al.*, 1996; Oliviero *et al.*, 2000; Vajsar *et al.*, 2000). Where laminin  $\alpha 2$  is associated with the basal lamina of epithelia it may be of mesenchymal origin (Sorokin *et al.*, 1997; Lefebvre *et al.*, 1999). This may be the case in follicles too. Why it is expressed in only some healthy antral follicles and a high



**Fig. 6.** Electron micrograph of a late basal atretic bovine follicle showing highly convoluted basal lamina, being breached by a fibroblast. Follicular antrum is towards the top. Immersion fixed follicle 2 mm in diameter. Asterisks indicate basal lamina. Star indicates a fibroblast. Scale bar represents 1  $\mu\text{m}$ .

proportion, but not all, atretic follicles has yet to be determined.

In small healthy follicles (< 5 mm in diameter), two types of basal lamina have been found, aligned or loopy, and these occur in follicles which have rounded or columnar basal granulosa cells, respectively (Irving-Rodgers and Rodgers, 2000). In larger antral follicles (> 5 mm in diameter), only an aligned basal lamina with rounded basal cells has been observed (Irving-Rodgers and Rodgers, 2000). In agreement in the present study, the large dominant follicles had rounded cells and an aligned basal lamina. On atresia they underwent antral atresia and retained an aligned basal lamina. In smaller follicles there was no link between basal lamina phenotype and atresia type, as there was in large follicles. Thus, one of two events had occurred in the small follicles. Both types of small healthy follicles could have undergone either type of atresia, unlike large follicles. Alternatively, if healthy follicles with a rounded basal cell phenotype underwent antral atresia and those with a columnar phenotype underwent basal atresia, the phenotype of the basal lamina must have changed on atresia.

In conclusion, the follicular basal lamina survives the early events of atresia, retaining the components that were present before atresia. Its ultrastructure and composition can change upon atresia. However, as these changes do not occur uniformly in every atretic follicle it is unlikely that the follicular basal lamina is a key initiator of events in early

atresia. Instead, it appears that the events of atresia have an effect on the basal lamina, which does not involve degradation.

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